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Article

Keywords: biodegradation; polyethylene wax, polyethylene, plastic pollution

Posted Date: September 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10665506/v1

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Additional Declarations: No competing interests reported.

Novel microorganisms screening assay for detection of the biodegradation activity against polyethylene-wax

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Abstract

Plastic overconsumption and inefficient plastic waste management requires new ways of its disposal and recycling. Polyethylene (PE) biodegradation studies are hindered by inefficient screening methods which would enable testing substantial amounts of isolates. The aim of this study was to develop new screening method to look for microorganisms with activity against PE derivatives. PE-wax was used as a model substrate for oxidized PE, as previous studies have highlighted that the identification of enzymes capable of degrading oxidized PE may be key to progress in PE biodegradation studies. This work presents a protocol to obtain PE-wax emulsion, which can be used to prepare PE-wax containing agar plates. The applicability of this approach was confirmed by successfully isolating consortia from soil samples that exhibited activity against PE-wax. Identification of consortia present in samples revealed occurrence of taxa which might have the potential to biodegrade PE, i.e. *Pseudomonas*, *Stenotrophomonas* genus and family *Alcaligenaceae*. The results indicate that the isolates obtained using these methods were able to grow on PE-wax as highly selective carbon source, demonstrating that this approach can be used for screening microorganisms capable of biodegrading PE-wax. This method allows for the rapid and efficient isolation of microorganisms with the potential to biodegrade PE-wax.

Keywords biodegradation; polyethylene wax, polyethylene, plastic pollution

1. Introduction

Many efforts are taken into search for microorganisms able to biodegrade synthetic polymers, as overconsumption of plastics placed the world in the “Plasticene era” [1]. In 2024 production of conventional plastics reached 430.9 Mt and with, inefficient in many regions of the world plastic recycling and waste management a massive amount of plastics will be released to the environment. In 2024 fossil-based PE production covered 26% of the plastics production market, making it the most widely produced plastic polymer [2]

PE is characterized by a stable carbon–carbon backbone, hydrophobic structure and absence of hydrolysable functional groups making it particularly resistant to enzymatic attack [3,4]. Biodegradation of PE proceeds through several stages: oxidative activation of the polymer surface by enzymes such as alkane monooxygenases and oxidoreductases, depolymerization into smaller hydrocarbon fragments, and mineralization, where these intermediates are metabolized to carbon dioxide and water. However as growing evidence suggests, oxidative activation is the principal bottleneck [5,6]. Despite this resistance, microorganisms capable of partial PE biodegradation have been isolated from various environments, including soil, and marine sediments [5,7]. Although promising, the discovery and verification of plastic-degrading microorganisms are limited by the lack of standardized, rapid, and reliable screening approaches [4,8] Recent findings by Sandholm et al. 2025 highlighted also that microbial growth on low-molecular-weight and pre-oxidized polyethylene substrates may be associated with the utilization of alkanes and their derivatives, rather than direct depolymerization of high-molecular-weight PE. This emphasizes the importance of careful interpretation of screening results [9]. Therefore, the development of robust high-throughput screening methods combined with complementary validation techniques is essential for the reliable identification of microorganisms with PE-degrading potential. Such advances are key to improving our understanding of PE biodegradation mechanisms and developing effective biotechnological solutions for plastic pollution.

Various methods were developed to perform screening for microorganisms able to degrade plastics, including PE. For instance, the use of the oxidation–reduction indicator 2,6-dichlorophenolindophenol (DCPIP) has been proposed to monitor microbial redox activity in the presence of PE. Reduction of DCPIP from blue to colourless suggests electron transfer during microbial metabolism, providing convenient, indirect proof for metabolic interaction with substrate. However, as emphasized by Kim et al. 2023, this method reflects metabolic activity rather than direct structural degradation of the polymer [5].

In many cases studies rely on prolonged incubations with polymers in the form of powders or films as the sole carbon source followed by analyses such as weight loss, Fourier-transformed infrared spectroscopy (FTIR) or Scanning electron microscopy (SEM) [7,10–12]. Another important approach in biodegradation research involves the observation of biofilm formation on polymer films, as microorganisms initiate colonization before enzymatic degradation occurs. Studies have demonstrated that biofilm development on polyethylene films indicates microbial adaptation to hydrophobic surfaces, yet it does not necessarily confirm polymer breakdown. Complementary techniques, including SEM and FTIR are therefore often employed to confirm both physical and chemical alterations associated with biodegradation [12,13].

Among these, respirometric measurements of CO_2 production provide the most direct evidence of polymer mineralization, although they cannot specify the degradation mechanism or exclude alternative carbon sources. Consequently, CO_2 measurements should be integrated with molecular or chemical analyses such as FTIR or isotope tracing to confirm polymer-derived mineralization [14].

In addition, polymer-containing agar plates provide a practical and cost-effective method for preliminary screening. This technique enables rapid visual screening of single colonies or microbial consortia capable of growing in the presence of polymers and the appearance clear zones serves as visual indicator of microbial activity. Similar approaches have previously been employed to prepare agar plates containing various synthetic polymers such as polyethylene terephthalate (PET) and biodegradable plastics including polycaprolactone (PCL), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), and polylactic acid (PLA) [15–17].

The current work shows a protocol to obtain PE-wax containing agar plates which can be used for screening single clones or populations of microorganisms with plastic-degrading capabilities and use of obtained oxidized PE-containing emulsion for preparation of liquid cultures useful in screening microorganisms with activity against PE derivatives. As previous studies have highlighted, identifying enzymes capable of breaking down oxidized PE may be the key to success in studies on PE biodegradation [3,18]. In this context, our approach provides a practical strategy for addressing this challenge. By using PE-wax as model substrate representing oxidized, low-molecular-weight PE fractions, our method enables efficient screening and identification of microorganisms capable of degrading oxidized PE which may support discovery of microorganisms capable of PE biodegradation.

2. Materials and Methods

99 2.1. Soil samples

100 Soil samples were collected from areas differing in land-use, also where high plastic
101 pollution was suspected. Samples were collected from the soil layer from 0 – 20 cm using a soil
102 rod. The sample analysed in the laboratory was an average sample prepared from 25 punctures
103 taken evenly over an area of 1 ha. Soil samples were stored at 4°C prior to analyses. The
104 description and location of the sampling sites is included in the table below (Table 1). All
105 sampling sites were located in Lower Silesia, Poland.

106 Table 1. Sampling sites locations and land-use types

Sampling site	Coordinates	Land-use
National road 352 near Zgorzelec	51°08'25.0"N 15°02'00.4"E	areas located along traffic routes
Legnica Economic Zone, Toyota Boshoku factory	51°09'48.4"N 16°10'47.6"E	areas in the neighbourhood of factories
Natura 2000 site PLH020066 Przełomowa Dolina Nysy Łużyckiej near Zgorzelec	51°07'29.9"N 15°00'09.9"E	areas with limited anthropogenic pressure
A4 motorway, at the Krzywa exit	51°16'30.7"N 15°47'28.8"E	areas located in the area of traffic routes

107 2.2. Isolation of microorganisms

108 From each soil sample a 1 g of soil was transferred into sterile 0.9% NaCl solution and
109 shaken for 1h at rotary shaker (Sartorius Certomat MOII) at 25 °C. Next, 100 µl was spread on
110 Luria-Bertani (LB), Yeast Extract-Peptone-Dextrose (YPD) and Martin agar plates for selection
111 of bacteria, yeast and fungi. The plates were incubated at 25 °C for 5–7 days in a bacteriological
112 incubator (Memmert). The developed colonies were sub-cultured in fresh medium and then
113 preserved in sterile glycerol at -80 °C [17].

114 2.3. Preparation of a PE-wax emulsion

115 To obtain 5 % polyethylene emulsion 10 g of oxidized PE granules (DEUREX® EO 40 K)
116 were heated to 98-112°C (drop point) to obtain liquified polymer. Then stirring at 700 RPM
117 was applied. First 0,175 g of NaOH (56992, WARCHEM, Poland) was added, then under
118 constant mixing 1,25 g of Sarcosyl powder (L5125, Sigma-Aldrich, Germany) was added. Then
119 stirring was raised to 1500 RPM and 150 ml of boiling distilled water was added. Obtained
120 emulsion was gradually cooled down (approx. 10°C for every 10 minutes) until it reached an
121 ambient temperature. Distilled water was then added to obtain 200 ml of emulsion. The
122 emulsion was adjusted to pH 7.0. Emulsion can be stored in room temperature for 30 days
123 without loss of its properties. Preparation of water-based PE-wax emulsion was adapted from
124 Sayadi et al. and manufacturer instructions [19].

2.4. Preparation PE-wax containing agar plates

Plates are prepared using two layers of agar medium: the bottom layer contains nutrients, and this is overlaid with a PE-wax containing layer. Each layer is made by pouring approximately 10 ml of each solution into sterile 9 cm diameter petri dishes.

Step 1 - preparation of nutrient agar layer: The bottom layer consists of Nutrient Broth (NB). Medium was prepared by dissolving 13 g of NB (CM0001B, Thermo Fisher Scientific™, USA) powder with 20 g of agar (2025-1000, A&A Biotechnology, Poland) into 1000 ml of distilled water. Later medium was heat sterilized by autoclaving. This medium was selected as it has a wide range of nutrients, and general-purpose mediums were previously successfully used to produce agar plates with polymers [15,17]

Step 2 – preparation of a PE-wax containing top agar layer: To obtain a 0,5 % PE-wax emulsion containing top agar layer 2 g of agar (2025-1000, A&A Biotechnology, Poland) was added to 180 ml of distilled water and autoclaved. When the agar in water cooled to approximately 80°C, a 20 ml of 5% PE-wax emulsion was added with constant stirring (700 RPM) and poured on previously settled plates with NB medium. Although the top agar was not sterile, growth of contaminants in the agar has not been observed. The plates were stored at 4°C prior to analyses for 30 days without loss of their properties [15].

2.5. Preparation of liquid medium with 1% (w/v) PE-wax emulsion

To prepare liquid minimal medium (FMM) supplemented with 1 % (w/v) PE-emulsion 2 g NaH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4, 13 g Yeast Extract (YE) was dissolved into 800 ml of distilled water. Obtained medium was heat sterilized by autoclaving. To that 200 ml of 5% PE-emulsion was added to reach 1% (w/v) concentration in the final medium. Liquid medium supplemented with 1% PE-emulsion was used within 24 hours after preparation.

2.6.Fourier-transform infrared spectroscopy (FTIR)

FTIR was used to confirm stability of PE-wax emulsion after autoclaving and to confirm chemical composition of Deurex PE-wax. Spectra were registered in ATR-mode using Nicolet iN10 integrated infrared microscope with the Nicolet iZ10 external FTIR module Thermo Fisher Scientific™, USA). Before scanning the samples, the background was recorded. FTIR-ATR spectra were acquired in the region 4000-480 cm^{-1} with a resolution of 4 cm^{-1} and performing 64 scans. Spectra were matched against a commercial polymer database of polymers, plastics, polymer additives, plasticizers and packing materials in OMNIC software (9.2.0.41).

2.7. Screening of microorganisms able to degrade PE-wax

PE-wax containing agar plates prepared as described above were used for screening microorganisms isolated from soil for degradation activity against PE derivatives. Inoculum cultures were first grown in LB or YPD medium for 24 h at 25 °C with continuous agitation at 200 rpm. After incubation, 2 µL of the resulting culture was inoculated onto the surface of the PE-wax agar plates. The inoculated plates were incubated at 25 °C for seven days, after which the formation of halo zones surrounding colonies was observed, which was interpreted as a positive indication of microbial activity toward PE-wax.

2.8.Adaptive Laboratory Evolution (ALE)

Adaptive laboratory evolution (ALE) was performed to promote the adaptation of the selected soil-derived microbial consortium to the utilization of PE-wax as a sole carbon source. The experiment was conducted in minimal medium (FMM) supplemented with 1% (w/v) PE-wax emulsion in two replicated. Inoculum cultures were first grown in LB medium for 24 h at 25 °C with continuous agitation at 200 rpm, harvested by centrifugation, washed twice with sterile Milli-Q water, and resuspended to an initial OD_{600} of 0.1 before the first transfer. Cultures were incubated at 25 °C with orbital shaking at 200 rpm. Every four days, 1 mL of culture was transferred into fresh FMM containing 1% (w/v) PE-wax emulsion, resulting in a total of 11 consecutive transfers that ensured gradual adaptation of the consortium to the polymer substrate.

2.9. Biopolymer emulsions and clear zone formation method

PBS and PBSA emulsions were prepared as previously described [17]. Inoculum cultures were first grown in LB medium for 24 h at 25 °C with continuous agitation at 200 rpm. After incubation, 2 µL of standardized to OD_{600} of 0.2 culture was inoculated onto the surface of the PBS or PBSA agar plates and incubated for 72h. Activity against bioplastics was identified by formation of clear zones on FMM agar plates supplemented with 0,5 % biopolymer emulsions [20].

2.10. NBD-H assay on microbial cultures with PE-wax emulsion

Inoculum cultures were first grown in LB medium for 24 h at 25 °C with continuous orbital agitation at 200 rpm. Cells were harvested by centrifugation, washed twice with sterile Milli-Q water, and resuspended to an initial optical density (OD_{600}) of 0.2. Biomass was transferred to 24-deep well plates with 4 ml minimal medium (FMM) supplemented with 1% (w/v) PE-wax emulsion. All assays were performed in triplicate, and blank wells containing uninoculated media served as controls. After 24 and 168 h 1 ml of culture was collected and centrifuged at 13 000 rpm for 5 minutes. Then 100 µL of supernatant was transferred to black bottom 96-well plate and processed using the method adapted from Klauer et al. 2025. In short 100 µL of 2-

fold concentrated 29:1 acetonitrile: aqueous reaction mixture consisting of 33,33 mM aqueous HCl and 0.033 µM NBD-H (4-hydrazino-7-nitro-2,1,3-benxoxadiozole hydrazine) was added. The plate was incubated at 40°C for 30 min before reading the fluorescence at excitation 470 nm and emission 530 nm using BioTek Synergy H1 (BioTek, USA) [21]. Results were presented as MFI (Mean Fluorescence Intensity).

2.11. A growth curve assay

Growth kinetics of the selected bacterial consortium isolated from soil were determined using a Spark Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). Inoculum cultures were first grown in LB medium for 24 h at 25 °C with continuous orbital agitation at 200 rpm. Cells were harvested by centrifugation, washed twice with sterile Milli-Q water, and resuspended to an initial optical density (OD_{600}) of 0.1. The growth curve assay was performed in sterile 96-well microplates containing 200 µL of either LB medium or a minimal medium (FMM) supplemented with 1% (w/v) glucose. Cultures were incubated at 26 °C for 48 h under orbital shaking. Optical density at 600 nm was automatically recorded every 30 min. All assays were performed in triplicate, and blank wells containing uninoculated media served as controls.

2.12. Identification of isolated microorganisms

Genomic DNA (gDNA) was isolated using the Genomic Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. The quality and quantity of gDNA were assessed with a Qubit 4 fluorimeter. Next, bacterial metabarcoding was performed by amplifying the full-length 16S rRNA gene and preparing libraries following the ONT 16S Barcoding Kit 24 V14 protocol. Libraries were cleaned, pooled, and sequenced on an ONT MinION using R10.4.1 flow cells (minimum 50,000 reads per sample). Raw data were basecalled with Dorado (GPU; super high accuracy, sup model). Read quality was evaluated with PycoQC and MinIONQC, and trimming/demultiplexing was performed with Porechop and Chopper according to QC results. Species identification and downstream statistical analyses were conducted using MetONTIIME (QIIME2 long-read implementation).

3. Results

3.1. Quality control of obtained emulsion and PE-wax containing agar plates

FTIR spectroscopy was used to compare the chemical structure of PE-wax (Deurex) with the PE-wax water-based emulsion before and after heat sterilization (Figure 1). The FTIR spectrum of PE-wax and obtained water-based emulsions exhibit characteristic absorption peaks for PE. In the PE-wax two intense peaks observed at 2915 cm^{-1} and 2848 cm^{-1} correspond to the asymmetric and symmetric stretching vibrations of CH, which are typical for long

aliphatic polymer chains. Another characteristic band appears at 1463 cm^{-1}, assigned to the in-plane bending (scissoring) vibration of CH. Additionally, a peak at 719 cm^{-1} is attributed to the rocking vibration of C-C in PE structure. These bands are widely recognized as diagnostic signals confirming the presence of PE as the main polymer backbone.

In addition to the typical PE bands, the spectrum of the PE-wax exhibits a weak absorption band at 1716 cm^{-1}, which corresponds to the stretching vibration of carbonyl groups (C=O). This signal indicates the presence of oxidized functional groups in the PE-wax, such as ketones, aldehydes or carboxylic acids, which are commonly formed during controlled oxidation of PE waxes.

In the spectrum of the PE-wax emulsion before heat sterilization, the characteristic PE bands at 2916 cm^{-1}, 2848 cm^{-1}, 1463 cm^{-1}, and 719 cm^{-1} remain clearly visible, confirming that the polymer backbone remains unchanged during the emulsification process. However, several additional absorption bands appear in the emulsion spectrum, indicating chemical interactions occurring during emulsification. A new band observed at 1608 cm^{-1} together with a second band at 1396 cm^{-1} can be assigned to the asymmetric and symmetric stretching vibrations of carboxylate groups (COO^-). The appearance of these bands is therefore consistent with the conversion of carboxylic acid groups into their corresponding salts, after addition of NaOH in the emulsion preparation process. Furthermore, a broad absorption band observed at 3432 cm^{-1} corresponds to O–H stretching vibrations, which are attributed primarily to water molecules in the aqueous emulsion and possibly to hydroxyl groups formed during oxidation of PE. Another band appearing at 1631 cm^{-1} may be associated with amide-related vibrations originating from the surfactant used in the emulsification process (Sarcosyl). This compound contains an amide functional group that typically exhibits absorption in the region around 1630–1650 cm^{-1}. This band may also partially overlap with the bending vibration of residual water.

Overall, the FTIR results confirm that the fundamental PE structure remains preserved during emulsification, while the additional bands observed in the emulsion spectrum originate from neutralized oxidized groups, the presence of the surfactant, and water. All spectra shown in the image below have been matched to oxidized PE with 99.15, 97.33, and 96.68 % fit respectively for PE-wax, PE wax emulsion before and after heat sterilization when compared to the database in the OMNIC software.

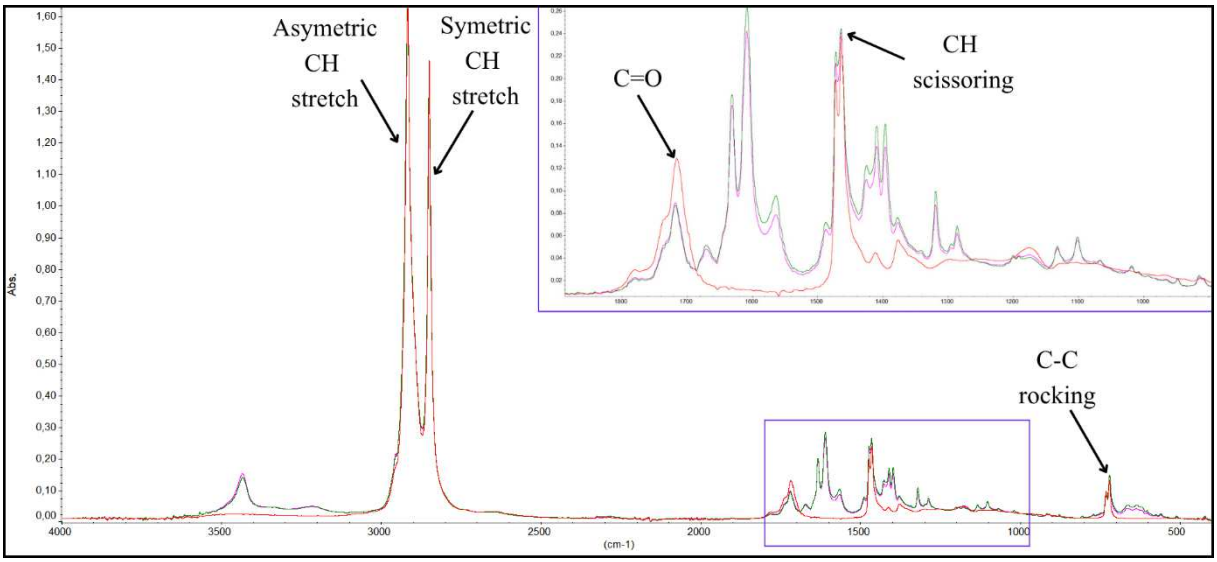


Figure 1. FTIR spectra of PE-wax granules (red) and PE-wax emulsion before (purple) and after (green) heat sterilization.

3.2. Isolation and screening of microorganisms

Four soil samples were collected from sites representing different land-uses including two areas located along traffic routes, one site in areas in the neighbourhood of factories and one area with limited anthropogenic pressure (for details see Table 1 in Materials and Methods). Sampling sites were chosen based on varying levels of anthropogenic pressure and, potentially, varying degrees of microplastics pollution. Environmental microbial consortia were isolated from these samples using nutrient-rich LB, YPD, and Martin agar media. In total, 96 mixed microbial cultures were obtained and stored in sterile glycerol at -80 °C.

The preliminary evaluation of biodegradation potential was conducted on PE-wax containing two-layer agar plates, as described in the Materials and Methods section. Among 96 mixed microbial cultures two consortia (78 and 81) exhibited visible clear zones surrounding the inoculation spots after seven days of incubation, indicating potential enzymatic activity toward PE derivatives (Figure 2). Microorganisms exhibiting activity against PE-wax were isolated from a sample collected near the A4 motorway. These active consortia were subsequently selected for further investigation of their biodegradation potential under liquid culture conditions with PE-wax emulsion in minimal medium.

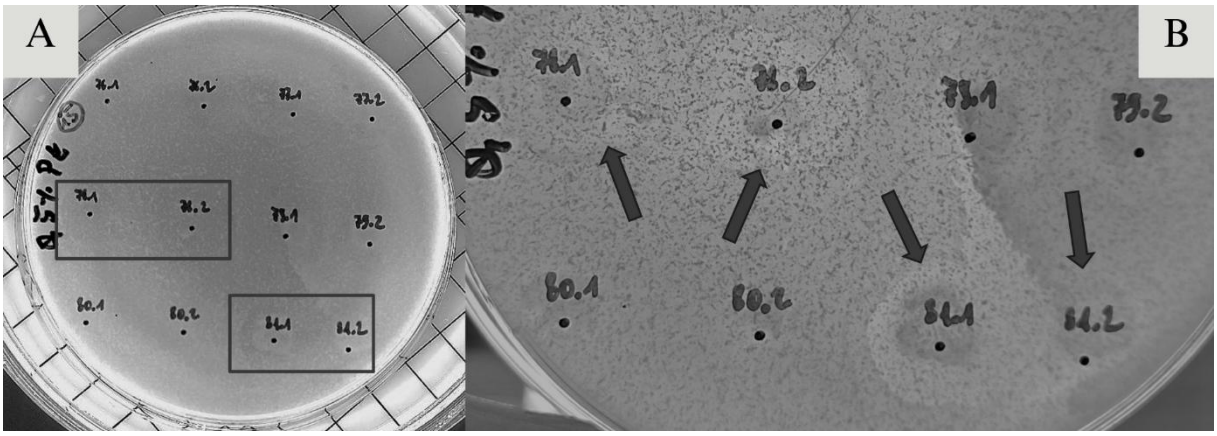


Figure 2. Halo zones formed after 7 days by isolates no. 78 and 81 on PE-wax containing agar plates (a) and close-up photograph of formed zones (b).

Both consortia were able to produce biomass in liquid cultures on FMM medium supplemented with 1% (w/v) PE-wax emulsion. In this media the limiting factor of growth is the carbon source in the form of PE-wax, however it should be considered that in the process of preparation of emulsion Sarcosyl is used, which may be metabolized by microorganisms and therefore could represent an additional source of organic carbon in the system [22]. To further evaluate if developed method with PE-wax containing agar plates is suitable for searching for microorganisms with activity against PE derivatives consortia from sample no. 78 and no. 81 were subjected to ALE as described in the Materials and Methods section. Throughout all passages there was a visible biomass production in all of the samples, indicating that consortia were able to grow on PE-wax emulsion. Consortia from sample no. 81 produced visibly more biomass after ALE as presented in Figure 3 and were chosen for further studies which aimed to confirm their ability to biodegrade PE-wax.

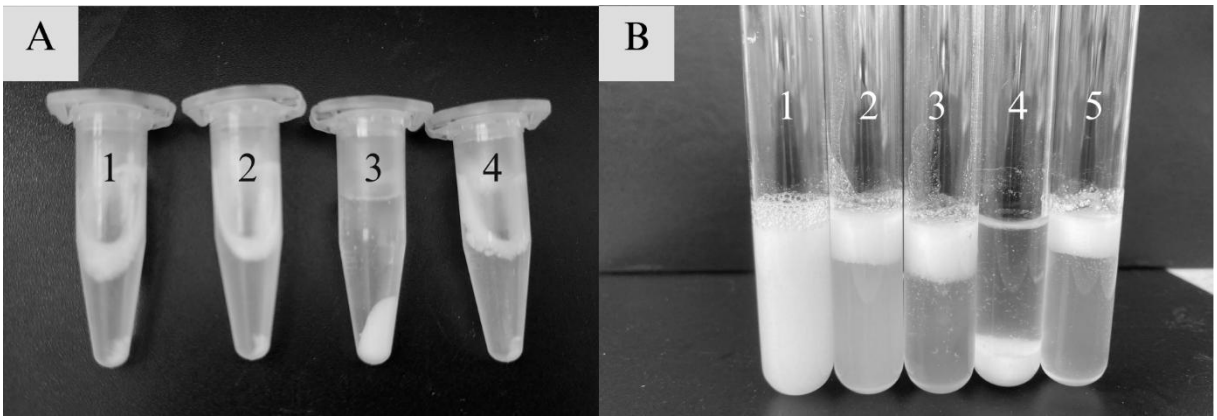


Figure 3.a) 1,2,4 - isolates showing minimal activity against PE-wax, 3- biomass production by isolate no.81 after ALE on FMM medium with 1% (w/v) emulsion, b) 1- FMM medium with

1% (w/v) PE-wax emulsion 2,3,5- isolates showing minimal activity against PE-wax, 4- settlement of emulsion in cultures with isolate no.81 after ALE on FMM medium with 1% (w/v) PE-wax.

To further evaluate enzymatic activity of isolated cultures against polymers clear zone formation method with bioplastics was used. Two biodegradable aliphatic polyesters - polybutylene succinate (PBS) and poly(butylene succinate-co-adipate) (PBSA) - were used. As presented on Figure 4 consortia before and after ALE showed activity against both PBS and PBSA. Two replicates of cultures after 11th transfers of ALE were labelled as 81.1A, 81.3 A (here and after named as 81.11A, 81.11B). There is a visible formation of clear zone around each of the colonies.

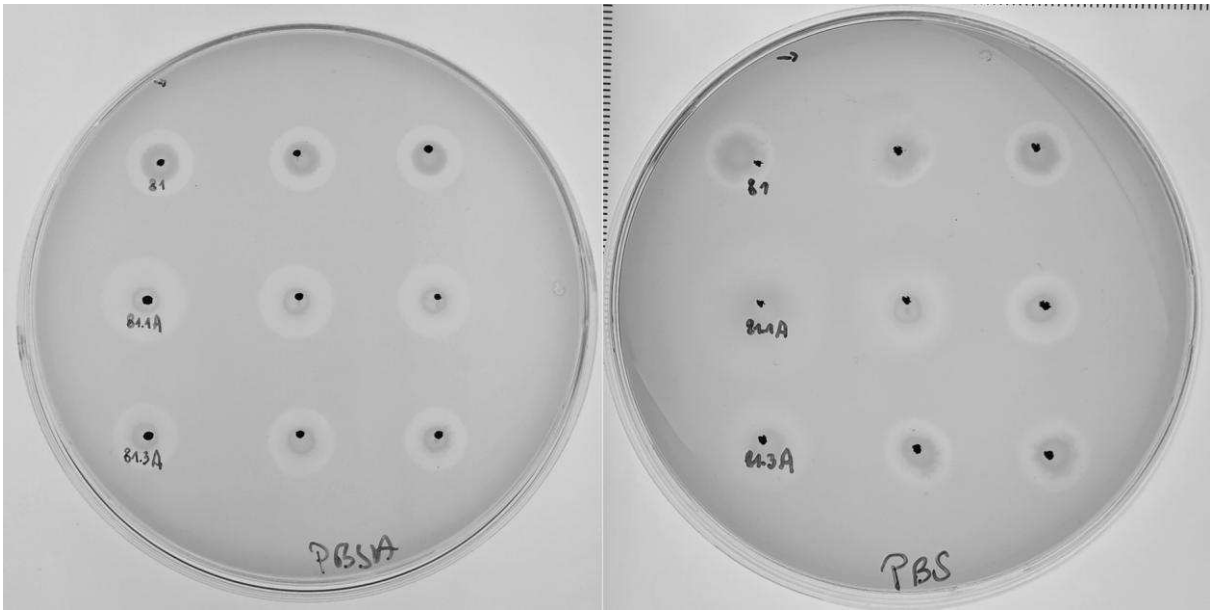


Figure 4. Halo zones formation by mixed cultures isolated from sample no. 81 before (81) and after ALE (81.1A, 81.3A) (three biological replicates in a row) on FMM medium with 0,1% (w/v) PBSA (left) and 0,1 % (w/v) PBS (right).

To confirm the method is suitable to screen for microorganisms with activity against PE-wax NBD-H assay was used [21]. This substance is highly selective, and it is used for detection of aldehydes on hydrocarbon substrates where it generates fluorescent hydrazones. NBD-H was proven to detect one of the first steps of polyolefin degradation which is an oxidation of the C-H bond and formation of aldehyde. Through NBD-H assay we confirmed changes in the polymer structure of PE-wax. As presented in Figure 5 there is substantially higher MFI in sample no. 81 before and after ALE compared to control (which was medium without microorganisms inoculation). MFI in the control remained relatively stable throughout

the incubation period. In contrast, a considerable increase in MFI was observed in sample no. 81, both before and after ALE, at both time points. Moreover, MFI increased between 24 h and 168 h, suggesting progressively increasing bacterial activity over time. Interestingly, sample no. 81 shows a higher MFI value before ALE compared to all samples after ALE. A possible explanation for this observation is that a substantial fraction of aldehyde groups generated during the biodegradation process in the cultures after ALE had already undergone further oxidation, most likely to carboxylic acids. As a result, these oxidized products could no longer react with NBD-H to form fluorescent hydrazones, leading to an apparently lower signal. The pre-ALE samples may be at an earlier stage of PE-wax depolymerization, in which a larger amount of aldehydes was still present and has not yet been oxidized. These aldehydes remain reactive toward NBD-H, leading to the higher MFI values observed before ALE. The results of NBD-H assay clearly indicate that polymer structure of PE-wax was changed in the presence of microorganisms isolated using a method developed in this study.

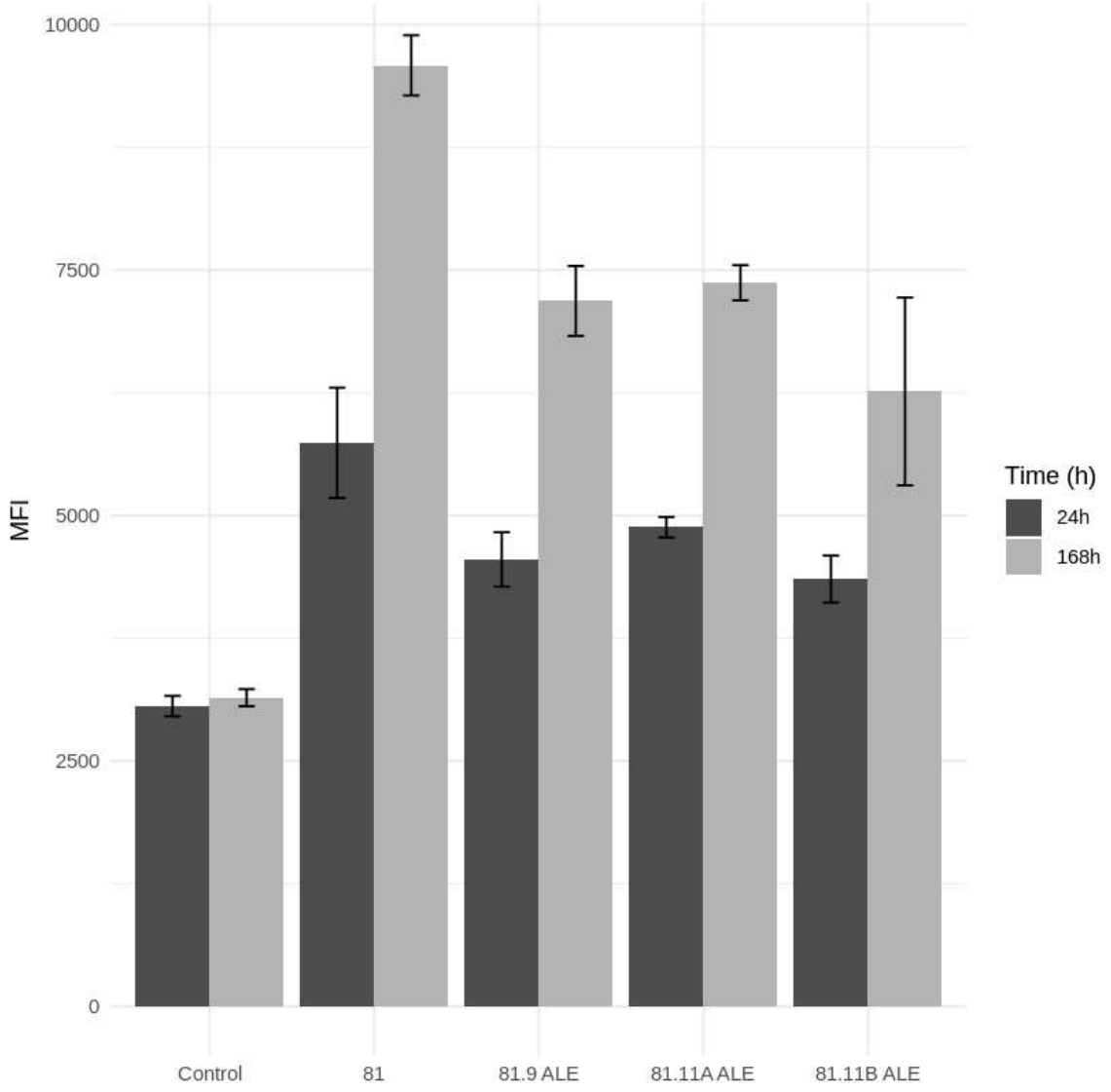


Figure 5. MFI of supernatant collected after 24 and 168h from cultures (control, sample no.81 and consortia after 9 (81.9 ALE) and 11 (81.11A ALE, 81.11B) transfers) on FMM medium supplemented with 1% (w/v) PE-wax emulsion. Results of the NBD-H assay showed the generation of fluorescent hydrazones in the presence of aldehydes, confirming structural changes in polymer chain.

3.3. Impact of ALE on cell growth and viability

Growth characteristics and viability of cultures before and after ALE were evaluated in LB medium and FMM supplemented with 1% (w/v) glucose as presented on figure below (Figure 6). In LB medium both cultures exhibited similar growth pattern, although culture after ALE had a slightly extended lag phase. Consortia after ALE reached a similar maximal optical density (OD_{600}= 1.7) compared to cultures before ALE (OD_{600}= 1.7). In minimal medium, after 32 hours of cultivation differences between the populations were more visible. Similarly to growth on LB medium, evolved strains showed a moderately prolonged lag phase. However, this was followed by enhanced overall growth. Evolved strains reached a substantially higher maximal optical density (OD_{600}= 1.5) compared to cultures before ALE (OD_{600}= 1.3). Further differences between populations were observed after around 40h of growth, as evolved strains progressed into stationary phare whereas culture before ALE entered the death phase. The evolved stains had increased maximal cell density in both rich and minimal media indicating improved cell viability and growth after ALE, at the cost of slight prolongation of lag phase. The overall condition of culture after ALE is higher in minimal medium compared to cultures before ALE, as cultures evolved to manage limited resources more effectively.

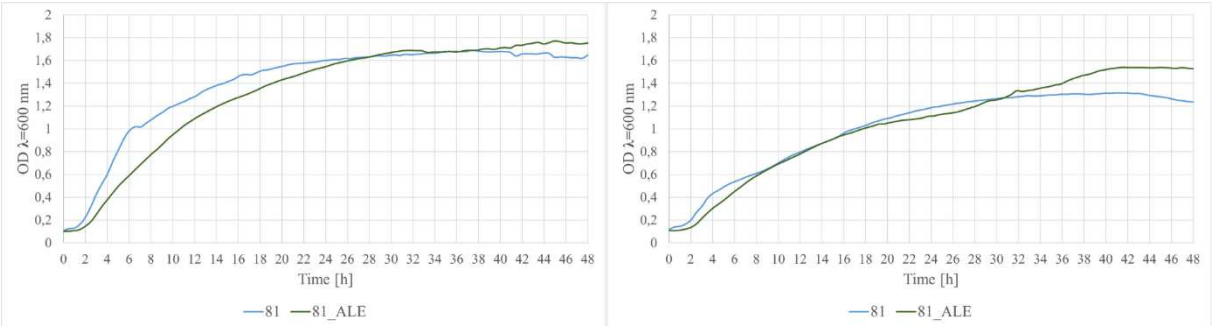


Figure 6. Growth curve of isolate no.81 before and after ALE on LB medium (left) and FMM supplemented with 1% (w/v) glucose (right).

3.4.Identification of the isolated microorganisms

The isolates were obtained from the soil consortium of sample no. 81 and the cultures after the ALE, as described in the 'Materials and Methods' section. Results presented on Figure 7a showed change in composition of communities before and after ALE, which demonstrated a clear pattern of ecological succession. The initial community before ALE was highly diverse and dominated by genus *Serratia* (~70%), with contribution from the genus *Pseudomonas* (~20%) and other taxa (~10%). In sample after 9th transfer (81.9 ALE) the relative abundance of *Serratia* decreased to ~50 %, while *Pseudomonas* increased to ~30 %. At this stage, the genus *Stenotrophomonas* (~15%) emerged, along with members of the family *Alcaligenaceae* (~5%), indicating a change in the structure of the community and increase in additional taxonomic groups. In first sample after 11th transfer (81.11A ALE) the community underwent a substantial simplification, as the *Serratia* genus was no longer detected and *Pseudomonas* became dominant (~60%). The relative abundance of the genus *Stenotrophomonas* increased (~37,5%) and members of the family *Alcaligenaceae* persisted only at low abundance (~2,5%), while no other taxa were observed. In sample 81.11B the community reached a highly simplified state, almost entirely dominated by genus *Pseudomonas* (~95%) with *Stenotrophomonas* remaining only at low abundance (~5%). Overall results indicate a strong ecological succession driven by highly specialized carbon source (PE-wax) during ALE. Results show a progressive reduction in taxonomic diversity and the eventual dominance of genus *Pseudomonas* in samples after 11 transfers.

In addition to the relative abundance profiles, the dynamics of community changes were further visualized using a heatmap representing relative changes in abundance across ALE stages (Figure 7b). This representation highlights the temporal progression of the microbial community, clearly illustrating the emergence and disappearance of individual genus and families. Overall, the heatmap complements the bar plot analysis by providing a dynamic view of taxonomic shifts, reinforcing the observed pattern of ecological succession and progressive community simplification during ALE. Although the final species composition differs between the 81.11A ALE and 81.11B ALE samples, a common trend towards the dominance of the genus *Pseudomonas* can be observed in both replicates.

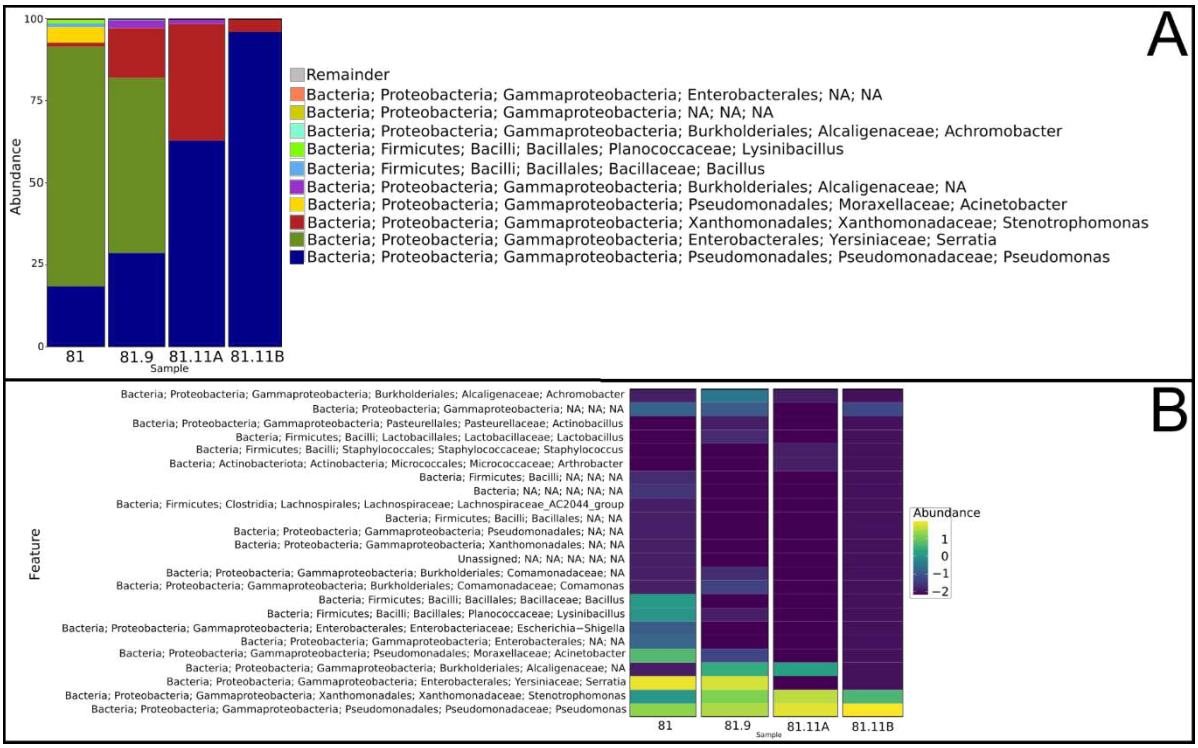


Figure 7. a) Relative abundance of microbial genera before and after ALE, b) Heatmap of change in relative abundance of microbial genera and families during ALE.

3.5.Discussion

The continuous increase in synthetic polymer production and the ubiquitous presence of plastic pollution emphasize the urgent need to isolate and identify novel microorganisms and enzymes able to biodegrade synthetic polymers. Among these, PE is the most widely produced. However, one of the key limitations in PE biodegradation studies is still limited availability of high-throughput screening methods for evaluating large numbers of naturally occurring and engineered strains and microbial communities.

In the present study, new screening method which used PE-wax, as a model substrate representing oxidized PE was developed and evaluated. This approach was motivated by the fact highlighted in previous studies that identifying microorganisms and enzymes capable of breaking down oxidized PE may be the key to success in PE biodegradation studies Kim et al., 2024; Shehbas et al., 2026). Mentioned above emulsion was also used to prepare a liquid FMM medium with 1% (w/v) PE-wax. Stability and chemical composition of PE-wax emulsion was confirmed using FTIR. All spectra were matched with high fit (>90 %) with oxidized PE. Fundamental structure of PE remains preserved as all spectra exhibit characteristic absorption peaks at ~2915 cm^{-1}, ~2848 cm^{-1}, ~1463 cm^{-1} and ~719 cm^{-1} which are widely recognized as

diagnostic signals for main polymer backbone of PE. Additionally peak at ~1716 cm^{-1} at all spectra can be assigned to carbonyl group present in oxidized PE. Additional peaks at spectra of PE-wax emulsion originate from emulsification process and addition of NaOH and Sarcosyl. Obtained using FTIR results show that DEUREX® EO 40 K can be used as a model substrate of oxidized PE.

To validate developed methods for screening microorganisms with activity against oxidized PE four soil samples from areas differing in land-use were collected. After initial screening two mixed cultures forming halo zones on PE-wax containing agar plated were obtained. Clear zones surrounding colonies of consortia no. 78 and 81 indicated potential enzymatic activity as formation of zone of clearance was previously proven to be a visual indicator of microbial activity for various polymers [15,20].

Later consortia from sample no. 78 and 81 were subjected to ALE. As reported in Results section both consortia were able to produce biomass in liquid FMM medium supplemented with 1% (w/v) PE-wax emulsion, however consortia from sample no.81 produced more biomass and were chosen for further studies. Consistent with previous studies describing ALE as a powerful selective tool for improving growth and substrate utilization, our results suggested that the observed growth reflects evolved adaptations suggesting the ability of the isolated microorganisms to utilize PE-wax. The viability of cultures from sample no.81 before and after ALE was accessed. Obtained results suggested that consortia before and after ALE were in good condition. Evolved strains exhibited slightly extended lag phase but ultimately achieved higher maximal optical densities and maintained stationary phase for longer period under minimal growth conditions. These results indicated overall enhanced cellular condition, allowing populations to better cope with nutrient limitations [23–25].

Later enzymatic activity against bioplastics (PBS and PBSA) of cultures from sample no.81 before and after ALE was assessed. There was a visible formation of halo zones around each of the colonies. The observed enzymatic activity of the isolated microorganisms toward biodegradable polyesters such as PBS and PBSA is consistent with literature reports highlighting the broad substrate specificity of polymer-degrading enzymes, which often enables activity across structurally related aliphatic compounds [26]. In this context, the ability of isolates to degrade PBS and PBSA suggested the presence of versatile enzymatic machinery, which may also enable activity against PE-wax. Although this does not directly prove PE-wax biodegradation, it supports hypothesis that the applied screening strategy was effective for identifying microorganisms with potential activity toward oxidized PE.

In the process of preparation of PE-wax emulsion Sarcosyl was used, and it might serve as an additional carbon source for growth of microorganisms. To confirm structural changes in PE-wax after cultivation with selected microorganisms an NBD-H assay was used. NBD-H was previously reported in literature as a substance proven to detect formation of aldehyde on LDPE films [21]. Aim of conducted experiment was detection of newly formed aldehyde groups, which would lead to formation of fluorescent hydrazones and increase of MFI. As presented in Results section MFI for control, which was an uninoculated media, remained at nearly constant level, whereas there was a substantial increase of MFI in sample no.81 before and after ALE. An increase in MFI for sample no.81 before and after ALE in consecutive measurements, i.e. after 24 and 168h was also observed. Interestingly, MFI values for cultures after ALE (81.9 ALE, 81.11A ALE, 81.11B ALE) were lower that before ALE (81). As mentioned above possible explanation of these results may be that a fraction of generated during biodegradation aldehyde groups were further oxidized to products no longer detected by NBD-H assay. The NBD-H assay results clearly demonstrate that the polymer structure of PE-wax was altered when exposed to microorganisms isolated using the methods developed in this study. This indicates that isolated microorganisms were able to use PE-wax as a carbon source for their growth.

Obtained results demonstrate a clear pattern of environmental succession where highly diverse communities before ALE were transformed into highly specialized communities. Final microorganisms composition between the 81.11A ALE and 81.11B ALE samples are dominated by genus *Pseudomonas, Stenotrophomonas* and family *Alcaligenaceae*.

Different species from *Pseudomonas*, *Stenotrophomonas* genus and family *Alcaligenaceae* were previously described in literature as microorganisms with activity against long chain hydrocarbons, bioplastics and synthetic polymers [18,27–30]. *Pseudomonas* genus has been proposed as a potential degrader of polyethylene (PE), especially oxidized PE, which is more prone to microbial utilization than non-oxidized high-molecular-weight PE. A putative degradation pathway of PE involves initial oxidation followed by enzymatic breakdown and assimilation via alkane- and fatty acid-related metabolic routes. However, the key limiting step is the oxidation of high-molecular-weight PE, which is highly resistant and largely determines the overall biodegradation potential [13,31–33]. *Stenotrophomonas* sp. and *Achromobacter* sp. were described for their LDPE biodegradation potential as they were able to oxidase unpretreated LDPE [34]. *Alcaligenes faecalis* ISJ128, which belongs to *Alcaligenaceae* family was used to biodegrade pre-treated LDPE [35]. Consistent with previous findings, the microbial

consortia that emerged after ALE were dominated by taxa previously described in the literature as having the potential to biodegrade oxidized PE. The persistence and enrichment of these taxa after ALE may indicate that PE utilization is supported by interactions among different members of the consortium rather than by the activity of a single microorganism. Nevertheless, the present study does not allow to determine the specific contribution of individual taxa. Further research involving isolation of consortium members and investigation of their metabolic interactions will be necessary to clarify whether synergistic cooperation contributes to the observed biodegradation potential.

4. Conclusions

PE-wax containing agar plates proved to be an effective high-throughput screening method for evaluating the potential of selected cultures to degrade PE derivatives, as evidenced by the formation of halo zones around colonies, which served as a visual indicator of ongoing biodegradation processes. The applicability of this approach was further confirmed by the successful isolation of consortia from soil samples exhibiting activity against the PE-wax. These isolated cultures were subsequently subjected to ALE to assess their ability to survive and grow with the PE-wax as a highly selective carbon source. The results demonstrated that the consortia obtained using this method were indeed capable of utilizing the polymer under such conditions.

Moreover, structural changes in the polymer following incubation with the isolated consortia were confirmed using the NBD-H assay. Identification of microbial communities present before and after ALE revealed the occurrence of taxa with known potential to biodegrade PE, such as *Pseudomonas*, *Stenotrophomonas* genus and family *Alcaligenaceae*. Altogether, these findings demonstrate that the applied approach is effective for initial screening microorganisms capable of PE-wax biodegradation and can serve as a reliable tool in the search for polymer-degrading consortia or individual strains.

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Acknowledgements

Authors would like to thank DEUREX AG company for providing sample of their product for biodegradation studies.

This work was supported by the Wrocław University of Environmental and Life Sciences (Poland) as the Ph.D. research program „Bon doktoranta SD UPWr" from the subsidy increased by the minister responsible for higher education and science for the period 2020-2026 in the amount of 2% of the subsidy referred to Art. 387 (3) of the Act of 20 July 2018 – Law on Higher Education and Science, obtained in 2019. The article is part of a PhD dissertation titled "The study of the impact of the contamination of soils with microplastics on the adaptation of microbes to their degradation", prepared while at the Doctoral School at the Wrocław University of Environmental and Life Sciences. The APC is financed by Wrocław University of Environmental and Life Sciences.

Author Contributions statement

Paula Przygoda-Kuś: Investigation, Data curation, Formal analysis, Methodology, Validation, Visualization, Funding acquisition, Writing – original draft Patryk Mierzejewski.: Investigation - ALE, identification of microorganisms Tomasz Strzała.: Data curation- , Formal analysis- , Visualization -identification of microorganisms, Writing – review and editing Paweł Wiercik.:

Validation - FTIR, Writing – review and editing Aleksandra M. Mirończuk.: Conceptualization, Supervision, Methodology, Validation, Investigation - ALE, identification of microorganisms, Funding acquisition, Writing – review and editing

During the preparation of this work P.P.-K. used Consensus AI model in ChatGPT to improve language and readability and tool NotebookLM to organize the literature. After using this tool/service, the author reviewed and edited the content as needed and take full responsibility for the content of the published article.

All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Data Availability Statement

The sequencing datasets generated during the current study are available in the NCBI Sequence Read Archive (SRA) under accession numbers SRR40195526, SRR40195527, SRR40195528, and SRR40195529.

Conflicts of Interest

The authors declare no conflicts of interest.

Table and Figure Legends

Table 1. Sampling sites locations and land-use types

Figure 1. FTIR spectra of PE-wax granules (red) and PE-wax emulsion before (purple) and after (green) heat sterilization.

Figure 2. Halo zones formed after 7 days by isolates no. 78 and 81 on PE-wax containing agar plates (a) and close-up photograph of formed zones (b).

Figure 3.a) 1,2,4 - isolates showing minimal activity against PE-wax, 3- biomass production by isolate no.81 after ALE on FMM medium with 1% (w/v) emulsion, b) 1- FMM medium with 1% (w/v) PE-wax emulsion 2,3,5- isolates showing minimal activity against PE-wax, 4- settlement of emulsion in cultures with isolate no.81 after ALE on FMM medium with 1% (w/v) PE-wax.

Figure 4. Halo zones formation by mixed cultures isolated from sample no. 81 before (81) and after ALE (81.1A, 81.3A) (three biological replicates in a row) on FMM medium with 0,1% (w/v) PBSA (left) and 0,1 % (w/v) PBS (right).

Figure 5. MFI of supernatant collected after 24 and 168h from cultures (control, sample no.81 and consortia after 9 (81.9 ALE) and 11 (81.11A ALE, 81.11B) transfers) on FMM medium supplemented with 1% (w/v) PE-wax emulsion. Results of the NBD-H assay showed the generation of fluorescent hydrazones in the presence of aldehydes, confirming structural changes in polymer chain.

Figure 6. Growth curve of isolate no.81 before and after ALE on LB medium (left) and FMM supplemented with 1% (w/v) glucose (right).

Figure 7 a) Relative abundance of microbial genera before and after ALE, b) Heatmap of change in relative abundance of microbial genera and families during ALE.