Original article

Response of selected microbial strains and their consortia to the presence of automobile paints: Biofilm growth, matrix protein content and hydrolytic enzyme activity

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ABSTRACT

The goal of the current study was to examine the effects of pollutants (White color – CP; Metallic red color – FM; Thinner – CN; Thinner for rinsing paint – MF; Basic color (primer) – FH) originating from the automotive industry on the biofilm growth, matrix protein content, and activity of the hydrolytic enzymes of selected microbial strains in laboratory conditions that mimic the bioreactor conditions. The chosen microorganisms (bacteria, yeasts, and fungi) were isolated from automotive industry wastewater. Pure microbe cultures and their consortia were injected into AMB Media carriers and developed into biofilms. The use of AMB media carriers has been linked to an increase in the active surface area colonized by microorganisms. Afterwards, the carriers were transferred to Erlenmeyer flasks with nutrient media and pollutants at a concentration of 200 µL/mL. The current study found that, depending on the microbial strain, development phase, and chemical structure, the assessed pollutants had an inhibitory or stimulatory influence on the growth of single cultures and their consortia. Statistical analysis found positive correlations between the protein content in the matrix and the biofilm biomass of *Rhodotorula mucilaginosa* and consortia in CP and FH media, respectively. The proteolytic activity of *Candida utilis* was very pronounced in media with MF and CN. The best alkaline phosphatase activity (ALP) was achieved in the CN medium of *R. mucilaginosa*. Acid invertase activity was the highest in the FM and CP media of *Escherichia coli* and consortia, respectively, whereas the highest alkaline invertase activity was measured in the MF medium of *E. coli*. A positive correlation was confirmed between ALP and the biofilm biomass of *R. mucilaginosa* in CP and CN media, as well as between ALP and the biofilm biomass of *Penicillium expansum* in FM medium. The findings provide novel insights into the extracellular hydrolytic activity of the investigated microbial strains in the presence of auto paints, as well as a good platform for subsequent research into comprehensive biofilm profiling using modern methodologies.

1. Introduction

The rapid increase in the human population and technological advancement greatly consume natural resources and pollute the environment. Every day, a large amount of water is consumed and a large amount of waste is generated in various industrial production processes. The manufacture of motor vehicles includes mechanical and chemical processes, and a large number of operations that generate an oversized amount of organic and inorganic waste on a daily basis, such as polycyclic aromatic hydrocarbons (PAHs), paints, solvents, heavy metals, etc. (Ghomi et al., 2018). The presence of these contaminants, even in very low concentrations, in aquatic ecosystems may cause huge environmental issues and pose a significant risk to public health. In support of this statement is the fact that the presence of even very small amounts (less than 1 ppm) of dyes in water, particularly synthetic dyes, has been linked to cancer (Rasalingam et al., 2014). Depending on the type and concentration used, PAHs can be hazardous, carcinogenic, and mutagenic to living beings. Volatile organic compounds (VOCs)

have application as a solvent in domestic and auto-repair paints (Song and Chun, 2021). They can have harmful consequences for human health, because they cause damage to the respiratory organs and the immune system, or allergies (Mendell, 2007). The continuous growth of dangerous contaminants in aquatic ecosystems has forced the advancement of cost-effective cleanup technologies. Converting pollutants into safe compounds and/or removing pollutants from polluted water are critical to a green environment. For efficient use of microorganisms in waste management, it is important to investigate autochthonous species that are resistant to pollutants and can partially or completely degrade them. However, there are conditions in the environment that limit access to nutrients or pollutants, which is reflected in the abundance and activity of microbes in the environment, so the cleaning processes do not take place at the desired speed (Edwards and Kjellerup, 2013). Biofilm-mediated remediation, on the other hand, is recognized as an ecologically suitable and economically viable method for cleaning up environmental pollution. The efficiency of microbes in bioremediation is reflected in their interaction with pollutants, immobilization, and finally decomposition. Bacterial biofilms exist within indigenous communities at extremely polluted places to enable them survive, continue to exist, and cope with the harsh environment. The industry typically uses biofilm-based bioremediation to clean contaminated groundwater and soil (Mitra and Mukhopadhyay, 2016; Mukherjee et al., 2021). Scientists have discovered and reported a large number of microbes that play a key role in the biodegradative process in the environment so far (Ugbogu et al., 2017; Phulpoto et al., 2021). Paints are complex compounds mostly composed of solvents, pigments, binder, and additives. The fate of each compound in the environment is influenced by their chemical and physical properties as well as environmental characteristics. To date, observations of the efficiency of microorganisms in the breakdown of paints and colored surfaces have uncovered a variety of fungi species (g. *Aspergillus, Fusarium, Trichoderma, Penicillium, Cladosporium,* etc.) (Ugbogu et al., 2017; Savković et al., 2021; Zucconi et al., 2022) and bacteria species (g. *Bacillus, Clostridium, Flavobacterium, Pseudomonas, Mycobacterium,* etc.) (Pandey and Kiran, 2020; Phulpoto et al., 2021) that have been quite successful. Nevertheless, the biodegradation of different dyes by microbial biofilms has been poorly investigated in the world.

Taking into account the above mentioned, the current study was designed in order to evaluate the biofilm biomass, amount of proteins, and hydrolytic activity of the matrix formed by various microorganisms isolated from automotive industry effluent as a reaction to the presence of contaminants, i.e., car paints. The stability of microbial biofilms and the activity of hydrolytically enzymes in the presence of these contaminants are important variables in determining their biodegradability. Thus, this study should shed light on the relationship between hydrolytic activity and biofilm biomass of pure microbial strains and their community, as a fact that supports their participation in car paint removal.

2. Materials and methods

2.1. Chemicals and materials

Tryptic soybean agar (Merck KGaA, Germany), Tween 20, Bradford reagent, bovine serum albumin, sodium hydroxide, sodium carbonate, sodium dihydrogen phosphate, potassium chloride, calcium chloride, Folin-Ciocalteu phenol reagent, Coomassie plus reagent, trichloroacetic acid, sucrose, citrate buffer, glucose, disodium *p*-nitrophenyl phosphate (tetrahydrate), beta-glycerophosphate, ammonium molybdate, were purchased from Sigma-Aldrich (St. Louis, USA). The pollutants used in this study

were: basic color (primer) (marked–FH), thinner for rinsing paint (marked–MF), thinner (marked–CN), metallic red color 108 (marked–FM), and white color 268 (marked–CP), all provided by an automotive manufacturing company (Kragujevac, Serbia).

2.2. Wastewater sampling, isolation, and identification of microorganisms

Indigenous species of microorganisms have been isolated from wastewater that flows into the effluent of an automobile manufacturing company. Sterile plastic bottles were used for sampling, after which the samples were transferred to the laboratory for microbiology and stored at a temperature of 4 °C. In order to assess the microbial diversity, the wastewater samples were serially diluted with sterile distilled water, followed by streaking on Petri plates with tryptic soybean agar prepared of peptone (1%), yeast extract (0.5%), pH 6.–6.8 and agar (1.5%). After incubation at 28 °C for 24 h, the colonies were transferred to a fresh medium under sterile conditions, and then pure cultures were isolated. The determination of enterobacteria was made by the API tests Microgen GnA + B-ID System (Microgen Bioproducts Ltd., UK), whereas the identification of pure, yeast strains, was performed by using the biochemical API 20 C AUX test (BioMérieux, France) (Radojević et al., 2019). With the goal of identifying pure filamentous fungal cultures, their morphological characters were explored and a fungal identification key was employed (at the laboratory for Algology, mycology, and lichenology, Faculty of Science, Kragujevac, Serbia).

2.3. Isolation of microbes and inoculum preparation

The subject of this study was the individual species of microorganisms and their community (Table 1). A suspension of selected microorganisms was made by the direct colony method (Radojević et al., 2019). Using a densitometer (DEN-1, Biosan, Latvia), the turbidity of the suspension was adjusted to 10^8 and 10^6 CFU/mL (colony-forming unit per milliliter) for bacteria and yeasts respectively.

2.4. Experimental design (Biofilm assay)

2.4.1. Biofilm formation on AMB carriers

The main role of AMB Media carriers (Assisting Moving Bad Media) is to increase the active surface area occupied by microorganisms. The carriers used in the current study are made of high density polyethylene (ρ = 1 g/cm^3) and had the following sizes: outer diameter $\geq$ 12 mm, length $\geq$ 13 mm, usable internal surfaces $\geq$ 500 m^2/m^3 and free areas $\geq$ 85%.

For the formation of biofilm, sterile plates with 6 wells at a volume of 15 mL (Tissue Culture Plate, 6 wells) with AMB media carriers were used, which imitated MBEC^{TM-HTP} (Minimum Biofilm Eradication Concentration-High Throughput Plates) plates with 96 wells at a volume of 250 μL, of which 96 wedges rest. Unlike the MBEC^{TM-HTP} plate, which forms the biofilm on wedges, this modified method forms the biofilm on AMB media carriers that

Table 1
The list of tested microorganisms used in the current study.

Tested microbial strains	Code identification
Escherichia coli	PMFKG-F1
Rhodotorula mucilaginosa	PMFKG-F8
Candida utilis	PMFKG-F9
Cladosporium cladosporioides	PMFKG-F11
Penicillium expansum	PMFKG-F14
Consortia	all species

mimic wedges. First, 10 mL of growth medium previously diluted with sterile distilled water until desired solution obtained was added to the six wells plate (Zurob et al., 2019). Afterwards, the plate wells were inoculated by adding 100 μL of a suspension of individual microorganisms and incubated at 25 °C for 10 days. For the preparation of mixed biofilms, an equal amount of suspension was used and further procedures were repeated as in the case with individual microbial strains. Every 3rd day of incubation, the AMB media carrier was aspirated and washed with sterile saline to remove dead cells from the surface and those into plankton, and the liquid media was replaced by the new one. At the end of this experimental phase, individual and mixed biofilms were formed on the AMB carrier.

For the purpose of creating appropriate circumstances which simulate conditions for moving-bed biofilm reactor (MBBR) bioreactors, biofilms made on AMB media carriers were moved to 250 mL Erlenmeyer flasks. Each Erlenmeyer flask contained 200 mL of diluted growth medium (TSB or SAB) and 7 sterile AMB media carriers. They were then inoculated by adding one AMB media carrier with formed biofilm (previously grown for 10 days in a modified MBEC plate). On day 5 of cultivation at 25 °C, the test substances at a concentration of 200 μL/mL were added to the inoculated Erlenmeyer flasks. To monitor the cumulative effect of all the test auto paints on the mixed biofilm of all tested microorganisms, a 2 L Erlenmeyer flask was used. It contained 1.5 L of medium (diluted 1:3) and 20 AMB media carriers. Inoculation of the Erlenmeyer flask was achieved by AMB media carriers with previously formed mixed biofilm. All test substances were combined at a concentration of 200 μL/mL after a five-day incubation period. The influence of auto paints on the enzymatic activity of microorganisms was measured after 15 days of incubation in static conditions.

2.4.2. Quantitative assessment of biofilm growth

Quantification of biofilm biomass was performed using the Bradford protein assay, adapted for use in 96-well microtiter plates as previously described (Lemire and Turner, 2015). By sonication, in the presence of 0.1% Tween 20, the microbial biofilm was removed from the AMB carriers. In order to obtain the pellet, it was necessary to insert 2 mL of sonicate into the tube and centrifuge it at 10,000 g for 10 min. The resulting pellet was resuspended in 100 μL of the buffer, after which the tubes were heated in a boiling water bath for 10 min. Each well of the microtiter plate received 20 μL of Bradford reagent, 2 μL of the protein sample and distilled H_2O up to 100 μL. Absorbance was measured on a Biotek EPOCH plate reader at 595 nm, with two measurements for each well. The experimental design included both positive and negative controls (wells with media formulation, but without cells or spores). Serial dilutions of BSA (from 1 to 100 μg) were used to create the standard calibration curve.

2.4.3. Biofilm structure

The examination of the biofilm structure of single microbial strains in control (TSB and SAB liquid medium) and polluted growth media (control with the addition of individual test substances: FH, FM, MF, CN and NP) was done by fluorescence microscopy, as detailed in the paper by Radojević et al. (2019).

2.4.4. EPS extraction

After 15 days of cultivation, AMB carriers with developed biofilms (control and test substances) were translocated to centrifugation tubes and exposed to an ultrasonic bath to remove the biofilms from the carriers. The tubes with biofilms were exposed to centrifugation at 20,000 rpm for 20 min after sonication. The obtained precipitate was blended with fresh prepared 50 mM potassium phosphate buffer (0.6 g NaH_2PO_4 and 0.323 g KCl dis-

solved in 100 mL distilled water) and before being returned to an ultrasonic bath for 15 min at 40 amps. The resulting supernatant was then filtered (0.22 um membrane filter) and used to determine enzyme activity and protein concentration in EPS.

2.4.5. Determination of the protein concentration in the EPS

A modified Lowry method was used to determine the protein concentration (Molobela et al., 2010), and the procedure included the following steps: 10 μL of EPS solution and 300 μL of Comassie plus reagent were introduced into the microtiter wells, after which the plate was incubated at room temperature (10 min). Control wells contained only phosphate buffer. Following the completion of the incubation time, the absorbances of the standard and sample solution were measured at 595 nm using a Multiskan Ascent V1.24 plate reader (Amersham). BSA was used as a standard.

2.5. Determination of activity of enzymes

2.5.1. Assays of alkaline protease activity (E.C 3.4.21-24)

The Anson's approach was used to calculate the activity of alkaline proteases (Anson, 1938). Casein was used as a substrate. The cuvettes with the reaction mixture were incubated under optimal conditions (37 °C for 10 min), and the enzymatic reaction was halted by adding 1 mL of 5 percent solution TCA. Following centrifugation at 125g to create a supernatant, 5 mL of 6 percent solution Na_2CO_3 and 1 mL of Folin-Ciocalteu phenol reagent (diluted in water) were added separately to the supernatant, after which the tubes were left at room temperature (about 30 min). At OD_{660}, the absorbance of colored solutions was measured and compared to the standard (Tyr). The results are expressed in IU/mL. Under the exam conditions, one unit of enzymatic activity (IU) represents the quantity of enzyme that releases 1μg of Tyr per min.

2.5.2. Assays of invertase activity (EC 3.2.1.26)

To determine the invertase activity, a 1 mL reaction mixture made from 60 mM sucrose (substrate), 20 mM citrate buffer (pH 5.0 and pH 8.0, for acid and alkaline invertase, respectively) and 300 μL of filtrate (enzyme source) was used. This cocktail was putted in a water bath at 37 °C (30 min). The amount of reducing sugar liberated during the reaction was estimated spectrophotometrically according to the procedure described by Somogyi–Nelson (Jakovljević and Vrvić, 2015). Glucose was used as the standard. The amount of invertase that catalyzes the production of 1 μmol of reducing sugar is proportional to one unit of invertase activity.

2.5.3. Assays of alkaline phosphatase activity (EC 3.1.3.1)

The cell-free supernatants were used for estimation of alkaline phosphatase activity (ALP) according to the method of Tabatabi and Bremner, as reported in the paper (Behera et al., 2017). The enzyme activity was detected spectrophotometrically, after the reaction of a colorless substrate, *p*-nitrophenyl phosphate (*p*NPP) with the enzyme, which produces a yellow-colored end product, *p*-nitrophenol (*p*NP). To the previously prepared mixture, containing 1 mL of supernatant and 4 mL of freshly prepared modified universal buffer (pH 11.0), a solution of (0.025 mM) disodium *p*NPP (*p*NPP tetrahydrate) in a volume of 1 mL was added. Microbial growth was prevented by the addition of toluene in drops, thereafter the prepared mixture was incubated in a water bath at 37 °C for 1 h. Upon completion of incubation, the further chemical reaction was stopped by the addition of 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$. Before measuring the absorbance at 420 nm, the mixture was filtered (Whatman No. 42). *P*-nitrophenol was used as a standard.

In assay with BGP as a substrate, the reaction mixture composed of equal volume of 0.05 mol/L glycol buffer (pH 9.0) with

an activator (Mg²⁺), substrate, and supernatant were mixed and incubated (Jakovljević et al., 2014). After 30 min of incubation at 37 °C, the chemical processes were halted by adding 10% TCA to the test tube and placing it on ice for 15 min. The amount of inorganic phosphate released was determined by adding an ammonium molybdate solution to give a blue colored product. A spectrophotometer (Perkin-Elmer Lambda 25 UV/Vis) was used for the colorimetric measurement of the absorbance of solution at 660 nm (Lemire and Turner, 2015). The quantity of enzyme which liberated 1 μg of inorganic phosphate (under the test conditions) represents one unit of enzyme activity (IU).

2.6. Statistical analysis

The measurement results were statistically processed using the SPS software package (SPSS for Windows, v. 13, 2004). The tests used for data analysis were: Mann–Whitney, Kruskal–Wallis, and the test for correlation coefficient. The statistical significance of a correlation was verified at the 0.05 and 0.01 levels of significance.

3. Results

3.1. The effect of tested pollutants on microbial biofilms growth

In order to investigate the effect of testing pollutants (components of auto paints) on the biofilm growth of chosen microbial strains and their community, the AMB media carriers assay was used. A total of five individual microbial strains and their consortia (defined in Table 1) were allowed to form biofilm in different culture media: control growth medium (C) and media supplemented with individual test substances (FH, FM, MF, CN, and NP) at a concentration of 200 μL/mL.

Fig. 1 (a-f) shows the growth of isolated microbial strains in C and polluted media after the 1st, 7th, and 15th days of incubation. All individual microorganisms as well as their consortia displayed the capacity to produce biofilm in control (C) medium. The most intensive growth of all cultures in C medium was observed during the 1st and 7th days of cultivation, which points to the exponential growth phase in this period. Afterwards, the microbial growth continued, but to a lesser extent, and achieved its maximum on the 15th day. This observation indicates an existing stationary growth phase. As Fig. 1.a shows, the microbial consortia revealed the highest growth, followed by *C. cladosporioides,* which had insignificantly slow growth. In contrast to the mentioned culture, *E. coli* had significantly slowed growth, whereas the lowest growth was achieved by *R. mucilaginosa, C. utilis,* and *P. expansum*.

In a medium with the addition of FH, the growth and biofilm formation of single microorganisms and their consortia were supported, but on a lower scale compared to C. In FH medium, the highest growth was displayed by consortia, whereas some lower growth was achieved by *E. coli*. Compared to microbial consortia, significantly lower growth was found in *C. utilis, P. expansum*, and *C. cladosporioides*, whereas *R. mucilaginosa* was distinguished as the strain with the lowest growth. As the results displayed in Fig. 1.b show, reduced microbial growth (biofilm production) was expressed at different growth phases, depending on the microbial strain. It was noted that the growth of consortia, *C. cladosporoides*, *C. utilis*, and *P. expansum* declined, particularly on the 7th day compared to the 1st day. Thereafter, the growth proceeding, and the peak was measured on the 15th day. FH revealed the most potent inhibitory impact on the growth of *C. cladosporioides*, compared to C. During the development growth phase, the most resistant strain was *E. coli*, whereas the microbial community was resistant on the 1st and 15th days. The addition of CP to the medium affected the microbial growth in such a way that the highest growth showed

R. mucilaginosa, followed by consortia and *C. cladosporioides* (Fig. 1.c). Notably, lower growth was recorded in the medium of *E. coli*, whereas the least growth had *P. expansum* and *C. utilis*. On the 15th day, all microbial cultures reached their peak of development. The gradual growth suppression was noted on the 7th day in the medium with *C. cladosporioides*. In FM polluted medium, the highest growth showed *P. expansum*, followed by *R. mucilaginosa*, consortia, and *C. cladosporioides* (Fig. 1.d). Unlike the mentioned strains, *C. utilis* and *E. coli* displayed a lower growth rate. Excluding *C. cladosporioides* which revealed slow growth inhibition, all microbiological cultures achieved their maximum growth on the 15th day of cultivation. In a medium with the addition of MF, *E. coli* had the highest growth, followed by *R. mucilaginosa* and microbial consortia (almost uniform growth), with a maximum noted on the 15th day. Compared to the mentioned strains, *C. utilis* had slightly less growth, whereas notably less growth had *C. cladosporioides* and *P. expansum*, with a maximum achieved on the 7th day (Fig. 1.e). In CN contaminated medium, the highest growth was achieved by *R. mucilaginosa*, followed by microbial consortia and *C. utilis* with a maximum observed on the 15th day. Remarkably lower growth was observed in the medium inoculated with *E. coli* and *P. expansum*. In CN medium, the growth of *C. cladosporioides* was the least, with the maximum achieved on the first day. As Fig. 1.f clearly presents, the growth inhibition was expressed on the 7th day of cultivation, and this process was most extensive in the medium of the microbial consortia.

3.2. Biofilm characteristic (structure)

With the intention of supporting the results of this study and justifying the choice of biofilm-forming microorganisms, we relied on the results of previous research (Radojević et al., 2019), which revealed an excellent capacity of tested single bacteria strains to adhere and form biofilms on carriers after incubation in C medium. Moreover, for comparative purposes, we supplemented the data with fungi biofilms and provided an explanation of the structure of the biofilms. The results are summarized in Table 2. The biofilm formed by *C. cladosporioides* was compact, whereas the distribution of cells was consistent on the biofilm surface. Several cells existed in a filamentous state in the form of very extensive and branched hyphae. Although filamentous growth is not essential for biofilm formation, it is known to participate in strengthening the biofilm architecture and provide adhesion and protection (Kassinger and van Hoek, 2020). Filamentation was also observed in *P. expansum* biofilm, but the hyphae were shorter and unbranched. The biofilm of *C. utilis* existed in the form of cells distributed heterogeneously on the surface and with a low extent of filamentation. The groups of colonies were distributed around the EPS matrix. *R. mucilaginosa* biofilm was constructed from smaller, separated cell colonies and subcolonies which were distributed around the EPS matrix. Biofilm *E. coli* was composed of cells connected to the grid, medium to low density, which envelops the EPS matrix.

In media with the addition of auto paints, a notably lower number of cells adhered to the carrier and, as evidenced by the green fluorescent material, the biofilm was markedly reduced compared to C. The effect of pollutants at concentrations very close to concentration used in this study was reflected in the inhibition (from total to moderate) of biofilm growth. The application of FH at a concentration of 125 μL/mL resulted in the lowest percentage of biofilm inhibition of *E. coli*, *R. mucilaginosa* and *C. utilis*. However, it fully prevented the biofilm development of *P. expansum* and *C. cladosporioides*. The inhibition of the biofilm formation of all test species was noted in the medium with 125 and 250 μL/mL of MF, apart from *R. mucilaginosa*, which formed the biofilm in both cases. Except for *E. coli*, CN added in a concentration of 125 μL/mL inhibited the biofilm development of all microorganisms. In
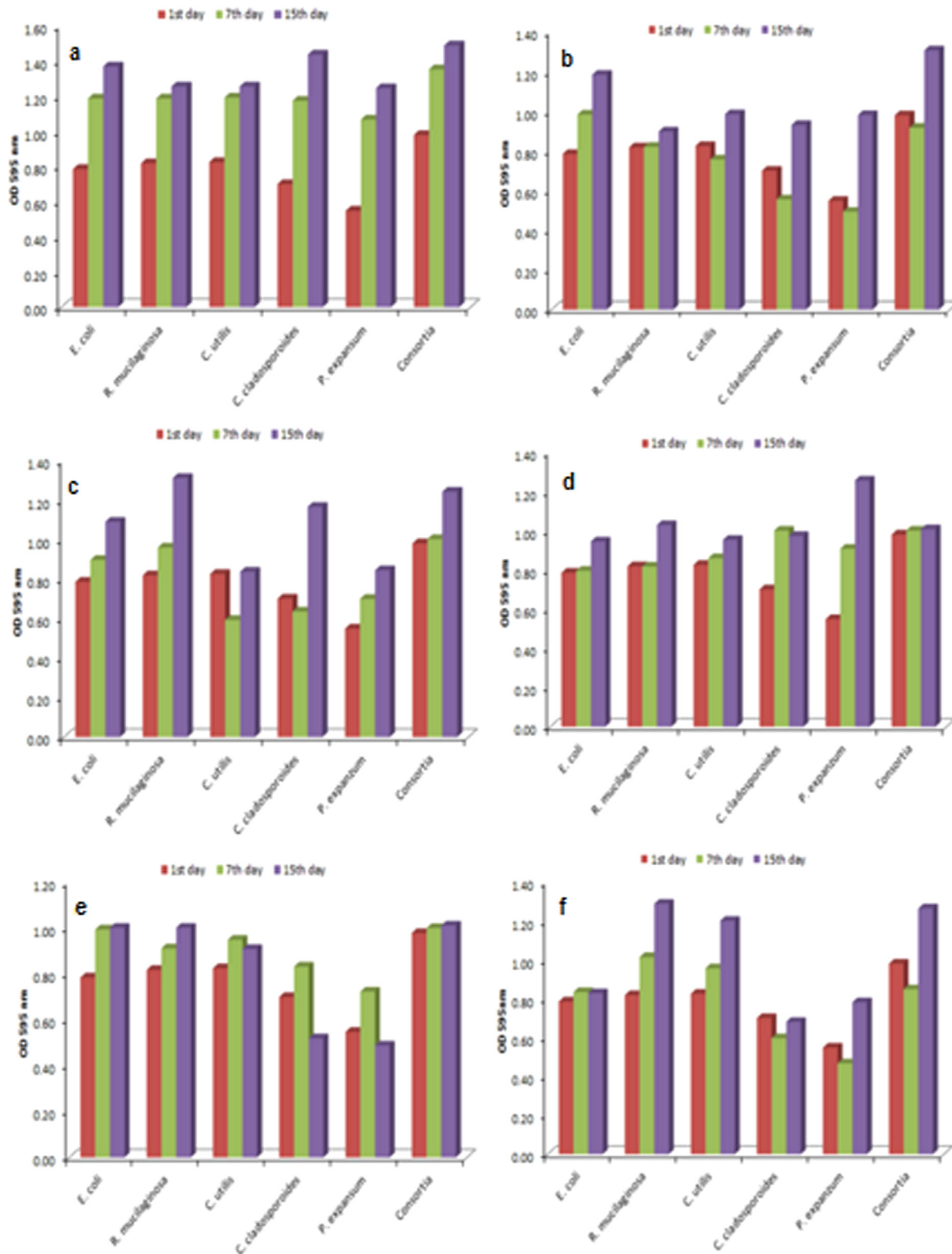
Fig. 1. The microbial growth in control (a) and media with tested pollutants: FH (b), CP (c), FM (d), MF (e), and CN (f) determined on the 1st, 7th and 15th days.

the presence of both concentrations of FM, the biofilm was generated by *C. cladosporioides*, *C. utilis*, *E. coli*, whereas development of *R. mucilaginosa* biofilm was prevented by a dose of 250 µL/mL. Unlike the previously mentioned pollutant, CP impacted the production of biofilms by *E. coli* and *R. mucilaginosa* while preventing

the formation of biofilms by the other microorganisms (Table 2). These results confirmed the different toxicity of tested auto paints depending on its concentration, chemical composition and tested microorganisms. Pollutants such as FH and FM showed the lowest extent of toxicity, whereas CP had the highest toxicity.

Table 2
Microbial biofilms detected at 24 h (bacteria), 72 h (yeast), and 96 h (fungi) of inoculation in control, FH, MF, CN, FM, and CP media.
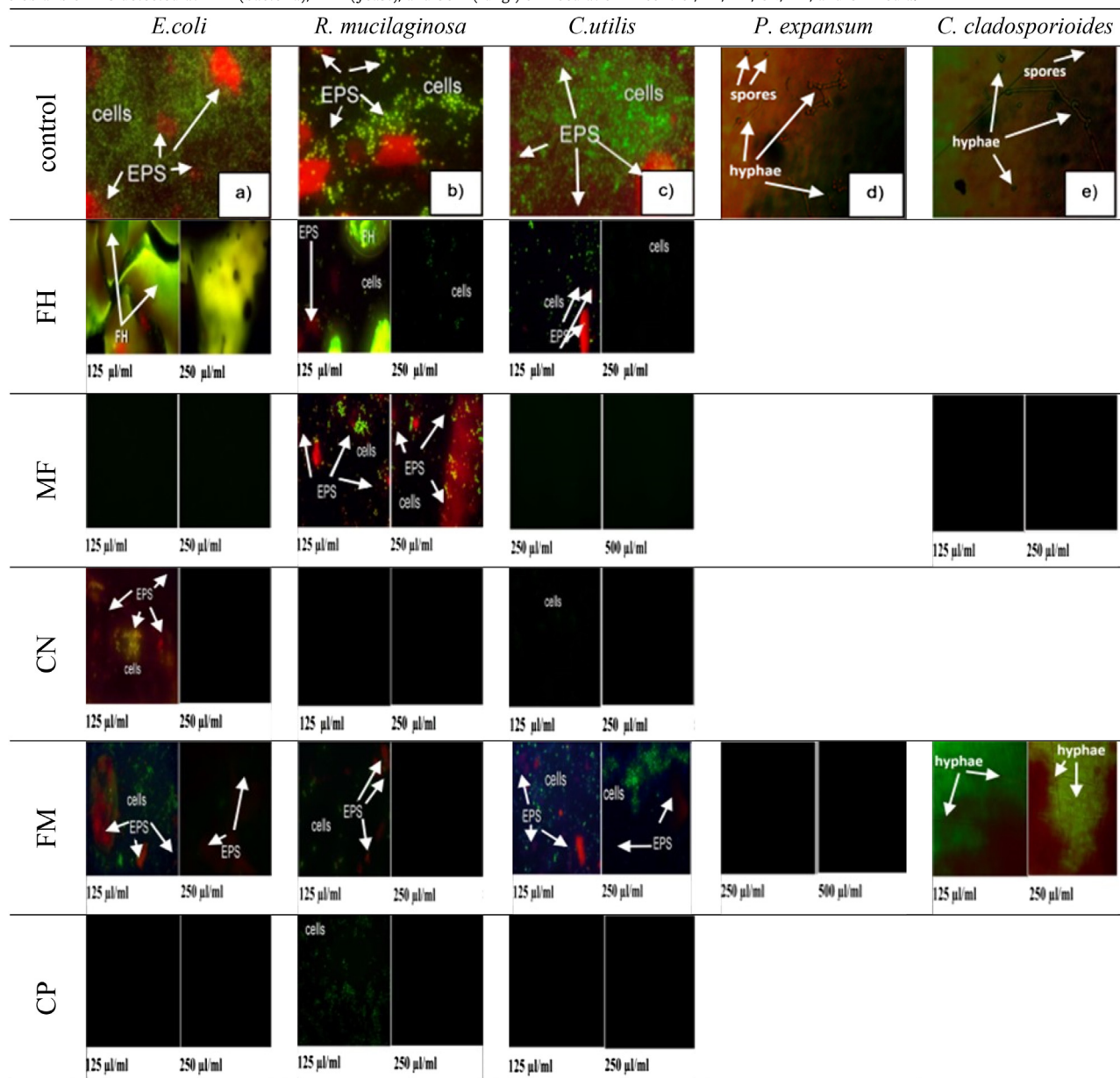
3.3. The content of proteins in EPS (matrix biofilm)

Besides biofilm capacity production, this study evaluated the amount of EPS proteins in the media with tested substances. As Table 3 shows, the amount of proteins measured in the polluted media was in the range of 0.03–0.33 mg/mL, depending on the type of pollutant and microorganism. In the medium with FH, the highest amount of proteins was produced by microbial consortia (0.33 mg/mL), whereas about a 2-fold lesser amount of proteins was produced by *C. utilis* and *E. coli*. In this medium, *C. cladosporioides* did not pro-

Table 3
Amount of EPS proteins (mg/mL) of microbial cultures measured in media with pollutants.

Microorganisms	Proteins (mg/mL)				
	FM	MF	FH	CN	CP
E. coli	0.47 ± 0.05	0.30 ± 0.05	0.13 ± 0.01	0.04 ± 0.01	0.22 ± 0.05
R. mucilaginosa	0.52 ± 0.15	0.56 ± 0.18	0.07 ± 0.01	0.06 ± 0.03	0.26 ± 0.05
C. utilis	0.22 ± 0.01	0.16 ± 0.02	0.16 ± 0.03	0.15 ± 0.05	0.20 ± 0.02
C. cladosporioides	0.09 ± 0.01	0.10 ± 0.01	0	0.01 ± 0.00	0
P. expansum	0.19 ± 0.03	0.17 ± 0.01	0.06 ± 0.01	0.02 ± 0.00	0.013 ± 0.01
Consortia	0.16 ± 0.05	0.37 ± 0.06	0.33 ± 0.08	0.06 ± 0.20	0.18 ± 0.05

Note: The mean ± standard deviation of a representative experiment (n = 3) is used to express the data.

duce proteins, whereas the other tested species produced a very small amount of proteins. In the MF medium, all cultures produced proteins (0.10–0.56 mg/mL). *R. mucilaginosa* produced the most proteins, while the consortia and *E. coli* produced a 2-fold lower amount of proteins. The least protein content was produced by *C. cladosporioides*. The addition of CN to the growth medium affected the production of a very small amount of proteins by all microorganisms (0.01–0.15 mg/mL), except for *C. utilis*, which produced the highest amount of proteins. In medium with FM, the largest amount of proteins was produced by *R. mucilaginosa* (0.52 mg/mL), slightly less *E. coli,* and significantly less *C. utilis*, *P. expansum*, and consortia. At least amount of proteins was produced by *C. cladosporioides* (0.09 mg/mL). The quantity of proteins measured in the medium with CP was in the range of 0.04–0.26 mg/mL. The amount of proteins was maximal in the medium inoculated with *R. mucilaginosa*, followed by *E. coli*, *C. utilis*, microbial consortia and *P. expansum*, whereas *C. cladosporioides* did not produce proteins (Table 3). Statistical analysis found a positive correlation between protein content in the matrix and biomass biofilm of *R. mucilaginosa* grown in medium with CP. Moreover, a positive correlation was found among protein content in the matrix and biofilm of microbial consortia in medium with FH.

3.4. Activity of hydrolytic enzymes (hydrolytic potential)

The activity of hydrolytic enzymes of microbial biofilms produced in media with tested paints was determined on the 15th day of cultivation and is displayed in Table 4.

The proteolytic activity of microbial biofilms was generally poorly expressed in media with tested pollutants, with some exceptions. A very pronounced protease activity was revealed by *C. utilis* in media with MF (0.219 IU/mL) and CN (0.167 IU/mL), followed by *E. coli* in medium with FH. *R. mucilaginosa* and consortia achieved very low proteolytic activity in the FM and FH media, respectively. The weakest proteolytic activity was measured in FH and CN media of *P. expansum* (Table 4).

Alkaline phosphatase activity (BGP substrate) of biofilms was completely inhibited in MF medium, whereas the enzyme activity in FM media was expressed only in the case of *P. expansum* (0.312 IU/mL). Enormously low enzyme activity was detected in the CP medium of *R. mucilaginosa* and consortia, as well as in the FH medium of *E. coli* and *R. mucilaginosa*. The best enzyme activity in the FH medium was achieved by *C. utilis* (0.12 IU/mL). Nevertheless, very pronounced enzyme activity was measured in the medium with CN. In this medium, *R. mucilaginosa* reached the highest enzyme activity (1.404 IU/mL), followed by *C. cladosporoides* (0.108 IU/mL), *P. expansum,* and consortia (0.006 IU/mL). Alkaline phosphatase activity (pNPP substrate) of microbial biofilms was expressed in MF and FM media, whereas it was totally inhibited in other types of polluted media. Among the tested media, the highest enzyme activity (0.128–0.421 IU/mL) was measured in MF media. The enzyme activities of biofilms were in the following order: *E. coli*, *C. utilis* (2-fold lower activity), *R. mucilaginosa*, and consortia (with about a 3-fold lower activity). Other microbial biofilms did not show any enzymatic activity in this medium. In FM

Table 4
Hydrolytic enzyme activity (IU/mL) of biofilm matrix measured after 15 days of inoculation in media with pollutants.

Microbial strain/ pollutants	Hydrolytic enzyme activity (IU/mL)				
	Alkaline protease activity	Acid invertase activity	Alkaline invertase activity	ALP activity (BGP substrate)	ALP activity (pNPP substrate)
E. coli					
FH	0.109 ± 0.240	0.004 ± 0.001	0	0.012 ± 0.003	0
MF	0	0	0.020 ± 0.005	0	0.421 ± 0.050
CN	0.005 ± 0.001	0.025 ± 0.002	0	0	0
FM	0.052 ± 0.015	0.043 ± 0.010	0.002 ± 0.001	0	0.037 ± 0.015
CP	0.002 ± 0.001	0	0.007 ± 0.003	0	0
R. mucilaginosa					
FH	0.012 ± 0.003	0.005 ± 0.002	0	0.018 ± 0.009	0
MF	0	0	0.008 ± 0.002	0	0.146 ± 0.072
CN	0	0.025 ± 0.005	0	1.404 ± 0.158	0
FM	0.035 ± 0.010	0.012 ± 0.008	0	0	0.146 ± 0.056
CP	0.003 ± 0.001	0	0	0.012 ± 0.003	0
C. utilis					
FH	0.002 ± 0.001	0	0	0.120 ± 0.065	0
MF	0.219 ± 0.076	0	0.011 ± 0.005	0	0.220 ± 0.048
CN	0.167 ± 0.042	0	0	0.006 ± 0.002	0
FM	0	0	0.005 ± 0.001	0	0.073 ± 0.032
CP	0.001 ± 0.000	0.007 ± 0.001	0.008 ± 0.001	0	0
C. cladosporioides					
FH	0	0	0	0	0
MF	0	0.022 ± 0.010	0	0	0
CN	0	0	0	0.108 ± 0.050	0
FM	0	0	0	0	0
CP	0	0	0	0	0
P. expansum					
FH	0.003 ± 0.001	0	0	0	0
MF	0	0.007 ± 0.002	0	0	0
CN	0.001 ± 0.000	0	0	0.006 ± 0.002	0
FM	0	0.020 ± 0.005	0	0.312 ± 0.026	0
CP	0	0	0.006 ± 0.001	0	0
Consortia					
FH	0.033 ± 0.018	0.011 ± 0.002	0	0	0
MF	0.002 ± 0.001	0	0.007 ± 0.002	0	0.128 ± 0.032
CN	0.004 ± 0.002	0.010 ± 0.001	0	0.006 ± 0.003	0
FM	0.004 ± 0.001	0	0	0	0
CP	0.009 ± 0.005	0.043 ± 0.020	0.005 ± 0.001	0.004 ± 0.001	0

Note: The mean ± standard deviation of a representative experiment (n = 3) is used to express the data.

medium, *R. mucilaginosa* achieved the highest enzyme activity (0.146 IU/mL), followed by *C. utilis* (about a 2-fold lesser activity) and *E. coli* (0.037 IU/mL). In this medium, the enzyme activity of the rest of the microbial biofilms was not detected (Table 4). The results of the correlation test between the variables showed a positive relationship between ALP activity and the biofilm biomass of *R. mucilaginosa* in CP and CN media. Likewise, the test pointed to a positive correlation between ALP activity and biofilm biomass of *P. expansum* in the medium with FM.

The acid invertase activity of microbial biofilms was noted in all media with tested substances, but it was weak. The highest enzyme activity (0.043 IU/mL) was measured in FM and CP media of *E. coli* and mixed culture, respectively. *E. coli* and *R. mucilaginosa* showed the best enzyme activity (0.025 IU/mL) in the medium with CN, whereas *C. cladosporioides* achieved the highest activity (0.022 IU/mL) in the MF medium. On the other hand, *P. expansum* was enzymatically active only in medium with FM and MF, whereas *C. utilis* was active only in medium with CP. The biofilm of mixed culture expressed the enzyme activity in CP, CN, and FH media. *R. mucilaginosa* expressed enzyme activity in CN, FM, and FH media (Table 4).

The alkaline invertase activity of biofilms was not detected in FH and CN media. In contrast, the highest enzyme activity was measured in MF, followed by in CP (about 2-fold lower) and FM (the lowest activity). In a medium with MF, *E. coli* (0.02 IU/mL) achieved the highest activity, followed by *C. utilis* (2-fold lower), *R. mucilaginosa* (about 3-fold lower) and finally, consortia (0.007 IU/mL). In CP supplemented medium, *C. utilis* had the highest enzyme activity (0.008 IU/mL), followed by *E. coli* (0.007 IU/mL), *P. expansum* (0.006 IU/mL) and consortia (0.005 IU/mL). In a medium polluted with FM, weak enzyme activity was achieved by *C. utilis* (0.005 IU/mL) and *E. coli* (0.002 IU/mL), whereas other microbial biofilms were enzymatically inactive (Table 4).

4. Discussion

Numerous studies have investigated the biofilm producing efficiency of single and mixed microbial cultures in different media. Generally, one of the main conclusions of these studies is that because mixed microbial cultures present functional and metabolic consortia of different cell types, their growth is enhanced compared to single cultures. The consumption of nutrients from the growth medium and the production of different metabolic products by each individual strain in biofilm, as a result of the diverse metabolic pathways, can enhance the diffusion of molecules and biofilm growth (Berlanga and Guerrero, 2016). Moreover, an investigation performed on the pure and mixed bacterial cultures of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* found that mixed culture of both species exhibited the existence of thicker biofilm compared to pure cultures. The results of the current study are in accordance with the above cited scientific results, since the microbial growth in the C medium was in the following order: microbial consortia > *C. cladosporioides* > *E. coli* > *R. mucilaginosa* > *C. utilis* > *P. expansum*. Evaluations of single microbial cultures have shown that their growth and biofilm formation differ considerably depending on the microbial strain and growth phase. Vasudevan's study (2014) drew attention to the role of genetic diversity, which varies from one organism to another, as well as environmental conditions, in the creation and nature of biofilm. The results obtained in the presence of the tested auto paints at a concentration of 200 μL/mL showed their inhibitory or stimulatory effects on the growth of single strains and consortia, depending on the microbial strain, growth phase, and chemical structure of the tested pollutants. *E. coli* was the most resistant to FH but the most sensitive to CN. The best resistant strain to CN was *C. utilis*

whereas it was the most sensitive to CP. *C. cladosporioides* was the most resistant to MF but the most sensitive to CP. The stimulation of *R. mucilaginosa* growth was observed in CP and CN media, compared to C. *R. mucilaginosa* showed some degree of resistance to FM and MF, whereas it was the most sensitive to FH. The stimulation of *P. expansum* growth was noted in FM medium (compared to C), whereas it was the most sensitive to MF. Microbial consortia had inhibited growth in media supplemented with all of the tested pollutants. The interaction between individual microbial strains in the presence of tested pollutants decreased the growth of consortia, indicating less consortium efficiency in pollutant biodegradation relative to individual strains. The strongest inhibitory effects on microbial consortia growth had FM and MF, whereas the least inhibitory effect had FH, compared to C. Differences in response to the assessed pollutants between single microbial strains are influenced by their morphological, physiological, and genetic characteristics. Certain processes occur inside biofilms that permit or prevent bacteria from developing tolerance mechanisms to various contaminants (Harrison et al., 2007). The study of microbial biofilm generating strains with high resistance to contaminants and environmental stress is critical for using biofilms in environmental bioremediation. An overview of the literature (Harrison et al., 2006; Mitra and Mukhopadhyay, 2016) provides strong evidence about the remediation of a broad range of contaminants (heavy metals, petroleum, explosives, etc.) by biofilms of individual microbial strains or consortia. Because there is no literature data on the degradation of car paints by microorganisms, this research will focus on the outcomes of surfactant degradation. The study by Chandran and Das (2011) showed a high biodegradation capacity (97%) of diesel oil polluted water by the yeast biofilm *Candida tropicalis* on gravel particles after 10 days. Abena et al. (2019) investigated the efficiency of five single microbial strains and their consortia to degrade crude oil during 10 days of incubation and established that the consortia had a significantly higher level of degradation (94%) compared to single strains (50–83.5%). Summarizing the obtained results, it is clear that the tested microbial biofilms can be used selectively for bioremediation (decontamination) of polluted wastewater in the automotive industry. The finding that all single microbial strains formed biofilms in C medium points to the significance of carbon sources and nutrient supplements for biofilm growth. The differences in biofilm structure among tested microbial strains in control growth medium can be explained by their different biochemical processes. This discovery is consistent with Shrout et al.'s (2006) claim that types of carbon sources have a significant effect on biofilm formation by influencing quorum sensing signals and regulating swarming motility inside a number of species. Andersson et al. (2008) speculate that the presence or lack of certain carbon sources may also affect the biosynthesis of additional signal chemicals, cell-surface appendages, and EPS. The addition of tested pollutants in the medium had an inhibitory effect on the microbial biofilm formation and affected the change in the structure of biofilm. Since the tested pollutants have a different chemical structure, they express a specific effect on the contacts (quorum sensing) between the microbial cells in the biofilm, their metabolic activity, the creation and arrangement of EPS, etc. These results provide the basis for future research on the functional activity of the investigated microbial strains and the quorum sensing mechanism of multiple signal molecules' interaction in waste water treatment systems.

In addition, the current study also investigated the protein content and hydrolytic activity of EPS. An examination of the literature reveals that extracellular polymeric substances (EPS) are the fundamental functional and structural constituents of microbial biofilms that are crucial for mechanical stability (Flemming and Wingender, 2010). Carbohydrates and proteins greatly influence the changes in the physico-chemical characteristics of the cellular

surface (Sheng et al., 2010). It is a well-known fact that the microbial biofilm matrix performs the function of the external digestion system. Moreover, their ability to supply extracellular enzymes to nearby biofilm cells and digest dissolved, colloidal or solid biopolymers is well documented in the literature. Numerous studies of the microbial extracellular proteome have revealed the presence of various enzyme classes, including proteases, phosphatases, esterases, glucosidases, and lipases (Tasić et al., 2016). Periasamy et al. (2007) verified the existence a strong positive correlation between biofilm formation and EPS generation by *P. ruthenica* SBT033. Zhang and colleagues (2019) discovered a large amount of cytoplasmic proteins in the EPS fraction, which they attributed to external membrane vesicles or cell lysis during biofilm formation. The papers of Sheng et al. (2010) and Shi et al. (2017) shed light on the role of EPS proteins in regulating the stability of the internal structure of biofilm and maintaining biomass. In line with the mentioned observations, the current study confirmed extensive variation in the production of EPS proteins by the tested microbial strains in the presence of pollutants, and more specifically, the significant role of EPS proteins in biofilm formatting of *R. mucilaginosa* and microbial consortia in CP and FH media, respectively. Therefore, further investigation could be directed toward examination of the relationship between these pollutants and secretion and the identification of specific proteins which regulate different metabolic reactions in microbial biofilm.

Studies (Mohlenhoff et al., 2001; Lebre et al., 2017) showed that pollutants, even when vulnerable to microbial colonization, present a restrictive substrate for microorganisms. Especially strains with distinct special metabolic (various hydrolytic enzymes) and physiological (production of spores, tolerance to food scarcity or very dry circumstances, etc.) capacities may grow or survive. According to this statement, the current study investigated the enzymatic activity of the tested microorganisms to find out whether, indeed, these microorganisms have the metabolic and degradation potential to be responsible for the biological degradation of the investigated pollutants. Among microbial enzymes that play a significant role in the bioremediation of a wide range of pollutants, proteases stand out. In the literature, bacteria and filamentous fungi are recognized as very good protease producers, but only a few of them have commercial application (Malik et al., 2017). In the study by Rodarte et al. (2011), the authors concluded that bacteria and fermentous fungi achieved significantly better activity than yeasts on the qualitative casein hydrolysis test. In this regard, *P. expansum* produced protease activity of 2.55–5.12 U, depending on pH value, whereas *P. mirabilis* had no expressed protease activity. Summarizing the scientific results, the effect of pollutants on protease activity can be twofold: inhibitory or stimulatory. Baweja et al. (2016) found that the enzyme produced by *Bacillus pumilus* MP 27 retained 100% stability in treatment with Tide (commercial detergent), whereas its compatibility in treatments with surfactant types Triton X-100, Tween-20, and SDS was in the range of 87.73–30%. The data published by Saggu and collaborators (2019) showed that various chemicals such as surfactants, alcohols, phenols, and heavy metals (mercury, silver, copper, zinc, and nickel) have been generally used for the removal of biofilms. This study confirmed very pronounced proteolytic activity of *C. utilis* in media supplemented with MF and CN as result of its resistance to each of these particular pollutants. Since the resistance of microbial proteases to the presence of various chemicals suggests their potential use in bioremediation, *C. utilis* could be exploited in waste management.

The main physiological role of ALP is the dephosphorylation of compounds in order to increase the rate of diffusion of the molecules in the cells and inhibit them from diffusing out. Based on literature data, the activity of ALP and its metabolic roles, such as growth, cell differentiation, etc., have been confirmed in many bac-

teria and fungi species. For example, this enzyme is known to be active in the periplasmatic space of *E. coli*. The production of ALP by *R. mucilaginosa* was described in the article by Oh et al. (2009). Chen and co-workers (2019) observed that the remarkable increase in ALP activity in *R. mucilaginosa* may improve growth and immunity status. Barad and colleagues (2016) discovered some homologues of the alkaline phosphatase (Pal) complex in the genom of *P. expansum* at pH levels ranging from 4.5 to 7. The scientific literature has confirmed that approximately 50% of microorganisms produce phosphatase activity optimally (0.0016–0.0161 IU/mL) between 25 and 37 °C. One study by Obidi et al. (2018) found that several microbial strains, isolated from discolored painted walls, produced ALP under different cultural conditions, which were used as biomarkers for their metabolic activity. The current study explored the ALP activity of the cultures toward BGP and PNPP substrates and displayed much better activity in the assay with BGP as a substrate. The results obtained in this assay displayed, for the first time, excellent ALP activity of *R. mucilaginosa* in CN medium and moderate activity of *P. expansum* and C. utilis in FM and FH media, respectively. However, *E. coli* grown in MF medium expressed the best ALP activity toward pNPP. In the same assay, *R. mucilaginosa* grown in FM medium achieved the highest enzyme activity. The results presented here could be useful for further research into the above-mentioned strain's ability to reduce phosphates.

According to the literature, various chemicals can alter the activity of bacterial invertase and act as inhibitors, modulators, or activators. Kaur and Sharma (2005) revealed that one type of anionic detergent (SDS) increased the invertase activity of *Streptomyces* ALKC 8, and pointed out that the release of extracellular invertase from this strain can be prevented by the detergent. In contrast, Lincoln and More (2017) reported the inhibitory effects of Hg^{2+}, Cu^{2+}, pCMB, β-ME, and DTT on enzyme activity as well as the roles of Fe^{2+}, Mg^{2+}, and Mn^{2+} as enzyme modulators on enzyme activity. Barbosa and co-workers (2018) found high structural stability of the enzyme produced by *R. mucilaginosa*. The data in the scientific papers clearly showed that the optimal conditions (pH and temperature) for invertase activity were 4.0 and 70 °C for *R. mucilaginosa* and 4.5 and 50 °C for *S. cerevisiae*. The current study examined the activity of both acid and alkaline invertase of microbial cultures in growth media supplemented with the pollutants. The highest acid invertase activity showed *E. coli*, and mixed cultures cultivated in FM and CP medium, respectively. According to the current study, selected microbial strains and their consortia extracellularly released hydrolytic enzymes, which have the ability to cleave different proteins, carbohydrates, and phosphate substrates. This finding demonstrates the importance of secretion of hydrolytic enzymes in biofilm formation and maturation.

5. Conclusions

This study provided new results about the effects of the tested pollutants (auto paints) on the biofilm growth, matrix protein production, and hydrolytic enzyme activity of single microbial cultures and their consortia in laboratory conditions that mimic bioreactor conditions. The pollutants had a selective effect on the extracellular enzyme activity of biofilms. The proteolytic activity of consortia was measured in all media regardless of pollutant type. The protease activity of consortia was best in FH medium but very inhibited in MF medium. *C. utilis* achieved very pronounced proteolytic activity in the media with the addition of MF and CN. In medium supplemented with CN, *R. mucilaginosa* reached the highest ALP activity. *E. coli* achieved the highest acid invertase activity in the medium with FM, whereas consortia had the best enzyme activity in the medium with CP. The best alkaline

invertase-producing strain in the MF medium was *E. coli*. Moreover, the current study clearly showed that biofilm formation of *R. mucilaginosa* in CP and CN media as well as *P. expansum* in the FM medium is positively associated with the secretion of hydrolytic enzymes such as ALP, which indicates their role in the hydrolyze of these specific pollutants. The provided findings significantly support the concept that the aforementioned microbial strains can be employed selectively for bioremediation of automotive wastewater. Considering that the tested strains had different hydrolytic potential in the presence of auto paints, it is necessary to investigate their relationship to the exhibited degradation. A future study could clarify the manner and extent to which these pollutants are degraded by enzymes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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