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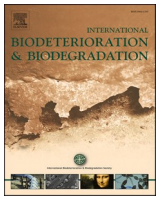

Oxidative deterioration of polypropylene by redox mediators and yeast expressing a fungal recombinant laccase

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ABSTRACT

Biodegradation of polyolefins is a multistep process that might integrate physical, chemical, enzymatic and/or microbial actions. In this work, we report the use of the laccase redox mediators ABTS and DMP (2,2' azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and 2,6-dimethoxyphenol, respectively), recycled by laccase activity in aerobic conditions, to induce oxidation of polypropylene film (PP): the laccase was produced in the reaction medium by a recombinant *Kluyveromyces lactis* yeast strain, expressing a fungal heterologous laccase gene. As a result, we evidenced the formation of oxidize groups on PP surface which increased its water wettablity. We also showed a clear modification of the polymer surface with the appearance of humped and exfoliated regions which favored the break of PP film.

1. Introduction

About four hundred million tons of plastics have been worldwide produced in 2022 (http://plasticseurope.org). Because of the nature of plastics, recalcitrant to naturally occurring biodegradation, plastic wastes have an enormous impact on the environment. Estimated half-lives of plastic polymers in the environment range from years to thousands years (Chamas et al., 2020). The first report of environmental pollution by plastic material dates back to 1972 (Carpenter and Smith, 1972). Most common synthetic polymers, produced from fossil feedstocks for industrial and civil uses, are polyethylene terephthalate (PET), polyethylene (high and low density, HDPE and LDPE, respectively), polypropylene (PP), polyurethane (PU), polystyrene (PS) and polyvinyl chloride (PVC). Plastic wastes are currently eliminated by landfilling, combustion or physical and chemical recycling. However, the formers generate environmental pollution and the latter new plastic material or different chemicals to dispose of. One challenging alternative is the biological degradation of plastics by means of enzymes and/or microorganisms, a procedure that allows to transform plastics in environmental friendly carbon molecules or biomass, to be directed into the biological carbon cycles or, possibly, to new added-value products. This issue is rising increasing interest in the scientific community, as demonstrated by proliferation of reports (Wei and Zimmermann, 2017; Danso et al., 2019; Mohanan et al., 2020; Amobonye et al., 2021; Singh Jadaun et al., 2022; Zhang et al., 2022).

Biodegradation of polyolefins has been preferentially oriented to polyethylene (PE) while a minor interest was addressed towards PP degradation (Mohanan et al., 2020; Singh Jadaun et al., 2022). Despite the fact that data are not always reliable and have to be interpreted with caution (Gu, 2021; Gu et al., 2024), microorganisms and microbial communities are assumed to be potentially able to degrade PE. Many examples have been reported from different taxa. Among bacteria, species belonging to both Gram-positive and Gram-negative groups have been found to be effective in PE degradation (Restrepo-Flórez et al., 2014; Sen and Raut, 2015). Fungi of different genera and algae were also shown to be capable of such process (Hasan et al., 2007; Zahra et al.,

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2010; Kumar et al., 2017). Degradation of plastics is based on the activity of extracellular enzymes (Gan and Zhang, 2019) and is facilitated by polymer pretreatment, presence of chemical oxidants and/or enzyme cofactors/mediators or by biofilm formation (Restrepo-Flórez et al., 2014; Zheng et al., 2005). Enzyme involved in degradation are especially those involved in lignin degradation by bacteria and fungi, in particular laccases (EC 1.10.3.2), manganese peroxidases (EC 1.11.1.13) and lignin peroxidases (EC 1.11.1.14) (Wei and Zimmermann, 2017; Restrepo-Flórez et al., 2014; Santo et al., 2013; Krueger et al., 2015; Gómez-Méndez et al., 2018; Zhang et al., 2020). Bacterial alkane hydroxylases (EC 1.14.15.3) is another class of enzymes capable of PE degradation (Yoon et al., 2012; Jeon and Kim, 2015). Partially purified enzymes or culture supernatants are routinely used although microbial cultures or communities also have potential applications.

Laccases (benzenediol:oxygen oxidoreductase, *p*-diphenol oxidase) are multi-copper redox enzymes present in plants, fungi and bacteria, with a very large range of substrates. Some substrates, also called mediators, have the ability to oxidize polymers which cannot be directly oxidized by the enzyme, thus enlarging further the actual range of action of laccases. These enzymes have several potential or actual applications in textile, paper, pharmaceutical and food industries as well as in bioremediation activities (Piscitelli et al., 2010).

In order to increase and facilitate enzyme production, recombinant protein production systems have been developed, as alternatives to the native enzyme sources. Recombinant protein expression in model systems also allowed a remarkable improvement in enzyme studies and optimization (Demain and Vaishnav, 2009). Laccase recombinant expression has been developed in bacteria, in plants, in filamentous fungi and in yeasts. In particular, tens of laccase expression systems have been set up in yeasts, especially fungal laccases expressed in *Saccharomyces cerevisiae* and *Pichia pastoris* (Piscitelli et al., 2010; Antošová and Sychrová, 2016), the latter being a better producer (Otterbein et al., 2000; Colao et al., 2006). Laccases have also been expressed in *Kluyveromyces lactis* (Piscitelli et al., 2010; Camattari et al., 2007; Faraco et al., 2008; Ranieri et al., 2009), that has the advantage of high biomass yield because of respiratory metabolism (Kiers et al., 1998), high protein secretion capacity accompanied by limited glycosylation extent, availability of strong and regulated expression systems (Spohner et al., 2016). In our laboratory, the highly expressed and regulated *KlPDC1* promoter (Destruelle et al., 1999; Micolonghi et al., 2011) of the Pyruvate decarboxylase (PDC) gene of *K. lactis* has been used to drive the expression of the *LCC1* laccase gene (cDNA) from the fungus *Trametes trogii* (Colao et al., 2003). The cassette *KlPDC1* promoter-*LCC1* gene has been employed both to assemble centromeric-replicative expression vectors (Camattari et al., 2007) and to generate integrative yeast strains harboring the expression cassette in the chromosome (Ranieri et al., 2009). Maximal enzyme productions were found in the integrative strain (s) rather than in the transformant clones containing the centromeric vector.

In the present study, we report the selection of an optimal yeast integrative strain harboring a laccase expression cassette to obtain oxidative deterioration of PP in the presence of different redox mediators and aerobic conditions. The effects of the incubation in yeast cultures on the properties of UV pre-treated PP films were investigated by ATR-FTIR spectroscopy, water contact angle and SEM analyses as well as stress-strain mechanical tests.

2. Materials and methods

2.1. Yeast strains and media

The used *K. lactis* strains have been described in Ranieri et al. (2009). Synthetic-Dextrose (SD) medium was composed of 0.67% yeast nitrogen base (Becton Dickinson, Sparks, Maryland USA), 2% glucose and auxotrophic requirements (adenine, arginine, leucine, lysine, methionine, tryptophane and uracil, as needed): SDA medium was SD medium containing also 2% case amino acids (Acid-hydrolized casein, Becton Dickinson). Tween20 was added at 0.1% final concentration to avoid adhesion of biological matter to PP samples. 2% agar was added for plate media. Media contained 0.2 mM 2,2' azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 0.2 mM 2,6-dimethoxyphenol (DMP), as redox mediators of the recombinant laccase produced by the yeast strains, and 0.1 mM $CuSO_4$.

Yeast growth has been measured as optical density (OD) at 600 nm. Opportune culture dilutions have been done to reduce OD within the linear range.

2.2. Laccase activity

Yeast culture samples were centrifuged at 8000×*g* for 10 min to separate cell biomass. Supernatants were pre-equilibrated with 0.1 mM $CuSO_4$ and used for photometric assay of enzyme activity which was performed at room temperature with 2 mM ABTS as a substrate in 0.1 M citric acid, 0.2 M K_2HPO_4 buffer pH 3.4 (Colao et al., 2006) or with 2 mM DMP in 0.1 M Na acetate buffer pH 4.5 (Slomczynski et al., 1995), in a final volume of 1 ml. ABTS oxidation was determined at 420 nm ($\varepsilon_{mM} = 36.0\ mM^{-1} \times cm^{-1}$) and DMP oxidation was determined at 477 nm ($\varepsilon_{mM} = 14.6\ mM^{-1} \times cm^{-1}$). The enzymatic activity was expressed as $mkat \times ml^{-1}$.

2.3. UV treatment

Strips of opportune size (120 × 7 mm^2) were cut from pristine commercial PP transparent sheets (thickness 0.04 mm and crystallinity $X_c = 62\%$, as evaluated by DSC calorimetric analysis reported in Fig. S1), washed with 95% ethanol, thoroughly rinsed with sterile distilled water and conserved sterile. Since polymer degradation is greatly facilitated by pretreatments that introduce oxidized residues on sample surface (Mohanan et al., 2020; Amobonye et al., 2021; Arkatkar et al., 2010; Jeyakumar et al., 2013; Jain et al., 2018), PP strips were subjected to photo-oxidation by a 16 UV lamps (350 nm, 336 W) photoreactor (Ryonetcon) for 24 h before the degradation experiments.

2.4. Yeast cultivation and experiment setup

Yeast strains were cultivated in SDA medium or in SDA medium plus 0.1% Tween20 at 28 °C with shaking (175 rpm). Cultures destinated to PP degradation were prepared with SDA plus 1% Tween20, 0.05 mM $CuSO_4$ and ABTS or DMP, and grown for 2 days. UV pretreated PP strips were placed in bacteriology glass tubes with metal stopper, allowing fully aerated conditions, and completely submerged in 5 ml of SDA (containing also 0.1% Tween20,0.1 mM $CuSO_4$ and 0.2 mM ABTS or 0.2 mM DMP as laccase mediators) aliquots of yeast culture. Tween20 was added to prevented adhesion of biological material to the strip surface. The tubes were then incubated at 28 °C with shaking (175 rpm) in opportune reclinate position to guarantee aeration. After defined incubation times (about 10 days), culture samples were withdrawn for laccase activity determination and to monitor cell growth by measuring optical density at 600 nm. Cells were refreshed after 10 days incubation time by withdrawing the old culture and by adding 5 ml of fresh SDA (+Tween20 + $CuSO_4$ + mediator) medium culture in the bacteriology test tube. PP strips were withdrawn after 1, 2, 3 and 4 months treatment with laccase/mediator corresponding to three culture refreshes for each month. In order to completely remove organic and/or biological matter from the PP surface before subsequent analysis, the sample strips were washed thoroughly with 0.1 M Tween20, with 70% ethanol and then with sterile water. Samples were then subjected to two sonication cycles in 2% SDS. After sonication, strips were finally washed with distilled water and 50% ethanol.

2.5. ATR-FTIR spectroscopy

FTIR spectroscopy in attenuated total reflection (ATR) mode was used to characterize pristine and UV-exposed PP strips (PP and PP_{ox}, respectively), along with those contacted with MW98-8C (control) and DR98 yeast strains for various time periods. All samples were thoroughly washed and dried as previously described. A FTIR Nicolet 6700 spectrophotometer equipped with a Golden Gate Single Reflection device (Specac) endowed with a diamond internal reflection element was employed. Spectra were recorded at 4 cm^{-1} resolution in 4000-600 cm^{-1} spectral range by co-adding 200 scans.

2.6. Contact angle

Static contact angle measurements were performed by dropping 5 μL of distilled water on the polymer sample surface. Photographs were acquired by a home-made system and the static contact angle was calculated by using the ImageJ contact angle plugin. Contact angle values are reported as the mean value obtained from at least triplicate measurements (n = 3, ±SD).

2.7. Morphology characterization

Morphology of the PP film surfaces was investigated by Scanning Electron Microscopy (SEM). The instrument adopted was a Field-Emission SEM (model AURIGA, manufacturer Zeiss). Prior to the measurements, PP films were dried under vacuum at room temperature and samples of suitable size (approx. 1 cm^2) were cut from the films. The samples were mounted on SEM-suitable sample holders using conductive copper tape. In order to avoid local charging phenomena of PP, mounted samples were covered with a chromium layer using a sputter coater (model Q150T, manufacturer Quorum Technologies) equipped with a film thickness monitor. The thickness of the applied chromium layer was 10 nm. SEM imaging was performed using a beam energy of 1.5 KeV in order to minimize charging phenomena. Images were acquired using an Everhart-Thornley detector.

2.8. Mechanical characterization

The mechanical properties of pristine, UV treated, MW98-8C (4th month) and DR98 (4th month) treated samples were studied by tensile stress-strain tests by using a universal testing machine Instron 4502. The measurements were performed on 40 × 0.7 × 0.04 mm^3 strips at room temperature, using a 2 kN load cell at a crosshead speed of 5 mm min^{-1}. All the experiments were carried out in triplicate (n = 3, ±SD).

Stress-strain curves were reported as the apparent stress *F/A* (Pa), where *F* is the tensile force and *A* is the initial cross-sectional area of each test specimen, versus the strain $(L\text{-}L_0)/L_0$, where L_0 and *L* are the initial and the deformed sample length, respectively. The Young modulus was calculated from the slope of the initial linear region of the stress–strain curves.

3. Results

3.1. Experiment design

The aim of this work was to oxidize olefins, in particular PP, to generate chemical and structural changes to the substrate surface, thus allowing or facilitating further degradative steps. The scheme of the proposed oxidative attack is illustrated in Fig. 1. Oxidation of PP was pursued with redox mediators of a laccase enzyme produced by a recombinant strain of the yeast *K. lactis* and mediator regeneration was ensured by incubation of the reaction medium in aerated conditions.

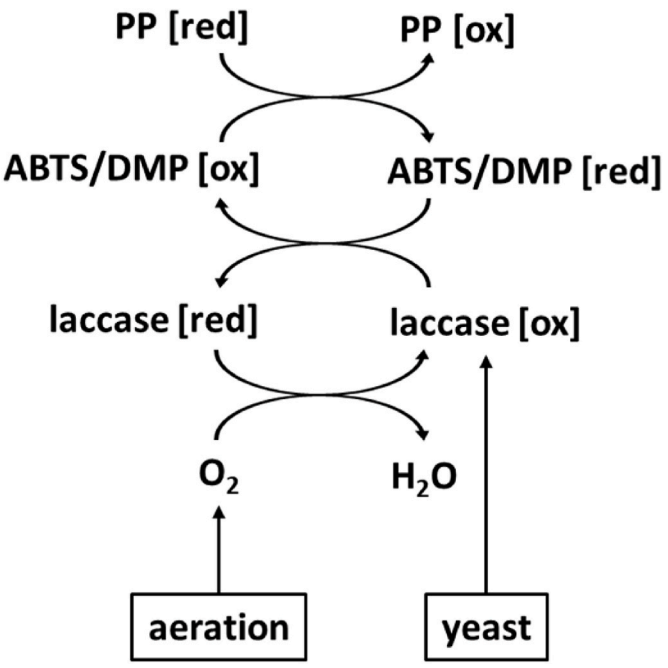

Fig. 1. Oxidation of PP. In figure are reported the reactions involved in the proposed PP oxidation system based on the laccase mediators. The final electron acceptor of the PP oxidation is the molecular oxygen supplied by aeration. Active laccase enzyme is supplied by refreshing of the recombinant yeast culture expressing the fungal *Lcc1* gene.

3.2. Strain and mediator selection

In a previous work (Ranieri et al., 2009) we constructed various yeast strains producing laccase by means of the yeast integrative vector pDRLCi containing cDNA of the *LCC1* laccase gene from the fungus *T. trogii* (Colao et al., 2003). Vector pDRLCi has been transformed in *K. lactis* strains MW98-8C, MW270-7B, JA6 and MW179-1D, and the corresponding integrative transformant strains DR98, DR270, DRJA6 and DR179 were obtained (Ranieri et al., 2009). Transcription of the laccase gene was driven by the regulated and highly inducible promoter of the *KlPDC1* gene from *K. lactis* (Camattari et al., 2007; Destruelle et al., 1999; Micolonghi et al., 2011; Bianchi et al., 1996; Ottaviano et al., 2014). Chromosomal integration of the plasmid ensured high stability along time of the recombinant sequences. Strains were maintained at 4 °C on SDA plates and/or in SDA liquid medium.

In order to select the optimal strain for our purposes, the four recombinant strains were inoculated in SDA medium and cultivated for about 250 h at 28 °C with shaking in aerated microbiology tubes. Culture samples were withdrawn at different time points and both cell density and laccase activity in the supernatants were measured with ABTS as substrate. Results, reported in Figs. S2A and S2B, showed that strain DR98 reached high cell density and the higher laccase activity: this strain was chosen for further experiments.

Laccases can use a very large variety of mediators to oxidize polymers. We took into consideration two mediators: ABTS and DMP. Strain DR98 was cultivated in SDA medium for 250 h and its activity was measured at different time points by using the two mediators. Results (Fig. S3) indicated that both mediators were used (oxidized) efficiently by the enzyme produced by the recombinant yeast strain at all time points.

Plastic oxidation is supposed to require long incubation times and, thus, laccase activity has to be maintained over long time periods. In order to verify the consistence of cell biomass and enzyme activity along time, cell growth (OD_{600}) and laccase activity were monitored in 12 days incubation periods of DR98 and the parental MW98-8C strains in SDA

medium at 28 °C with shaking. Results (Figs. S2C and S2D) showed that high values of cell density and laccase activity were maintained up to day 12: an incubation period of 10 days was thus choose to ensure the maximal efficiency of the PP sample treatment with the biological material. Tween20 was added to the SDA medium in order to keep PP surface clean and obstacle adherence of biological/organic matter.

3.3. Experiment set up and incubation of PP_{ox} with yeast cultures

Oxidation of PP by the recombinant laccase expressed by yeast was studied by incubating strips of photo-oxidized polymer in microbiology tubes with synthetic rich medium (SDA medium plus 0.1 % Tween20, 0.1 mM $CuSO_4$ and 0.2 mM DMP or ABTS as redox mediator) containing proliferating yeast cultures that synthesizes and secrete the active enzyme (Fig. S4A). 12 UV pretreated PP_{ox} strips were put into 12 tubes: four of them were used for treatment without laccase in cultures with the control strain MW98-8C; eight strip-containing tubes were destinated to duplicated treatments with laccase-expressing DR98 strain cultures, obtained from two independent pre-cultures (DR98#1 and DR98#2), in order to ensure consistency of the results. MW98-8C and DR98 cultures were pre-cultivated in flasks at 28 °C with shaking (175 rpm) for two days and then added to the PP_{ox} strips in tubes. Tubes with PP_{ox} strips and yeast cultures were then incubated for 10 days at 28 °C with shaking (175 rpm), opportunely reclined, to ensure proper aeration. Tubes were incubated for 1, 2, 3 and 4 months, corresponding to 3, 6, 9 and 12 consecutive 10-days culture refreshes. Before discarding each culture, optical density and laccase activity were measured (Fig. S4B). Average optical densities (OD_{600}) of MW98-8C, DR98#1, DR98#2 and laccase activities in supernatants of DR98#1 and DR98#2 cultures of the entire experiment are reported in Fig. 2 (experiment with DMP mediator).

The duplicated experiments with DR98 strain gave essentially identical results for both growth and activity. Differently, growth of the reference strain MW98-8C was slightly, but significantly, lower than DR98 strain. Data corresponding to the experiment with mediator ABTS, reported in Fig. S5, were similar to those obtained with DMP. Figs. S5A and S5B also show the effects of Tween20 on yeast cell growth and on laccase activity, and indicated that the presence of this compound prevented to reach higher biomass yields in the cultures but had little effect on enzyme activity. This finding suggested that Tween20 could not be used as an additional carbon source by yeast cell.

3.4. Analysis of PP strips

3.4.1. FTIR spectroscopy

The ATR-FTIR spectra of pristine (pristine PP) and UV pre-treated (PP_{ox}) PP strips are reported in Fig. 3A. The exposure of PP strips to UV radiation brought about the appearance of a large absorption band between 1800 cm^{-1} and 1650 cm^{-1}, due to the C=O stretching of different oxidized groups. The ratio between the integrated intensity in the ranges 1800-1650 cm^{-1} and 1500-1400 cm^{-1} (C-H bending, taken as reference band) is called carbonyl index (CI) and represents a reliable index of PP oxidation degree (Almond et al., 2020). CI increased from 0.01 of the pristine PP to 0.13 after UV treatment (PP_{ox}). The crystallinity of PPox was evaluated by DSC analysis and resulted to be Xc = 56%, slightly lower than the pristine sample (Fig. S1). This reduction could be likely attributed to the decrease of the ordered phase fraction involved in the oxidation reaction.

The PP_{ox} strips were withdrawn after each scheduled monthly incubation period in the cultures with the MW98-8C and DR98 strains, washed thoroughly and dried. Extended treatments with Tweeen20, washing with SDS and ethanol, sonication were finalized to exclude any interference by biological material with sample analysis. Selected spectra of PP_{ox} (0 month of incubation) and those of the samples incubated with DR98, reported between 1850 and 1400 cm^{-1}in Fig. 3B, clearly shows the progressive intensity increase of the band related to the oxidized groups with the sample immersion time. On the other hand, the spectra of PP_{ox} strips immersed in cultures of the control strain MW98-8C, without laccase expression, did not show any significant variation with respect to the starting UV oxidized sample (Fig. S6). Differently from the samples incubated in MW98-8C culture, the progressive oxidation of PP_{ox} surfaces triggered by the activity of laccase from DR98 is clearly evidenced by the increase of the carbonyl index values as a function of the incubation time (Fig. 3C). Moreover, the absence of amide II band at about 1550 cm^{-1} indicates a lack of biological contamination on incubated samples (Sandt et al., 2021).

3.4.2. Water contact angle

Progressive oxidation of the PP_{ox} strip promoted by DR98 strain, as suggested by FTIR results, introduces polar oxidized functional groups to the surface. Then, water contact angle (WCA) determination is an effective analysis to investigate the resulting changes in hydrophilicity of the outermost sample layer. The pristine PP strip exhibited a water contact angle of 86 ± 2°. This value decreased to 82 ± 2° as a result of UV exposure. The results of the analysis carried out on the sample incubated in presence of MW98-8C and DR98 strain yeasts and DMP mediator for different time periods is reported in Fig. 4A.

This figure shows that the MW98-8C culture did not modify the surface properties of PP_{ox} strip, as evidenced also by the ATR-FTIR analysis. In contrast, the WCA of the PP_{ox} strip in contact with DR98 progressively decreased as a function of incubation time, indicating the formation of a highly wettable hydrophilic surface. However, it must be emphasized that surface roughness, besides surface chemical composition, also affects WCA. As we will see later, increased roughness can further enhance the wettability of already hydrophilic surfaces.

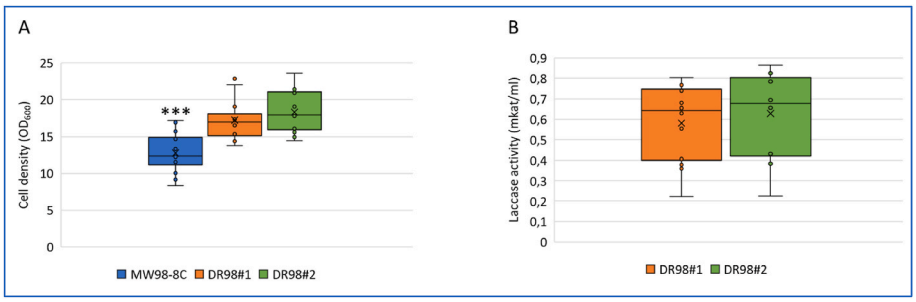

Fig. 2. Cell density and laccase activity in PP_{ox} DMP treatment cultures. In panel A, the cell densities reached by the cultures used for PP treatment with DMP, before refreshing with new cultures, are reported. Values are averages of all the cultures used in the experiments, from the 1st to the 4th month (three cultures each month). Statistical significance (***) of MW98-8C was $P < 0.001$ respect to DR98#1 and DR98#2. Difference between DR98#1 and DR98#2 was not significant. In panel B, the laccase activities measured in DR98#1 and DR98#2 cultures before refreshing are reported. Values are averages of all the used cultures along the experiment.

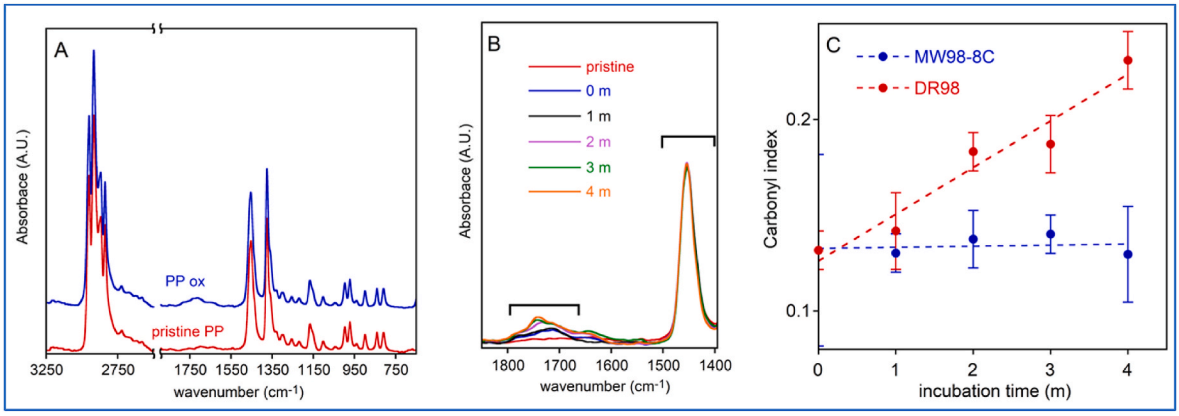

Fig. 3. Spectroscopic analysis. ATR-FTIR spectroscopy characterization: ATR-FTIR spectra of pristine and UV pre-treated PP_{ox} samples are reported in panel A. Evolution of the spectra of the samples incubated in presence of DR98 strain with DMP mediator recorded at different time is shown in panel B. The pristine PP is displayed for sake of comparison. In panel B, the spectral ranges where the carbonyl index was calculated are highlighted. 'm' indicates the sample immersion time (months). Variation of the carbonyl index as a function of the incubation time of PP_{ox} (immersion time = 0) in the DR98 and MW98-8C cultures is shown in panel C.

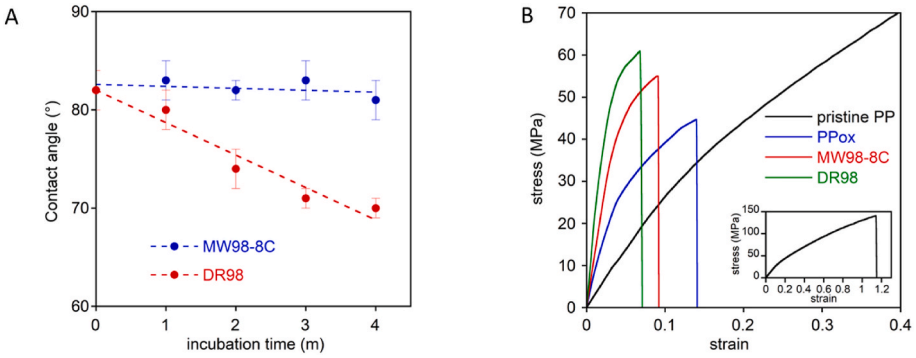

Fig. 4. Water contact angle (WCA) analysis and mechanical characterization. WCA variation is reported in panel A as a function of the sample incubation time in MW98-8C and DR98 cultures (DMP mediator). Stress-strain curves of pristine and UV-treated PP film before (PP_{ox}) and after incubation for 4 months in MW98-8C and DR98 yeast strain cultures are reported in panel B.

3.4.3. SEM microscopy

Surface morphology of PP strips was investigated by SEM analysis. Images acquired at 10K X and 25K X magnifications are shown in Fig. 5. At the higher magnification, it is possible to observe that UV exposure caused just a barely noticeable roughness increase with respect to the pristine sample (Fig. 5B and D *versus* Fig. 5A and C). A further roughness increases is displayed by the PP surface of the sample incubated with MW98-8C, presumably due to the solubilization of oxidized product formed in the previous treatment and removed by the thorough cleaning of the sample (Fig. 5E and F). In contrast, the surface morphology of the sample treated with the laccase-producing DR98 strain showed significant modifications (Fig. 5G and H) due to the appearance of humped regions, indicating the lifting of the outermost layer. Notably, this exfoliation or delamination occurred below the grooves occasionally present on the pristine sample. This result suggests that the uneven surface is not caused by the deposition of debris or biological contaminants. Similar alterations of the PP surface morphology after the 4 months treatment with DR98 strain were also found using ABTS as redox mediator instead of DMP (Fig. S7).

3.4.4. Mechanical properties

The possible influence of different treatment subjected by pristine PP strips on mechanical properties were investigated by stress-strain experiments. It is well-known, in fact, that polypropylene has a low resistance to tearing and any imperfection on the surface, such as nick or flaw, can initiate the fracture of the sample (Billham et al., 2003). The stress-strain curves of selected samples are displayed in Fig. 4B and the calculated mechanical features are reported in Table 1. The oxidation of PP by UV irradiation brought about a dramatic decrease of the PP_{ox} toughness, as a result of the large decrease of the elongation at break. This phenomenon was already reported in a number of researches and is attributed to the formation of crack precursors rather than the variation of the material properties in bulk (Raab et al., 1982). The progressive increase of sample stiffness and the decrease of elongation at break in the early stage of exposure to UV radiation has been attributed to further crystallization favored by the chain scission taking place mainly in the amorphous phase (Gupta et al., 2007; Ainali et al., 2021). Moreover, both the incubation in the two yeast stains for 4 months brought about a further increase of the PP_{ox} brittleness, more pronounced for the sample treated with DR98 strain. This could indicate that the immersion in water favor the formation of flaw on the surface of oxidized strip, mainly for those samples which underwent a further extensive oxidation by the action of the yeast expressing laccase.

4. Discussion

In this work we focused on the oxidative degradation/deterioration of the polyolefin PP, a plastic material very diffused but rarely used as substrate for microbial degradation studies (Mohanan et al., 2020; Singh Jadaun et al., 2022). Polyolefin degradation is strongly facilitated when the starting material is subjected to pretreatments such as mechanical attrition and UV light exposure in the presence of oxygen, either through intentional processes or natural environmental factor, to thermal pretreatment or by the presence of chemical additives in the polymeric

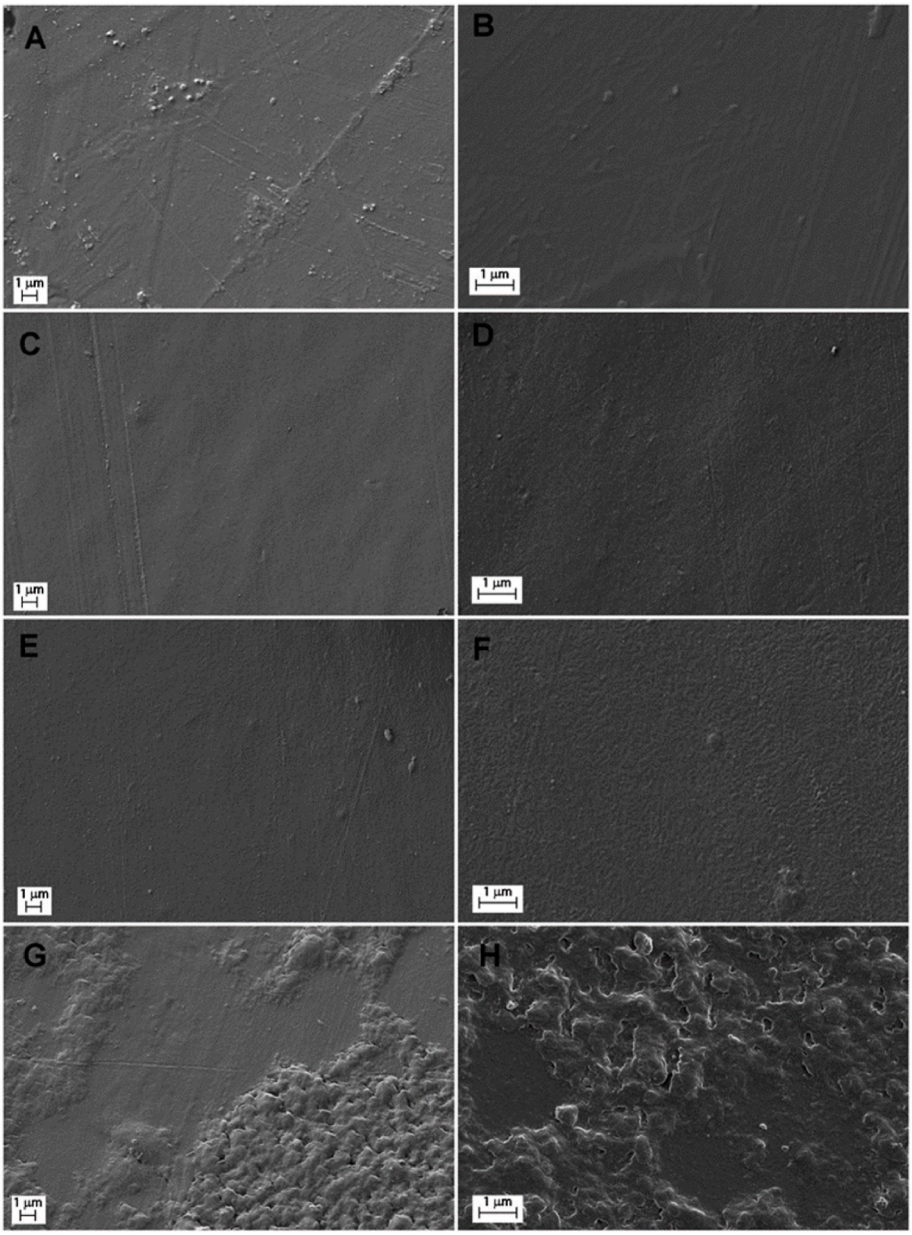

Fig. 5. SEM analysis. SEM micrographs acquired after chromium coating of pristine PP film (panels A, B); UV treated PP_{ox} (panels C, D); PP_{ox} incubated for 4 months with MW98-8C (panels E, F) and DR98 (panels G, H) strains. Micrographs acquired at 10K X (panels A, C, E, G) and 25K X (panels B, D, F, H) magnifications.

Table 1
PP mechanical properties.

Sample	Young modulus (Mpa)	Elongation at break	Stress at break (Mpa)
Pristine PP	270 ± 10	1.15 ± 0.06	140 ± 10
PP_{ox}	750 ± 10	0.14 ± 0.06	40 ± 8
PP-MW98-8C (4m)	1200 ± 20	0.11 ± 0.02	52 ± 7
PP-DR98 (4m)	1800 ± 20	0.07 ± 0.03	63 ± 2

Mechanical properties of pristine and UV-treated PP films before (PP_{ox}) and after incubation for 4 months (4m) in MW98-8C and DR98 yeast strain cultures. Values reported are the average of three independent measurements ± SD.

material. These conditions modify the physical and chemical properties of the material surface, generate microparticles or supply compounds that favor microbial activities and are of particular importance especially when recalcitrant polymers, like PE and PP, are considered (Arkatkar et al., 2009, 2010; Jeyakumar et al., 2013; Cacciari et al., 1993; Fontanella et al., 2013; Aravinthan et al., 2016; Auta et al., 2017, 2018).

Polyolefin biodegradation consists of three major steps (Danso et al., 2019; Mohanan et al., 2020; Amobonye et al., 2021; Singh Jadaun et al., 2022; Gu et al., 2024). Firstly, the surface of the material has to be modified by the introduction of oxygen atoms in the carbon backbone of polymers (oxidative degradation/deterioration): successively the covalent bonds of the carbon backbone are broken and small oxidized molecules are generated (oxidative fragmentation/depolymerization). Finally the small molecules are assimilated by the microbial cells (natural consortia or selected species) and metabolized to produce biomass and carbon dioxide. Microbial consortia able to degrade PP are predominantly isolated from soils (Arkatkar et al., 2009, 2010; Cacciari et al., 1993; Auta et al., 2017, 2018; Skariyachan et al., 2018; Jeon et al., 2021), however even unusual niches can be the sources of PP-degrading microbes (Skariyachan et al., 2021; Yang et al., 2021).

Our approach was based on the use of the redox enzyme laccase and its substrates (mediators) to introduce oxidized groups along the PP backbone and to generate surface structure modification of the sample films, in order to further facilitate access to substrate and oxidative degradation. Laccase are widely diffused enzymes (Giardina et al., 2010) with both physiological and ecological (carbon cycle) roles in nature and active on a large variety of substrates (Kunamneni et al., 2008). Small molecules substrates allow to bypass steric hindrance of polymeric substrates and hence to extend the oxidative activity of laccases. In this work, we demonstrated the oxidative activity of two laccase mediators, DMP and ABTS, on PP substrate. These mediators are oxidized by two different mechanisms: ABTS by Electron Transfer (ET) and DMP by Hydrogen Atom Transfer (HAT) (Fabbrini et al., 2002). The presence of the yeast-produced and secreted recombinant laccase in the reaction medium allowed the continuous recycling of the active oxidant mediator and the generation of consistent (chemical, structural, morphological) changes of the PP substrate. These changes included the introduction of oxidized groups, detected by FTIR; the increase of surface hydrophilicity, demonstrated by the reduction of water contact angle, and large modification degradation of the surface morphology and sample homogeneity, as shown by SEM analysis and mechanical characterization.

Our results suggest the possibility to increase the efficacy of the first step of polyolefin degradation and, hence, favor and accelerate the subsequent degradation steps. The presented effects of polyolefins treatment with the laccase/mediator system could possibly be reinforced by some process improvements. For example, the use of other mediators more efficient in polymer oxidation; longer incubation times to obtain a higher oxidation level of the polymer; higher oxygenation during sample treatment to accelerate the rate of mediator recycling by laccase. More profound process improvement/implementations might be the mechanical pre-grinding, to increase the surface to mass ratio of the plastic substrate and to generate more accessible sites of action for the laccase/mediator system, or incubation at higher temperature in the range of physiological limits of yeast, as well as the use of mixed microbial cultures including microbes able to sustain the successive steps of biodegradation, *i.e.* plastic depolymerization and assimilation of depolymerized products. Finally, the expression of the heterologous laccase gene might be opportunely regulated to higher levels by adjustment of the incubation conditions or by changing the promoter sequence. The promoter used in the recombinant strains used in this work is the promoter of the *KlPDC1* gene, which is positively regulated by glucose and hypoxia, and negatively regulated by ethanol and the KlPdc1 enzyme itself (autoregulation) (Destruelle et al., 1999; Micolonghi et al., 2011; Ottaviano et al., 2014). Hypoxia cannot be used as expression inducer since laccase recycling requires oxygen. On the contrary, the expression of heterologous genes by the *KlPDC1* promoter in strains deleted for *KlPDC1*, has already been proved to be successful (Camattari et al., 2007; Porro et al., 1999; Salani and Bianchi, 2006) ad could be applied also in overexpression of the laccase gene for plastic deterioration.

5. Conclusions

A critical step of plastic biodegradation is the initial attack of material surface. Our study demonstrated that incubating UV-pretreated polypropylene films with yeast recombinant strain expressing a fungal laccase, in the presence of a laccase redox mediator under aerobic conditions, induced significant changes to the PP surface. In particular, an increase of the carbonylic index and of water wettability, exfoliation of polymer surface as well as variation of the mechanical properties were observed.

CRediT authorship contribution statement

G. Sabellico: Investigation. **A. Baggetta:** Investigation. **E. Sandrucci:** Investigation. **G. Zanellato:** Investigation. **A. Martinelli:** Writing – review & editing, Supervision, Resources, Conceptualization. **A. Montanari:** Writing – review & editing, Supervision. **M.M. Bianchi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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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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Authors have no competing interest to declare. No AI or AI-assisted technologies have been used. We thank A.M. Girelli and O. Russina (Dept. of Chemistry, Sapienza University of Rome) for useful discussions about the experimental design and result analysis, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2024.105947.

Data availability

No data was used for the research described in the article.

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