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Identification of the best-performing novel microbial strains from naturally-aged graffiti for biocleaning research

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ABSTRACT

Microorganisms that can act as biocleaning agents can potentially be isolated from existing graffiti. In this study, a total of 30 different isolates of bacteria, fungi and yeasts were obtained from black, blue, red and silver graffiti coatings exposed outdoors conditions for 10 years. An additional strain was obtained from samples of black powdered graffiti exposed under laboratory conditions for 5 years. Each of the isolates from the most prolific sources (i.e. red and silver graffiti coatings and black powdered graffiti) was evaluated alone and in combination with Tween® 20, to determine their potential as biocleaning agents, in test tube assays. Two strains isolated from the red graffiti coating (identified as *Candida parapsilosis*) and one strain isolated from the black powered graffiti (identified as *Rhodococcus erythropolis*) performed best alone. The quantity of live cells throughout the trials and biofilm formation indicated that both strains were able to utilize the paint as a substrate. When the microorganisms isolated were combined with Tween® 20, the quantity of live cells increased notably throughout the incubation time, and turbidity and graffiti discoloration were observed. Infrared spectroscopic examination revealed that *R. erythropolis* performed the best alone, while the addition of Tween® 20 *to C. parapsilosis* enhanced the oxidative degradation of the graffiti.

1. Introduction

In the last two decades the use of naturally occurring bacterial, fungal and yeast cells and enzymes to remove undesired substances such as salts and organic matter from cultural heritage assets has produced some successful results (Ranalli et al., 2005; Lustrato et al., 2012; Bosch-Roig and Ranalli, 2014; Sanmartín et al., 2018). Graffiti paint, which is usually successfully removed by laser techniques (Costela et al., 2003; Gómez et al., 2006; Rivas et al., 2012; Pozo-Antonio et al., 2016, 2018), has recently been added to the list of bioremovable material. Biocleaning is a relatively novel approach to graffiti removal that has been developed in the last decade. However, the process is complicated and although the results are promising, they are not entirely successful. The identification of strains of microorganisms capable of degrading paint and maximization of the biocleaning potential are important steps in developing this complex approach (Bosch-Roig and Sanmartín, 2021).

Most microorganisms found in paints are aerial contaminants that are not involved in degradation processes (Gaylarde et al., 2011). Nevertheless, old paintwork has proven to be a good source of putative biodegradative microbial populations. In an earlier study (Sanmartín et al., 2015) involving the search for natural strains of bacteria and fungi capable of degrading graffiti, a total of 54 different strains were isolated from recent and old graffiti, the bodywork of a car in a scrapyard, the underlying soil, an acrylic wall painting and the interior of spray paint cans. Among these strains, 10 displayed some degradative capacity and caused visual damage to graffiti paint. Half of these strains were isolated from old paint coatings. In other studies, some strains were also isolated from paint stripping waste remains (*Comamonas* sp. ATCC 700440) and from soil containing paint waste (a mixture of *Bacillus* sp., *Delftia lacustris*, *Sphingobacterium caeni* and *Ochrobactrum anthropi* (ATCC 53922), and also proved promising for graffiti bioremoval (Sanmartín and Bosch-Roig, 2019; Cattò and Sanmartín et al., 2021).

The advantage of isolating bacteria, fungi and yeasts from the coatings to be removed may not be clear, considering that many strains can be purchased from international collections of microorganisms (ATCC, DSMZ, CECT, etc.). However, it has been demonstrated that if the strain

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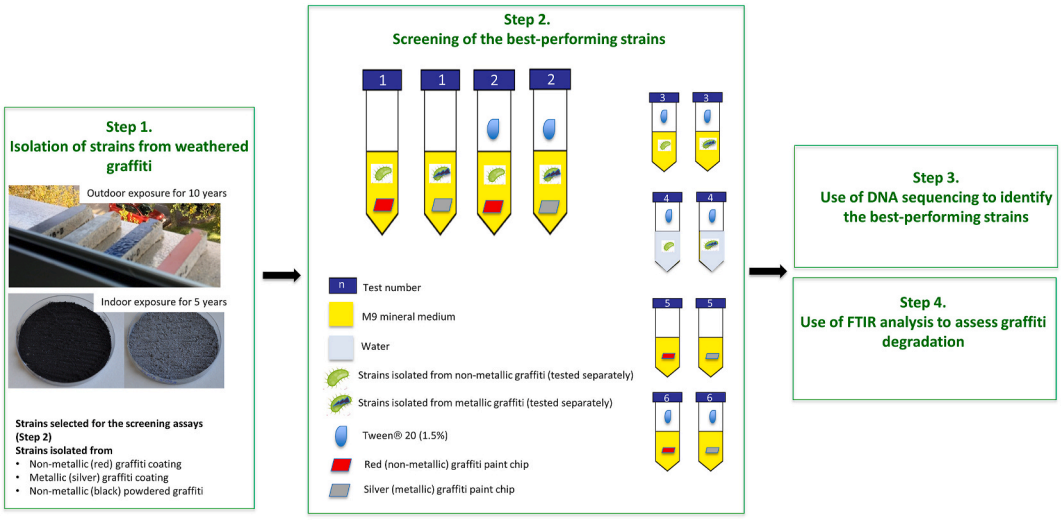

Fig. 1. Schematic diagram of the methodology used in the study.

Table 1
Strains isolated from naturally-aged graffiti samples. Those selected (13 strains) for the screening study are indicated in bold and underlined.

Source	Sample Code	Putative bacterial strains	Putative fungal strains	Total number of strains isolated
Silver graffiti coated granite	S1	**5**	0	5
	S2	**1**	2	3
Red graffiti coated granite	R1	**2**	2	4
	R2	**4**	2	6
Black graffiti coated granite	B1	2	0	2
	B2	3	0	3
Blue graffiti coated granite	Bu1	3	1	4
	Bu2	3	0	3
Black powdered graffiti	–	**1**	0	1
Silver powdered graffiti	–	0	0	0

is adapted to the material to be degraded, it will have a greater biocleaning capacity (Sanmartín and Bosch-Roig, 2019). The bioremoval capacity of a commercially available strain of the bacterium *Pseudomonas stutzeri*, which is widely used in biocleaning research (Lustrato et al., 2012; Sanmartín and Bosch-Roig, 2019), was compared with that of A29 strain of the same bacterium isolated from the coating to be removed (Antonioli et al., 2005). These researchers demonstrated that the isolated strain grew optimally and displayed higher enzymatic (protease) activity than the commercial strain.

Other ways of maximizing the biocleaning performance of microbial strains may be possible. The non-ionic detergent Tween® 20 is known to favour the separation of bacterial cells without affecting the membrane proteins, while also promoting the growth of microorganisms (Ando et al., 1959). In developing a biocleaning treatment for the removal of black crusts, Troiano et al. (2013) combined the method with a gentle chemical treatment with Tween® 20, with successful results.

In the present study, a method was developed for detecting potential graffiti biocleaning agents (Fig. 1). In step 1, microbial strains were isolated from black, blue, red and silver graffiti coatings exposed outdoors under ambient conditions for 10 years and also from black and silver powdered graffiti exposed indoors under laboratory conditions for 5 years. In step 2, screening assays were carried out with a group of strains selected (considering most prolific graffiti sources from both outdoor and indoor-exposed samples) from those isolated in the previous step, to test their potential biocleaning capacity alone and in combination with Tween® 20. In step 3, the best-performing strains were identified by DNA sequencing. In the final step, 4, chemical changes in fragments of the graffiti coatings related to degradation by microorganisms alone and in combination with Tween® 20 were characterized by infrared spectroscopy (FTIR).

2. Materials and methods

2.1. Isolation of microbial strains from old graffiti

Samples consisted of (i) granite blocks coated with a graffiti paint layer and exposed between January 31, 2010 and January 31, 2020 (10 years) to natural conditions (outdoors) in Vigo (Spain), and (ii) powdered graffiti exposed for 5 years to laboratory conditions (indoors) in Valencia (Spain).

The samples exposed outdoors were eight blocks (of dimensions 3 cm × 10 cm x 3 cm) of Rosa Porriño granite (for further information, see IGME, 1981) each with a coating (on one of the largest surfaces) of a non-metallic (ultramarine blue [R-5002], devil red [R-3027], graphite black [R-9011]) or a metallic (silver chrome) graffiti paints from Montana Colors Mtn®, previously characterized by Rivas et al. (2012). The blue, red and black graffiti paints were composed of alkyd and polyester resins or varnishes, and the silver graffiti mainly contained polyethylene-type polymers. Rutile (TiO_2) was detected in red and blue paints and small traces of barite ($BaSO_4$) in the red paint (Rivas et al., 2012); these minerals are usually used as extenders in paints (Abel, 1999). The blocks (Fig. 1, Step 1) were placed horizontally (with the coated surface facing upwards) on windowsills in the Mining and Energy Engineering School (42°10′8″N, 8°41′22″W, sea height: 420 m) of the University of Vigo (Vigo, Spain). The samples were facing S45°W at a distance of 10 m from the car park of the School of Mining and Energy Engineering and at a distance of 8.2 km from the Atlantic Ocean. Sample codes were S1 and S2 for the silver graffiti coated granite samples, R1

and R2 for the red graffiti coated granite samples, B1 and B2 for the black graffiti coated granite samples and Bu1 and Bu2 for the blue graffiti coated granite samples (Table 1).

The samples exposed indoors comprised 5 g of the black and silver powdered graffiti described above. The powdered graffiti (Fig. 1, Step 1) samples were spread out in Petri dishes and exposed for 5 years in an air-conditioned laboratory in the Polytechnic University of Valencia (Spain).

Depending on the type of sample, two different protocols were used to collect the microorganisms for subsequent isolation: (a) sterile swabs moistened with sterile buffer solution (2 mL) were used to remove samples from graffiti painted granite blocks exposed outdoors, and (b) 10 mL of M9 minimal medium liquid culture (M9 broth: 75.2 g/L $Na_2HPO_4.2H_2O$, 30 g/L KH_2PO_4, 5 g/L NaCl, 5 g/L NH_4Cl) + 1M $MgSO_4$ + 1M $CaCl_2$) was added to the powdered graffiti (0.1 g) exposed indoors. The outdoor-exposed samples were serially diluted, and the different dilutions were spread on culture plates containing Tryptic Soy Agar (TSA), Tryptone-Glucose-Yeast extract Agar (TGYA) and Malt Extract Agar (MEA). The culture plates were incubated for 24–72 h at 28 °C. Morphologically different colonies of microorganisms were then selected and sub-cultured. The process was repeated until pure cultures of individual isolates were obtained. The samples exposed indoors were incubated for 50 days at 28 °C. Aliquots of 100 μL of each sample were spread on culture plates containing Nutrient Agar for isolation of individual colonies.

2.2. Screening assays to detect strains with potential biocleaning capacity

For the screening assays, the putative bacterial strains (because bacteria are easier to handle and are also more common in biocleaning studies) isolated from the most prolific graffiti sources were selected. For best results, isolates from non-metallic (black, red and blue) graffiti coatings were tested on red, non-metallic graffiti paint chip samples, while isolates from metallic (silver) graffiti were tested on silver metallic graffiti paint chip samples.

One colony of each selected isolate was resuspended in sterile saline solution (1 mL), and a 50 μL aliquot was added to a test tube containing M9 liquid culture (10 mL). Different tests were conducted (in triplicate) for each of the isolates (Fig. 1, step 2):

1. M9 liquid culture + sterilized red (non-metallic) or silver (metallic) graffiti paint chips. The corresponding sample codes (Table 2) are: RS(X)M and SS(X)M, where X is the number of the strain identified.
2. M9 liquid culture + sterilized red (non-metallic) or silver (metallic) graffiti paint chips + Tween® 20 (1.5% in distilled water). Hereinafter, RS(X)MT and SS(X)MT, where X is the number of the strain identified.
3. M9 liquid culture + Tween® 20 (1.5% in distilled water). Hereinafter, MTS(X), where X is the number of the strain identified.
4. Water (instead of M9 liquid culture) + Tween® 20 (1.5% in distilled water). Hereinafter, WTS(X), where X is the number of the strain identified.

Furthermore, the following tests were carried out without the isolated strains, also in triplicate (Fig. 1, step 2):

5. M9 liquid culture + sterilized red (non-metallic) or silver (metallic) graffiti paint chips. Hereinafter, RM and SM.
6. M9 liquid culture + sterilized red (non-metallic) or silver (metallic) graffiti paint chips + Tween® 20 (1.5% in distilled water). Hereinafter, RMT and SMT.

All test tubes were incubated at 28 °C in an incubator with natural convection (Binder-BD). Microorganism viability was analysed after incubation of the cultures for 6, 9, 14 and 23 days, by serial dilution and counting the number of colony forming units (CFUs mL^{-1}). In addition, similarly to the results of Sanmartín et al. (2015), visually detectable effects, such as turbidity, graffiti discoloration and biofilm formation, were recorded at the same times as the CFUs were counted. Moreover, at the end of the experiment, contact-type colour measurements were made using a portable spectrophotometer (Konica Minolta CM-700d) equipped with CMS100w (SpectraMagicTM NX) software in order to quantify the visually observable graffiti discoloration. The measurements were taken with the specular component excluded (SCE) mode using a CIE standard daylight illuminant D65, a small area view (SAV) of 3 mm and an observer angle of 2°. A total of 6 measurements per sample, 3 on each of the surfaces of the graffiti paint chips were made following Sanmartín et al. (2020). Colour measurements were analysed using the CIELAB colour system (CIE S014-4/E: 2007) which represents each colour by means of three scalar parameters or Cartesian coordinates: L*, lightness, which varies from 0 (absolute black) to 100 (absolute white); a*, associated with changes in redness-greenness (positive a* is red and negative a* is green); and b*, associated with changes in yellowness-blueness (positive b* is yellow and negative b* is blue).

Table 2
Visually detectable effects throughout the incubation period of the screening study on both proven treatments: simple (biological treatment only) and combined (plus addition of a chemical treatment with the non-ionic detergent, Tween® 20) for the three strains that showed potential biocleaning capacity.

Test number	Sample Code	Incubation Period (days)			
		6	9	14	23
1	RS1M	–	+	+ Bio	+ Bio
1	RS2M	–	+	+ Bio	+ Bio
1	RS3M	–	+	+ Bio	+ Bio
2	RS1MT	Dis	+Dis Bio	++ Dis Tur Bio	+++ Dis Tur Bio
2	RS2MT	Dis	+ Dis Bio	++ Dis Tur Bio	+++ Dis Tur Bio
2	RS3MT	Dis	+ Dis Bio	++ Dis Tur Bio	+++ Dis Tur Bio
3	MTS1	–	+	+	+
3	MTS2	–	+	+	+
3	MTS3	–	–	–	+
4	WTS1	–	+	+	+
4	WTS2	–	+	+	+
4	WTS3	–	–	–	+
5	RM	–	–	–	–
6	RMT	Dis	Dis	Dis	Dis

R: Red graffiti paint chip; M: M9 mineral medium broth; S1, S2, S3: strain 1, 2 and 3 respectively, those that yielded positive potential biocleaning response; T: Tween® 20; W: water.Tur: turbidity; Dis: graffiti discoloration; Bio: biofilm formation; -: absence of bacteria; +: few plate count colonies (<30 CFU mL^{-1}) of bacteria; ++: countable plate count colonies (between 30 and 300 CFU mL^{-1}); +++: uncountable plate count colonies (>300 CFU mL^{-1}) of bacteria.

For the experiments, the graffiti paint chips were sterilized by exposure to formaldehyde 37% v/v vapour for 48 h (Sanmartín et al., 2015). Tween® 20 was added in half the trials to test its effect on microorganism growth (Ando et al., 1959) and to confirm the findings of previous biocleaning studies, in which addition of the detergent reduced the treatment time by 38% (Troiano et al., 2013).

2.3. Identification of the best performing strains by DNA sequencing

To identify the best performing strains (Fig. 1, Step 3), genomic DNA was purified from cell cultures during the exponential growth phase and stored on Whatman Indicating FTA™ Micro Cards for bacterial and fungal isolates by STAB Vida Lda. (Portugal). Fragments of the bacterial 16S rDNA and the fungal ITS region plus the D1/D2 region of the 28S rDNA gene were amplified using previously described primers (White, 1990; Lane, 1991) and previously reported PCR conditions (Sanmartín and Carballeira, 2021). Sequences were aligned to generate consensus sequence and checked against the GenBank by BLAST of consensus sequence against NCBI database, applying a similarity score of ≥99.0% (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Assessment of graffiti degradation by FTIR analysis

Chemical changes in the graffiti caused by the best-performing strains, with and without Tween® 20 added, were characterized by infrared analysis (Fig. 1, Step 4). At the end of the screening study, red graffiti paint chip samples were dried in an oven at 40 °C for 24 h, before being analysed by Attenuated Transmittance Reflectance–Fourier transform infrared spectroscopy (ATR-FTIR) (Thermo Nicolet 6700). Infrared (IR) spectra were recorded at 2 cm^{-1} resolution over 100 scans, from 400 to 4000 cm^{-1}.

3. Results

3.1. Isolation assays

A total of 31 different strains were isolated: 24 were putatively identified as bacteria and 7 as fungi (Table 1). Almost all strains (30 of 31) were derived from the outdoor environment, and only one was isolated from the black powdered graffiti exposed indoors under laboratory conditions. The most prolific sources of the strains were the coated granite blocks, specifically the red graffiti coating, from which 10 strains isolated (of these, 6 were putatively identified as bacteria and 4 as fungi), and the silver graffiti coatings, from which 8 strains were isolated (of these, 6 were putatively identified as bacteria and 2 as fungi). The blue graffiti yielded 7 strains (6 were putatively identified as bacteria and 1 as a fungus), and the black graffiti yielded 5 strains, all putatively identified as bacteria.

3.2. Screening assays

From the 31 strains isolated, the putative bacteria isolated from the

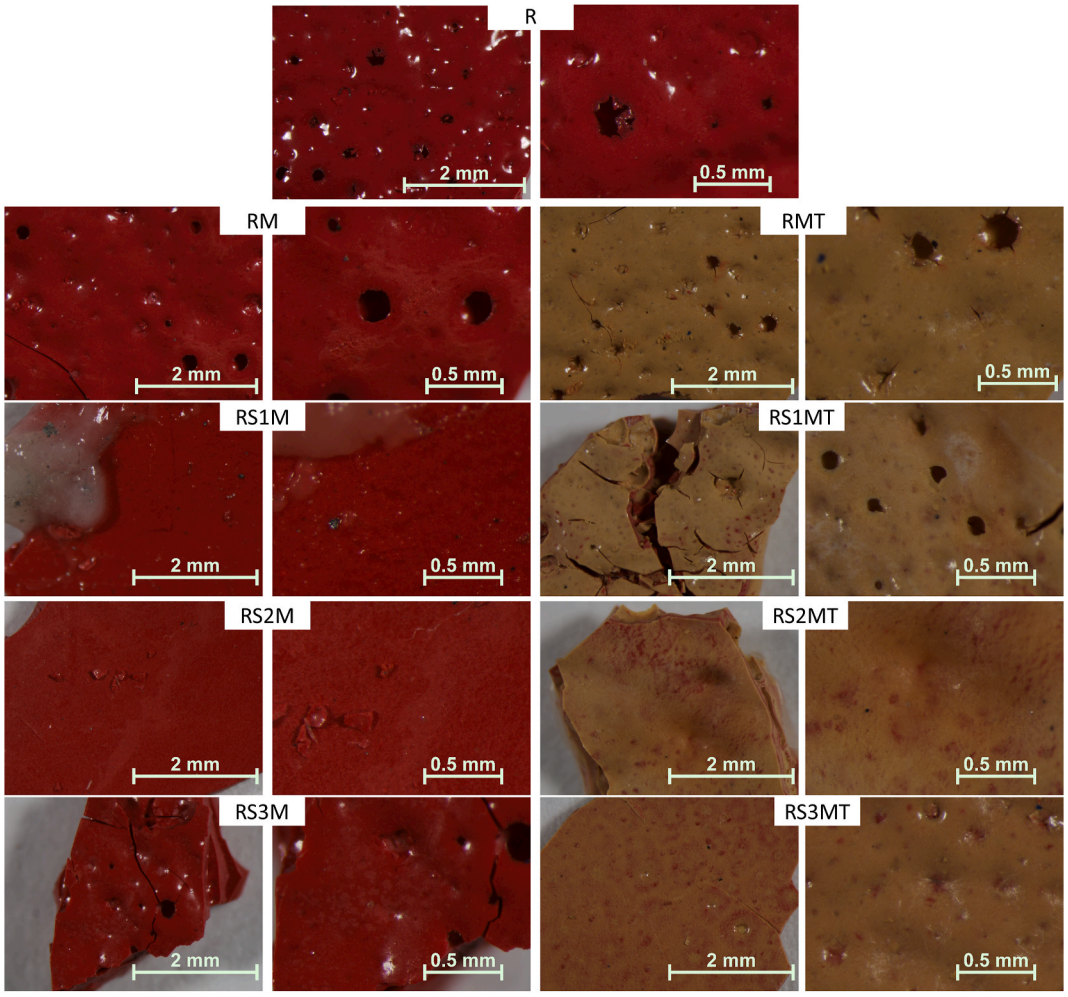

Fig. 2. Stereoscopic micrographs of the red graffiti paint chips treated with the microbial strains that yielded positive biocleaning responses (S1–S3) in the screening tests. R: Red graffiti paint chip; M: M9 mineral medium broth; T: Tween® 20.

most prolific graffiti sources, considering both outdoor and indoor-exposed samples, were selected for the screening assays. A total of 13 putative bacterial strains (6 isolated from the silver graffiti coating on granite, 6 isolated from the red graffiti coating on granite and 1 isolated from black powdered graffiti: Table 1) were thus tested.

Throughout the 23-day incubation, only 3 of the 31 strains in the test tube assays showed visually detectable effects, such as turbidity, graffiti discoloration or biofilm formation, due to the treatment. These 3 strains included 2 of the strains isolated from red graffiti coated granite exposed outdoors under ambient conditions (hereinafter referred to as S1 and S2) and the strain isolated from black powdered graffiti exposed indoors under laboratory conditions (hereinafter, S3).

For the best-performing strains (S1–S3), viable microbial cell concentrations, estimated from the number of CFUs and appreciable turbidity, graffiti discoloration and biofilm formation in the different trials (section 2.2) at the different measurement times (6, 9, 14 and 23 days of incubation) are shown in Table 2.

All three strains (S1, S2 and S3) grew in the presence of graffiti after 9 days of incubation and formed a biofilm on the surface of the graffiti after 14 days of incubation (Table 2, Fig. 2, Video 1 in supplementary material). When combined with Tween® 20, each of the three strains decolorized the graffiti (Fig. 2) before cell growth occurred (Table 2). This discoloration was confirmed by L* decreases of 30 CIELAB units for the graffiti with Tween® 20 without bacteria (RMT) and 25 CIELAB units when bacteria were present [R (S1–S3) MT]. However, when the detergent was not added, the L* change was lower than 6 CIELAB units [R (S1–S3) M]. Regarding the chromatic parameters (a* and b*), Tween® 20 caused changes around 40 and 20 CIELAB units respectively, while without it, the variations of a* and b* did not exceed 2 and 5 CIELAB units respectively. The detergent also accelerated formation of the biofilm on the graffiti samples (occurring after 9 days of incubation) and strongly favoured cell growth in the medium, increasing the turbidity and the number of cells over 300 CFU mL^{-1} (after 23 days of incubation) (Table 2). In addition, in both cases (strains alone and combined with Tween® 20), all three strains produced the same results.

In the absence of graffiti, no biofilms were formed in the test tubes and no turbidity appeared (Table 2). Growth of all three strains (S1, S2 and S3) was also lower than in the tubes containing the graffiti samples, especially S3, which did not grow until the end of the incubation period (day 23).

3.3. Identification of the best-performing strains

Sequencing analysis identified strains S1 and S2 as the yeast *Candida parapsilosis* (100% and 99.77% similarity in nucleotide identity, with accessions MH545914.1 and MK638869.1 respectively) and strain S3 as the actinobacterium *Rhodococcus erythropolis* (99.41% similarity in nucleotide identity, with accession CP050124.1).

3.4. Assessment of graffiti degradation by FTIR analysis

The FTIR spectra of the graffiti samples are shown in Fig. 3. For the reference red paint (R), the spectrum shows a broad band centered at 3465 cm^{-1} and a weak band at 3295 cm^{-1} corresponded to the O–H stretching vibration of water (Socrates et al., 2001). Strong bands were observed at 2925 cm^{-1}, attributed to (C–H)CH_2 asymmetric stretching, and at 2855 cm^{-1}, attributed to C–H symmetric stretching vibrations of alkanes (Socrates et al., 2001). A strong peak at 1721 cm^{-1} and a less intense band at 1625 cm^{-1} corresponded to C═O stretching vibration (Socrates et al., 2001). Bands at 1449 cm^{-1}, 1400 cm^{-1} and 654 cm^{-1} were attributed to out-of-plane bending of aromatic C–H bonds (Socrates et al., 2001). A strong peak at 1254 cm^{-1} (probably due to the esters) and several strong peaks at around 1200–1000 cm^{-1} were attributed to C–O group vibration stretching (Socrates et al., 2001). Bands at 755 cm^{-1} and 705 cm^{-1}, corresponding to aromatic out-of-plane bending, were attributed to the polyester portion of an alkyd (Knuutinen and Kyllonen, 2006). A band detected at around 542 cm^{-1} was associated with the presence of TiO_2 (Rivas et al., 2012), which overlaps with the C–H stretching and bending vibrations of the aromatic ring of the alkyd base of the paint. Compared with R, no changes were detected in RM (graffiti with M9) or RMT (graffiti with M9 and Tween® 20).

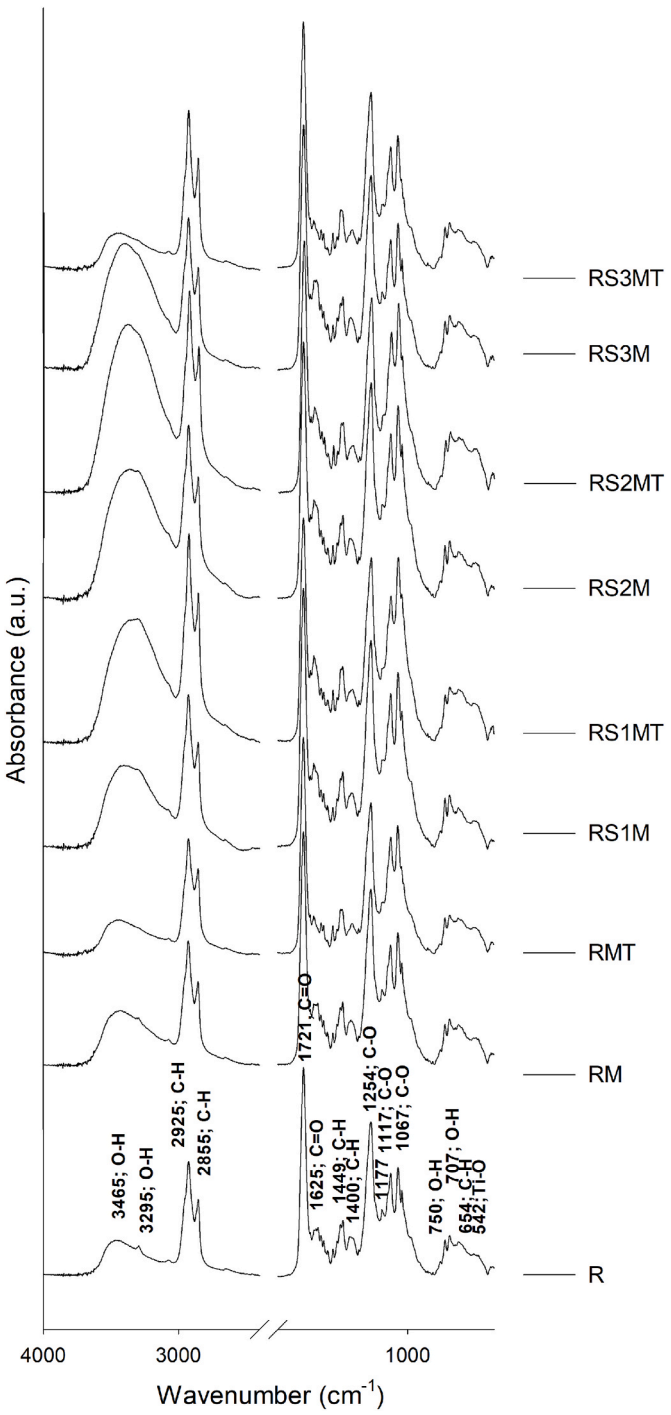

Fig. 3. FTIR spectra of the red graffiti paint chips treated with the strains that yielded positive biocleaning responses (S1–S3) in the screening tests. R: Red graffiti paint chip; M: M9 mineral medium broth; T: Tween® 20.

In the FTIR spectra of the graffiti treated with microorganisms, changes were identified in the intensity of the bands attributed to O–H stretching and C═O stretching, with an important increase in the band at 3400-3300 cm^{-1} (O–H stretching) and slight increases at 1625 cm^{-1}

(C═O stretching). These were observed in the graffiti fragments treated only with the bacterium, either *Candida parapsilosis* (S1 and S2) or *Rhodococcus erythropolis* (S3). When Tween® 20 was added to *C. parapsilosis*, the changes were enhanced, and when the detergent was added to *R. erythropolis*, the effects disappeared (spectrum similar to that of RMT).

4. Discussion

The presence of microorganisms on heritage surfaces is usually associated with biodeterioration. However, these colonies may also represent a source of new bioagents for biocleaning, regarded as a green conservation strategy. Biocleaning of graffiti shows great potential but has been developed by a small number of interdisciplinary research groups in relatively few studies (Bosch-Roig and Sanmartín, 2021, and references therein). Thus, very few microorganisms with the potential capacity to degrade graffiti have been identified and, to the best of our knowledge, this is only the second study (after Sanmartín et al., 2015) involving the microbial ecology of graffiti paintwork.

From the 31 putative bacterial isolates originally obtained, 13 were tested by screening, and 3 of these displayed biocleaning potential as they used the graffiti paint as the sole carbon and energy source. Of these, two (both from the outdoor environment, from which 30 strains were isolated) were identified as the same species, the yeast *Candida parapsilosis,* and one (from the indoor environment, from which only 1 strain was isolated) was identified as the species actinobacterium *Rhodococcus erythropolis*. The chances of discovering a strain that can potentially be used for biocleaning by isolation from an environment not exposed to environmental parameters seem much higher than if the isolation is done in an outdoor environment, although the deposition of bioaerosols and particles outdoors may increase the microbial population (Sterflinger et al., 2018). In outdoor environments compared to indoor places, nutrients and humidity are higher for microorganisms, however most of microorganisms on painted surfaces outdoors are actually aerial contaminants and are not involved in degradation processes (Gaylarde et al., 2011; Sanmartín et al., 2015). Graffiti exposed indoors are more limited in available nutrients, which can make viable only those microorganisms that can produce the particular enzymes able to use graffiti components as nutrients sources (Bosch-Roig and Sanmartín, 2021).

Candida parapsilosis is a yeast that has been isolated from nonhuman (domestic animals, insects and soil) and human (skin, wounds, etc) sources. It is classified as belonging to risk group 2 according to the CECT (Spanish Type Culture Collection classification based on INSHT (National Institute of Occupational Safety and Health) technical guide. Although it is not considered a significant risk to laboratory workers or environment, it has been identified as an opportunistic human pathogen. *C. parapsilosis* can have cytotoxic effects on cells, i.e. due to its adhesion and biofilm formation capacity (Kuhn et al., 2004). It is also capable of attacking xenobiotic (synthetic) compounds, such as aged Paraloid B-72 (Bellucci et al., 1999), but there are no previous references to graffiti paint.

Rhodococcus erythropolis is an aerobic, non-sporulating, Gram-positive bacterium typically isolated from soil. It is classified as risk group 1 according to the CECT classification, based on INSHT technical guide, i.e. a low individual and community risk and unlikely to cause disease. Very few cases of infections with *R. erythropolis* have been described and only in immunosuppressed patients (Vernazza et al., 1991). *Rhodococcus erythropolis* has been used in the field of bioremediation due to its ability to break organic C–S bounds, allowing the removal of sulphur from fossil fuels and reducing petroleum viscosity (Olmo et al., 2005). *Rhodococcus* sp. is widely considered a potential bioremediation agent, with the capacity to metabolize organic pollutants such as hydrocarbons (Alvarez, 2010).

Viable microbial cell concentrations and visually detectable effects, such as turbidity, graffiti discoloration and biofilm formation, were markedly increased by the addition of Tween® 20. It caused changes between 20 and 40 CIELAB units for the three colour parameters (L*, a* and b*), while the changes without Tween® 20 for L* and b* slightly exceeded 5 CIELAB units, which is the threshold above which an unexperienced observer notices two different colors (Mokrzycki and Tatol, 2012) and for the a* parameter, the change was lower than 2 CIELAB units, below which only an experienced observer could notice the difference (Mokrzycki and Tatol, 2012).

This was expected because Tween® 20 is a non-ionic surfactant and emulsifier that is widely used as an additive in microbiological growth media, providing microorganisms with exogenous fatty acid, which can be used as an additional energy source and thus promoting and accelerating growth (Ando et al., 1959; Castro et al., 2003; Harterreiten-Souza et al., 2011). According to several authors (Castro et al., 2003; Partanen et al., 2001; Nielsen et al., 2016), Tween® 20 has a growth-promoting effect in bacterial and yeast strains due to i) the change in nutrient availability caused by the reduction in nutrient particle size and homogeneity, ii) the fact that microorganisms with lipase enzymes can use Tween® 20 as a nutrient, and iii) the fact that the surfactant helps nutrients enter cells by permeabilization of the cell membrane.

Although the visible effects of both *C. parapsilosis* and *R. erytropolis* were very similar, the FTIR results suggest that different degradative processes took place. The increase in the bands associated with O–H stretching vibration (mainly at 3400-3300 cm^{-1}) is usually related to oxidation processes in paints, with the subsequent formation of alcohols, carbonyls and peroxides (Melo et al., 1999; Pintus et al., 2016). In the photo-oxidative degradation of paints, the changes in this infrared region are mainly due to the formation of new alcohol groups along the fatty acid portion, either through β-scission or Norrish type I reactions. The increase in the carbonyl group band at 1625 cm^{-1} indicates the appearance of carboxyl acids and ketones and aldehyde production (Ploeger et al., 2008; Duce et al., 2014).

In the present study, the addition of Tween® 20 appeared to enhance the degradation caused by *C. parapsilosis* and to cancel out that caused by *R. erytropolis*. This may indicate that *R. erytropolis* is more versatile regarding the substrates it can utilize for growth (broad catabolic diversity). Therefore, when graffiti is the only available nutrient, *R. erytropolis* may activate the highly specific enzymatic pathways that enable it to biocatalyze and thus oxidise the graffiti compounds. However, when Tween® 20 is present, *R. erytropolis* may activate another high specific enzymatic pathway that allows it to use the detergent as a nutrient. This hypothesis is supported by the findings of previous studies, in which different catabolic pathways and unique enzyme functions (oxidation, dehydrogenation, epoxidation, hydroxylation, hydrolysis, dehalogenation and desulfurization) increase the biodegradation potential and versatility of substrate utilization in this bacterium (De Carvalho and Fonseca 2005; Laczi et al., 2015).

Furthermore, the visually notable graffiti discoloration and the associated FTIR changes were not correlated. A similar finding was also observed in a study of weathering of graffiti paints where chemical changes related to oxidation processes and characterized by infrared analysis and perceptible colour changes were not correlated (Sanmartín and Pozo-Antonio, 2020).

5. Conclusions

This feasibility study was oriented towards the search for novel strains of microorganisms, from naturally-aged graffiti, capable of acting as biocleaning agents of graffiti spray paints. Selected strains were assessed alone and in combination with Tween® 20. The latter involved a new strategy relied on the potential effect of the non-ionic detergent, which was successful because Tween® 20 enhanced the cell growth and caused a remarkable graffiti colour fading. According to findings, one bacterium (phylogenetically identified as *Rhodococcus erythropolis*) and one yeast (phylogenetically identified as *Candida parapsilosis*) were

selected for their potential capacity to degrade alkyd and polyester-based spray paint graffiti. As *Candida* strains are not considered safe for the purposes of this type of research, the authors recommend that future experiments should only be carried out with *R. erythropolis*.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2021.105206.

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