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ORIGINAL PAPER

Degradation of nitrocellulose-based paint by *Desulfovibrio desulfuricans* ATCC 13541

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Abstract Nitrocellulose is one of the most commonly used compounds in ammunition and paint industries and its recalcitrance to degradation has a negative impact on human health and the environment. In this study the capability of *Desulfovibrio desulfuricans* ATCC 13541 to degrade nitrocellulose as binder in paint was assayed for the first time. Nitrocellulose-based paint degradation was followed by monitoring the variation in nitrate, nitrite and ammonium content in the culture medium using Ultraviolet–Visible spectroscopy. At the same time cell counts and ATP assay were performed to estimate bacterial density and activity in all samples. Infrared spectroscopy and colorimetric measurements of paint samples were performed to assess chemical and colour changes due to the microbial action. Microscope observations of nitrocellulose-based paint samples demonstrated the capability of the bacterium to adhere to the paint surface and change the paint adhesive characteristics. Finally, preliminary studies of nitrocellulose degradation pathway were conducted by assaying nitrate- and nitrite reductases activity in *D. desulfuricans* grown in presence or in absence of paint. We found that *D. desulfuricans* ATCC 13541 is able to transform nitrocellulose as paint binder and we hypothesised ammonification as degradation pathway. The results suggest that *D. desulfuricans* ATCC 13541 is a good candidate as a nitrocellulose-degrading bacterium.

Keywords Nitrocellulose · Paint · Microbial degradation · *Desulfovibrio desulfuricans* · Sulphate-reducing bacteria

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Introduction

Nitrocellulose, one of the most important and oldest cellulose derivative, is principally used as binder in paint and a bulk ingredient in gunpowder formulations (Auer et al. 2005). In addition, many materials commonly used in daily life, such as printing inks, decorative films, and pharmaceuticals, contain nitrocellulose (El-Diwani et al. 2009; Souza et al. 2005). The explosive and toxic properties of nitrocellulose and its recalcitrance to degradation make the compound harmful for human health and the environment

(El-Diwani et al. 2009; Freedman et al. 1996; Souza et al. 2005). In this respect, although nitrocellulose wastes from industries (e.g. ammunition material and paint industries) and household hazardous waste, which consists of waste from a number of household products, contain increasing nitrocellulose content, only limited research effort into nitrocellulose waste treatment have been reported (Auer et al. 2005; El-Diwani et al. 2009; Freedman et al. 2002; Petrova et al. 2002; Slack et al. 2005; Souza et al. 2005).

Currently, the disposal of nitrocellulose is carried out by different processes, including membrane separation processes to enhance recovery of the fines, detonation, incineration and controlled low temperature thermal denitration, but these treatments produce dangerous airborne particulates and pan residues containing toxic materials (Auer et al. 2005; Freedman et al. 2002). Biological methods are used in the decontamination of pollutants because biotransformation could produce environmentally safe products at potentially lower costs in comparison to physical and chemical treatment processes (El-Diwani et al. 2009; Freedman et al. 1996) and therefore could be promising for nitrocellulose degradation (Freedman et al. 1996). Microorganisms are able to degrade nitrocellulose by two pathways: (i) cleavage of β-1,4-glucoside bonds that produces nitrooligosaccharides of various length, normally carried out by fungi (Auer et al. 2005; Freedman et al. 2002; Petrova et al. 2002; Tarasova et al. 2004), and (ii) nitrocellulose denitration that reduces the degree of nitro substitution, generally performed by bacteria (El-Diwani et al. 2009; Petrova et al. 2010; Tarasova et al. 2004, 2005). Since nitrooligosaccharides have mutagenic properties (Petrova et al. 2002), the second pathway is preferred over the first for exploitation as a biodegradation pathway. It has been reported that nitrocellulose undergoes degradation by methanogenic or sulphate-reducing bacteria under anaerobic conditions (Freedman et al. 2002; Petrova et al. 2010; Tarasova et al. 2005). Sulphate-reducing bacteria of the genus *Desulfovibrio* were reported to decrease the amount of nitrocellulose powder in media containing this compound (Petrova et al. 2010; Tarasova et al. 2009). The study by Petrova et al. (2006) reported the presence of nitrate in the culture media containing nitrocellulose, due to the nitroesterase activity of *Desulfovibrio desulfuricans* 1388. The subsequent nitrate disappearance and ammonium appearance indicated the dissimilatory reduction of nitrate to ammonium (Bursakov et al. 1997; Petrova et al. 2006; 2002; Tarasova et al. 2004), a process involving nitrate reduction to nitrite and the subsequent nitrite reduction to ammonium by nitrate- and nitrite reductases (Bursakov et al. 1997; Kraft et al. 2011; Moura et al. 2007). There are several sulphate-reducing bacteria able to reduce nitrate to nitrite (Marietou et al. 2009; Moura et al. 1997), but it appears that this is not a shared feature across the genus *Desulfovibrio* (Marietou et al. 2009; Tarasova et al. 2009). In contrast, the dissimilatory reduction of nitrite to ammonium seems to be widespread in *Desulfovibrio*, which can synthesise a very active, constitutive nitrite reductase (Marietou et al. 2009; Moura et al. 2007).

To date, studies on nitrocellulose biodegradation have been performed on nitrocellulose powder, with a percentage of nitro groups of being more than 12%, the percentage generally used in the military sector (Auer et al. 2005; Freedman et al. 2002; Petrova et al. 2002; Tarasova et al. 2009, 2004). To the best of our knowledge there are no reported studies on the biodegradation of nitrocellulose as component of daily life materials, like nitrocellulose-based paints. The biodegradation of paints is also interesting because substances other than nitrocellulose are present that could interfere with the degradation process. Although the bulk of a paint is pigment and binder, which is the film-forming material in which the pigment particles are dispersed and forms the matrix that binds the pigments on the painted surface, other components like metals, solvent and additives, are present in paint formulations (Cappitelli et al. 2005).

The main aim of this work was to study the capability of *D. desulfuricans* ATCC 13541 to attack nitrocellulose as binder in paint. Ultraviolet–Visible and infrared spectroscopy, microscope observations and reflectance colour techniques were used to correlate nitrocellulose degradation to specific chemical changes in the composition of the paint material. In addition, a preliminary study on the nitrocellulose degradation pathway in *D. desulfuricans* ATCC 13541 was conducted.

Materials and methods

Nitrocellulose-based paint and paint sample preparation

The 20th century paint binders are generally made of synthetic and semi-synthetic polymers including

nitrocellulose (Buzzini and Massonnet 2004; Govaert and Bernard 2004; Zieba-Palus 2005). The red spray paint by Motip–Dupli® Autocolor (colour 5-0200) was selected in this study since most of red spray paints are composed of alkyd-nitrocellulose-based binders, including those produced by Motip® (Govaert and Bernard 2004; Segalini et al. 2000). Autocolor paint samples were prepared spraying the paint on plain glass slides (Prestige, 7.6 × 2.6 cm). The can was firstly shaken for 3 min per manufacturer's instruction and then the paint sprayed by a moving rate of about 20 cm/s, maintaining nozzle at a distance of 30 cm above the glass slide and the manual pressure on spray valve fixed to allow a uniform spread (Segalini et al. 2000). The amount of paint sprayed was 5.15 ± 0.93 mg with a paint layer thickness <50 μm. The painted slides were dried for 4 months prior to their use in degradation experiments. For each experiment a constant ratio of 4.55 mm^2 of Autocolor paint per 1 ml of cultural medium was used.

Degradation experiment

The sulphate-reducing bacterium *D. desulfuricans* ATCC 13541 was selected for all the degradation experiments. *D. desulfuricans* was maintained anaerobically in DSMZ 63 medium (Cappitelli et al. 2006), while experiments were performed in modified medium containing half the standard sulphate content. Sulphates were not completely omitted as they may help nitrocellulose degradation (Marietou et al. 2009; Tarasova et al. 2009). Degradation experiments were performed in triplicate with the three following samples: *D. desulfuricans* cells in the culture medium and an Autocolor painted slide (sample A); the culture medium without microorganisms and an Autocolor painted slide (sample B), analysed to detect any contamination occurrence, and *D. desulfuricans* cells in the culture medium (sample C). The initial cell concentration was 10^7 cells/ml and the Autocolor painted slide was held static at 90°.

All samples were incubated for 49 days at room temperature under anaerobic conditions with a normal daily light–dark cycles and analysed at the beginning and at the end of the incubation period. To monitor bacterial density and activity in all samples, a cell count using the Thoma counting chamber and an ATP assay, performed according to Ranalli et al. (2003, 1998), except for the Tris-HCl buffer solution (10 mM, pH 7.75), were achieved.

Nitrate, nitrite and ammonia concentration tests and pH measurements

Nitrate, nitrite and ammonia contents in the culture medium were evaluated by Ultraviolet–Visible spectroscopy using a UV/Vis 6705 Spectrophotometer (Jenway, UK). Samples were firstly filtered using a 0.22 μm membrane (Millipore, Italy) to eliminate bacterial cells and precipitated FeS particles produced by them that could interfere with the readings. Nitrate and nitrite content was assayed according to APHA et al. (1998) except for nitrite assay, where the sample volume used has been 1 ml. Ammonia concentration was determined using the Nessler reagent (Merck, Germany). The colourimetric reaction was performed by adding 2 μl sodium–potassium tartrate and 20 μl Nessler reagent to 1 ml of sample. After 15 min, absorbance was evaluated at 420 nm. The nitrate, nitrite and ammonia nitrogen (NO_3^-–N, NO_2^-–N and NH_3–N, respectively) concentrations were calculated using calibration curves previously obtained.

pH measurement was performed using a pH indicator strip (Merck, Germany) at 25°C, in order to confirm the variation of the nitrogen forms in the culture medium of all samples.

Analysis of variance (ANOVA) via MATLAB software (Version 7.0, The MathWorks Inc., Natick, USA) was applied to evaluate statistically significant differences among samples A–C at the beginning (0 day) and at the end (49 days) of the experiment. Tukey honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Statistically significant results were depicted by p values <0.05.

FT–IR spectroscopy

To detect nitrocellulose presence, Autocolor painted slides not subjected to any treatment (sample D) were analysed. Autocolor painted slides, held in the medium with and without cells (sample A and B, respectively), were analysed using a Nicolet 6700 spectrophotometer equipped with a DTGS detector or coupled with a Nicolet Continuum FTIR microscope equipped with an HgCdTe detector cooled with liquid nitrogen. Samples were analysed in attenuated total reflectance (ATR) and as KBr pellets to verify the presence of organic compounds related to bacterial metabolism and changes in the paint chemical formulation.

FTIR analyses were recorded between 4,000 and 700 cm^{-1} (HgCdTe detector) or 4,000 and 400 cm^{-1} (DTGS detector) with 128 acquisitions and 4 cm^{-1} resolution. The spectra were baseline corrected using the Omnic software, and normalised, when necessary, by setting the absorbance of a specific peak to a desired value.

Prior to the analysis, Autocolor painted slides were washed three times with phosphate buffer, to remove any traces of culture medium and then dried for 1 month at room temperature, to further remove any surface-bound water molecules that could interfere with the spectroscopic measurements.

Determination of colour variations

Reflectance colour measurements of Autocolor painted slides from sample A were performed to assess the capability of *D. desulfuricans* to modify the colour of the spray paint. Autocolor painted slides not subjected to any treatment (sample D) were considered in this experiment. This sample, prepared in triplicate as described in "Nitrocellulose-based paint and paint sample preparation" subsection, was considered as the colour reference. Colour measurements were performed according to Sanmartin et al. (2011), carrying out five measurements at random positions on an area of approximately 9.88 cm^2 (3.8 × 2.6 cm) of samples A and D, by use of a Konica Minolta colourimeter with a CR–300 measuring head (8-mm-diameter viewing area). The colour measurements were analysed by considering the CIELAB colour system (CIE 1986), which represents each colour by means of three scalar parameters or Cartesian coordinates: L*, lightness or luminosity of colour, 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). Alternatively, each colour is represented by means of three angular parameters or cylindrical coordinates, most closely related to the psychophysical perception of the colour: L*, lightness or luminosity of colour, also defined in both scalar and angular colour sets; chroma $C*_{ab} = \sqrt{a*^2 + b*^2}$ related to the intensity of colour or saturation and hue angle $h_{ab} = \arctan(b*/a*)$ or tone of colour which refers to the dominant wavelength and indicates redness, yellowness, greenness, or blueness on a circular scale, starting at 0° and increasing counter clockwise (Wyszecki and Stiles 1982). Furthermore, the partial (ΔL^*, Δa^*, Δb^*, ΔC^*_{ab} and ΔH^*_{ab}) and the total (ΔE^*_{ab}) colour differences between samples A and D of the Autocolor painted slides were calculated. The total colour difference was expressed as $\Delta E*_{ab} = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2}$ or $\Delta E*_{ab} = \sqrt{(\Delta L*)^2 + (\Delta C*_{ab})^2 + (\Delta H*_{ab})^2}$, where ΔL^*, Δa^*, Δb^* and ΔC^*_{ab} represent respectively the differences between the values of L^*,a^*,b^* and C^*_{ab} belonging to samples A and D, and ΔH^*_{ab} is given by $\Delta H*_{ab} = 2 \cdot \sqrt{C*_1 \cdot C*_2} \cdot \sin(\Delta h_{ab}/2)$, where Δh_{ab} is the difference in hue angle $\Delta h_{ab} = \tan^{-1}(b*_2/a*_2) - \tan^{-1}(b*_1/a*_1)$ and the subscripts 1 and 2 represent samples D and A respectively.

Multivariate analysis of variance (MANOVA) and the Tukey-B multiple comparison test, via SPSS software (version 15.0) were applied to compare separately each CIELAB colour coordinates (L^*, a^*, b^*, C^*_{ab} and h_{ab}) among Autocolor painted slides of samples A and D. Statistically significant results were depicted by p values <0.05.

Stereomicroscope and epifluorescence microscope observations

Stereomicroscope observations of the Autocolor painted slides (samples A and B) were performed using a Leica M205C stereomicroscope equipped with a Leica DFC 290 video camera, to visualise macroscopic changes in the paint film and its adhesion on the surface of the glass slide. Morphological studies of the film were carried out at different magnifications.

To evaluate the *D. desulfuricans* capability to attach onto the paint surface, a DAPI staining of samples A and B was performed according to Giacomucci et al. (2011). Epifluorescence observations were carried out with a Leica DM4000B digital epifluorescence microscope equipped with Cool-Snap CF camera (Photometrics, Roper Scientific) and pictures were acquired using RS Image ver. 1.7.3 software (Roper Scientific).

Nitrate- and nitrite reductase activity evaluation

To evaluate nitrate- and nitrite reductase activity in *D. desulfuricans* incubated in the presence of

nitrocellulose as binder of Autocolor paint, the bacterium grew for 49 days at 25°C in DSMZ 63 medium modified as previously described in "Degradation experiment" subsection with or without Autocolor painted slides.

Total protein extraction and enzyme assay

Desulfovibrio desulfuricans cells were harvested by centrifugation at 7,000 rpm for 15 min at 4°C washed two times in phosphate buffer and suspended in 0.1 M phosphate buffer, pH 7.6, in a ratio 1/8 (weight/volume). Total proteins were extracted by sonication performed in five cycles of 30 s at 55% power and after 1 min of stop. The extract was centrifuged at 11,000 rpm for 30 min at 4°C to eliminate cellular debris. Protein concentration in the crude extract was determined using the Bradford method (Bradford 1976).

The nitrate reductase activity was assayed at 37°C in 0.5 M phosphate buffer at pH 7.6. The reaction mixture and the protocol were conducted as reported by Bursakov et al. (1997), except for the use of 15 mM KCN. The nitrate concentration was then measured as described in "Nitrate, nitrite and ammonia concentration tests and pH measurements" subsection. The nitrite reductase activity was assayed as by Liu et al. (1994), except for the termination of the reaction that was achieved by vigorously shaking of the reaction tube. The nitrite concentration was then measured as described in "Nitrate, nitrite and ammonia concentration tests and pH measurements" subsection. The enzymatic unit is defined by μmol of substrate reduced per minute. To evaluate statistically significant differences among enzymatic activities the *t* test, via Microsoft Excel tool for Windows, was applied. Statistically significant results were depicted by *p* values <0.05.

Results

Characterisation of Autocolor® paint

Autocolor film (Sample D) was analysed by FTIR spectroscopy for the identification of the main component of the polymeric blend. The main component of the resin is nitrocellulose and an alkyd fraction (a modified polyester resin) was also present. The characteristic and diagnostic FTIR peaks of nitrocellulose are related to the N–O vibration at 1,655 and 1,280 cm^{-1}, while the polyester resin has the characteristic vibration peaks at 1,735 cm^{-1} (ν C=O) and 1,073 cm^{-1} (ν C–O), both related to the ester group of the polymer.

The complete characterisation of other components, like pigment, stabiliser or other additives, was beyond the aim of this work.

Degradation experiment

Cell counts and ATP content of samples A and C carried out at both the beginning and at the end of the experiments were not significantly different (Fig. 1a, b). Investigations performed on sample B showed that there was no occurrence of contamination during the incubation period (Fig. 1b). UV–Vis spectrophotometric measurements performed in the culture medium showed a decrease in nitrate (Fig. 1c) and nitrite (Fig. 1d) concentration in samples A and C in comparison to sample B, where they were significantly higher and an increase in ammonia concentration in all three samples (Fig. 1e). The final ammonia concentration was not significantly different across all three samples (Fig. 1e). These results were in agreement with a pH increment in the same sample (data not shown).

FTIR and FTIR–ATR spectroscopy

Bacterial growth and the degradation phenomenon were analysed by FTIR spectroscopy in KBr transmission and ATR. Since transmittance FTIR on KBr pellets shows the absorbance of the bulk material, this was the most adequate technique to investigate the bacterial degradation of the Autocolor paint. As FTIR–ATR analyses give information about surface phenomena they showed that bacteria have colonised the polymeric surface.

Acquired spectra were baseline corrected and normalised on the C–H stretching area using the 2,960 cm^{-1} peak, related the CH_3 asymmetric stretching vibration of the polyester component, which is expected to be the more stable component.

The paint was partially affected by the prolonged immersion in the aqueous culture medium, visible by the slow reduction in the intensity of signal related to the nitric substituent (peaks at 1,655 and 1,280 cm^{-1}),

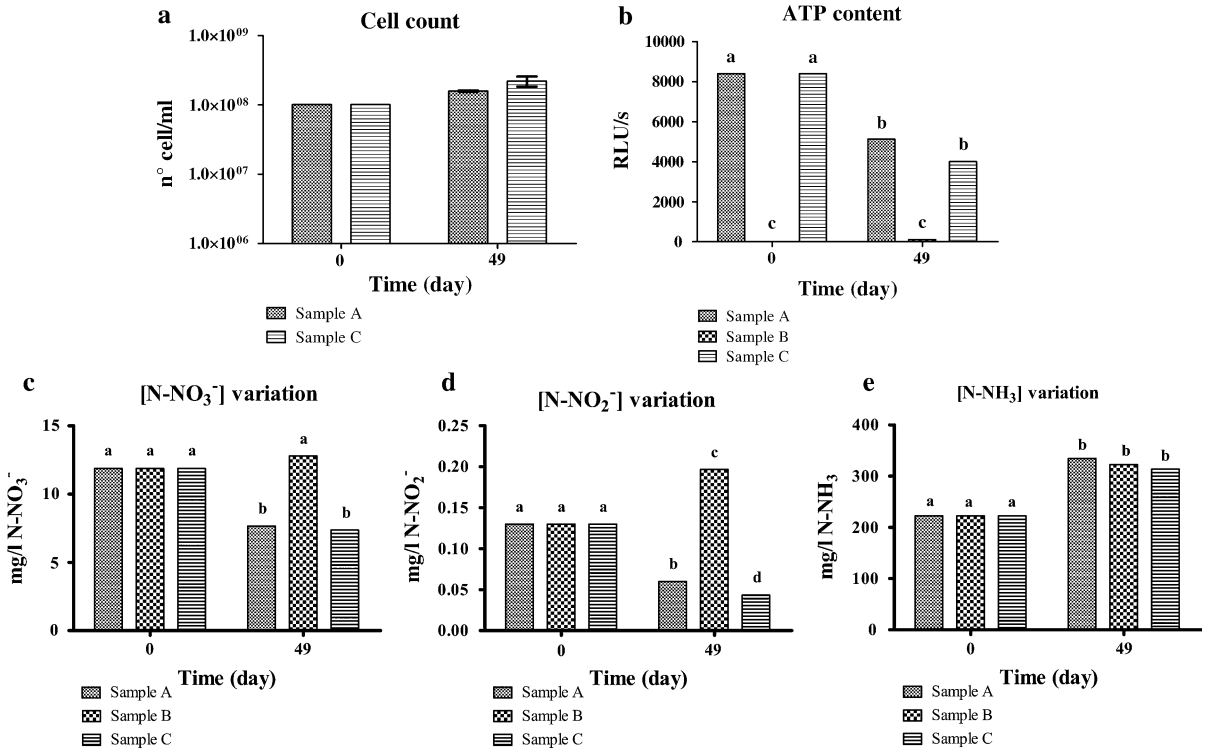


Fig. 1 Variation of cell concentration of samples A and C, ATP content and nitrogen forms concentration in the culture medium of samples A, B and C at the beginning (0 day) and at the end (49 days) of the experiments. **a** Cell count (*error bars* standard deviation), **b** ATP content (p value $= 5.55 \times 10^{-16}$), **c** nitrate nitrogen (p value $= 1.30 \times 10^{-13}$), **d** nitrite nitrogen (p value $= 0$), and **e** ammonia nitrogen (p value $= 2.45 \times 10^{-11}$) concentrations. The histograms represent average values from three independent replicates. According to post-hoc analysis, means sharing the same letter are not significantly different from each other

suggesting a partial abiotic denitrification process. Furthermore, the polyester component was also slightly affected by the prolonged contact with the buffer as was evident by the reduced intensity of the C=O stretching peak at 1,735 cm^{-1} and the C–O stretching vibration at 1,073 cm^{-1}, both related to the ester group of the main chain.

The comparison between sample D and sample A FTIR–ATR spectra showed an increase in signal related to amide groups at 1,540 cm^{-1} (see black arrow in Fig. 2a). The presence of proteinaceous material was also confirmed by the increase of signal in the area between 900 and 1,100 cm^{-1}, related to C–N and N–N stretching vibrations in nitrogen compounds. Furthermore, the relative intensity of the peak at 1,280 cm^{-1} (amide I signal) decreased due to degradation of the N–O substituent. This decrease was not observed for the peak at 1,655 cm^{-1} (amide II signal) for the concurrent increase of the amine products which signal falls in the same range (see green arrows in Fig. 2a). A noticeable increase in signal in the area from 3,000 to 3,700 cm^{-1} must be related to NH and OH stretching vibrations due to organic material.

The slight abiotic degradation recorded in the FTIR analysis of the KBr pellets was enhanced by the presence of bacteria which decreased the rate of nitrification of the cellulose-based paint leading to a considerable decrease of the signals at 1,655 and 1,280 cm^{-1} (see black arrow in Fig. 2b). The high specificity of the bacterial activity was also confirmed by the invariance of the C–N stretching peak visible at 840 cm^{-1} which remained stable during all degradation experiments. The degradation of the modified polyester component (alkyd resin) was slightly enhanced suggesting a partial bacterial degradation which was evident by the depletion of the signals related to the ester group (ν C=O at 1,735 cm^{-1} and ν C–O at 1,073 cm^{-1}).

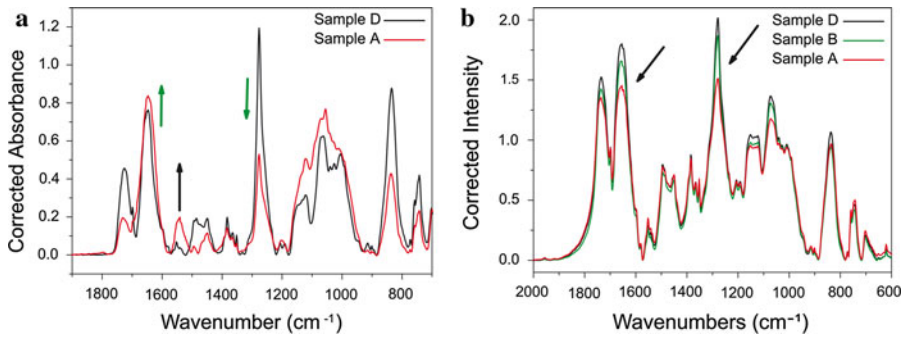


Fig. 2 **a** FTIR ATR spectra of the Autocolor film (sample D) and the degraded sample A in the range 1,900–700 cm^{-1}. *The arrows* variation in intensity related to bacterial compounds on the film surface. **b** FTIR spectra of the Autocolor film (sample D), of the paint incubated with the culture medium (sample B) and incubated in the presence of bacteria (sample A). *The arrows* diagnostic peak for the degradation of the nitro-substituent in the nitrocellulose molecules

Reflectance colour measurements

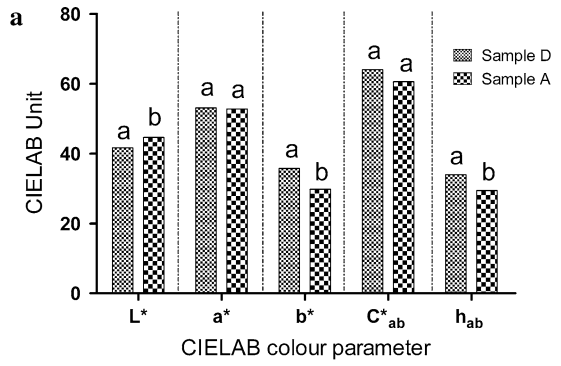
Reflectance colour measurements of Autocolor painted slides (samples A and D) were performed in order to study *D. desulfuricans* ATCC 13541 capacity to change the spray paint colour. The mean values of the CIELAB colour coordinates, using the Cartesian ($L^*a^*b^*$) and cylindrical ($L^*C_{ab}{}^*h_{ab}$) coordinates, in samples A and D are shown in Fig. 3a. Bacterial treatment led to a significant increase in lightness (L^*, from 41.6 ± 0.6 to 44.6 ± 0.7), a significant decrease in yellowness with a concomitant increase in blueness (b^*, from 35.8 ± 0.6 to 29.8 ± 1.0) and a significant decrease in the tone of colour (h_{ab}, from 34.0 ± 0.3 to 29.4 ± 0.4), which confirmed that *D. desulfuricans* ATCC 13541 caused Autocolor spray fading. To evaluate if these changes in colour were visible to the naked eye, the partial colour differences (ΔL^*, Δa^*, Δb^*, $\Delta C_{ab}{}^*$ and $\Delta H_{ab}{}^*$) and total colour difference ($\Delta E_{ab}{}^*$) in presence of *D. desulfuricans* cells were calculated (Fig. 3b). The visual colour difference threshold or just noticeable difference (JND), which constitutes the lower limit of perception in an individual with normal colour vision (Brown 1957; Macadam 1942) established as one CIELAB unit (Wyszecki and Stiles 1982), was not overcome by the partial difference of the redness–greenness parameter (Δa^*: 0.3 CIELAB units). The remaining colour differences exceeded this limit and also the value considered the general limit of perceptibility: three CIELAB units (Berns 2000; Prieto et al. 2010; Völz 2001), which is the upper limit of rigorous colour tolerance (Fig. 3b). Even taking into account a higher threshold of perception of 6 CIELAB units, considered an evident colour change (Hardeberg 1999), the overall change in colour or total colour difference ($\Delta E_{ab}{}^*$: 6.7 CIELAB units) overcame also this value, and therefore the colour change as a result of *D. desulfuricans* ATCC 13541 can be considered noticeable at first glance.

Stereomicroscope and epifluorescence microscope observations

Pictures of Autocolor painted slides (samples A and B) at the end of the degradation experiment are shown in Fig. 4. After bacterial treatment modifications on the surface of painted slides were visible (Fig. 4a, d), and further investigated by microscope observations (Fig. 4b, e). In particular, Autocolor painted slides treated with *D. desulfuricans* (sample A) showed paint detachment (Fig. 4b) while biologically-untreated Autocolor painted slides (sample B) showed no alteration in the adhesive characteristic of the paint (Fig. 4e). Moreover, epifluorescence microscopy observations of Autocolor painted slides after DAPI staining showed that *D. desulfuricans* was able to adhere to the surface of the paint layer of sample A (Fig. 4c) while in sample B (Fig. 4f) there was no fluorescence signal, confirming the absence of a microbial contamination.

Nitrate and nitrite reductase activity

The nitrate reductase activity was similar for *D. desulfuricans* incubated with or without Autocolor painted



b

	L*	a*	b*	C^*_{ab}	h_{ab}	
Sample D	41.6	53.1	35.8	64.0	34.0	
Sample A	44.6	52.8	29.8	60.6	29.4	
	ΔL*	**Δa***	**Δb***	**ΔC^*_{ab}**	**ΔH^*_{ab}**	**ΔE^*_{ab}**
	3.0	0.3	6.0	3.4	4.6	6.7

Fig. 3 **a** Mean CIELAB colour coordinates (CIELAB units). The histograms represent average values from five measurements from three independent Autocolor painted slides. *Different letters showed on the top of the histograms* statistically significant differences (p value <0.05). **b** Mean CIELAB colour coordinates (CIELAB units) and partial and total colour differences between samples D and A

slides, while nitrite reductase activity was significantly higher (p value <0.05) when *D. desulfuricans* was incubated with Autocolor painted slides (Table 1).

Discussion

Nitrocellulose wastes are recalcitrant to degradation and have a negative impact on human health and the environment (El-Diwani et al. 2009; Freedman et al. 1996; Souza et al. 2005) but there are bacteria that are able to remove the nitro groups from nitrocellulose, which renders the polymer residuals safer (El-Diwani et al. 2009; Petrova et al. 2010; Tarasova et al. 2004). To date, few studies have been conducted on nitrocellulose biodegradation and furthermore all those performed with bacteria have been conducted using nitrocellulose powder. In this study the capability of *D. desulfuricans* ATCC 13541 to degrade nitrocellulose as binder in paint was assayed for the first time. We selected *D. desulfuricans* ATCC 13541 principally because *Desulfovibrio* has been reported to be active against nitrocellulose (Petrova et al. 2002; Tarasova et al. 2004) and has been used in metal biosorption (Chen et al. 2000), and therefore the strain is resistant to the high concentrations of metals that can be encountered in paints. Long incubation times were used because Tarasova et al. (2005) observed a large decrease in nitro group content in nitrocellulose after 30–60 days of incubation.

In our studies, paint degradation was initially followed by evaluating changes in nitrate, nitrite and ammonium concentrations as previous studies of nitrocellulose degradation by *Desulfovibrio* spp., correlated these variations in nitrate and ammonium content to a nitroesterase activity (Petrova et al. 2006, 2002; Tarasova et al. 2009, 2004).

Infrared spectroscopy is a very good technique for paint analysis (Buzzini and Massonnet 2004; Cappitelli et al. 2005) and was previously used to investigate paint components and also nitrocellulose changes during degradation by *Desulfovibrio* spp. (Govaert and Bernard 2004; Segalini et al. 2000; Tarasova et al. 2005; Zieba-Palus 2005). The paint used was firstly selected as putative nitrocellulose-based paint and then confirmed as a nitrocellulose- and alkyd-based paint. The capability of *D. desulfuricans* ATCC 13541 to adhere onto the surface of the paint, assessed by epifluorescence microscopy observations was further confirmed by FTIR–ATR spectroscopy that showed the presence of proteinaceous material on the painted surface. *D. desulfuricans* degradation activity against Autocolor paint was assessed even if slight abiotic degradation of the paint, as a consequence of the long immersion time in the culture medium, was recognisable. The presence of bacteria acted with high specificity on the N–O bond of the nitro-substituted cellulose and implied a pronounced depletion of the related signal, suggesting that bacterial degradation favoured this chemical group compared to others.

After incubation with *D. desulfuricans*, we observed paint detachment and fading of Autocolor paint slides, both noticeable at first glance. Bacteria on painted surfaces can lead to paint detachment and discolouration (Cappitelli et al. 2009; Pepe et al. 2010). Although reflectance colour measurements were used to characterise paint colour objectively (Prieto et al. 2011), to date, no study concerning the colour change during paint biodegradation is available in the literature. In our case, although lightness increased significantly, greater changes occurred in other colour coordinates. The slightly increase in colour lightness can be explained by the ability of the metabolism of *D. desulfuricans* to convert sulphate to

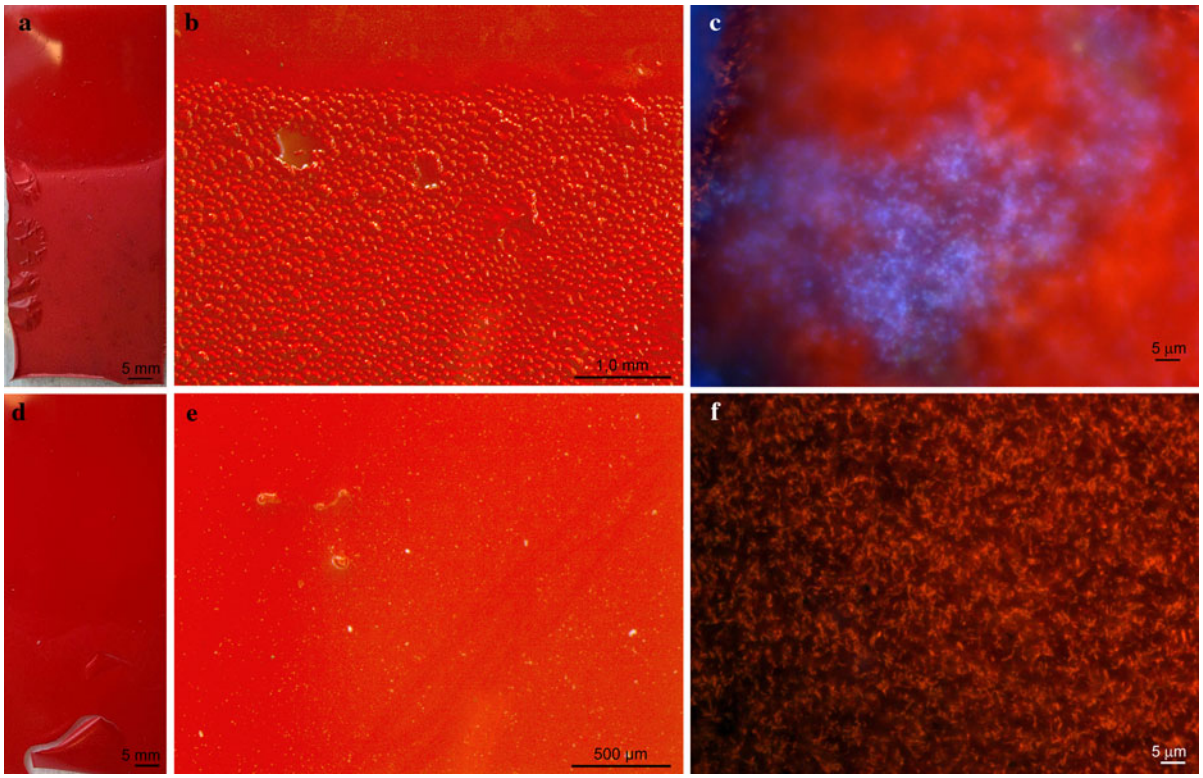


Fig. 4 Autocolor painted slides after 49 days of incubation with (**a**–**c**) or without (**d**–**f**) *D. desulfuricans* ATCC 13541 (samples A and B, respectively). **a**, **d** Pictures of Autocolor painted slides, the bottom half of the slide corresponds to the immersed part, **b**, **e** stereomicroscope observations and **c**, **f** epifluorescence observations after DAPI staining

Table 1 Enzyme activity values expressed in µmol of substrate reduced per minute

	Autocolor + *D. desulfuricans*	*D. desulfuricans*
Nitrate reductase activity	1089.91 ± 29.10	1059.33 ± 34.13
Nitrite reductase activity	87.09 ± 23.88*	35.18 ± 17.92

* Result of *t* test (p value <0.05), which was performed separately for nitrate and nitrite reductases activities

sulphides which, in the presence of iron (II) as a component of modified DSMZ 63 medium, form a black colour precipitate (Cappitelli et al. 2006). The colour fading of Autocolor paint, proved by changes in CIELAB colour parameters, could have been caused by the degradation of the paint binder, the removal of nitro groups from the nitrocellulose molecule and the degradation of other ingredients of the paint formulation.

According to Petrova et al. (2006, 2002) and Tarasova et al. (2004) nitrocellulose degradation by *Desulfovibrio* spp. occurs via dissimilatory nitrate reduction to ammonia. In this study, we found that the activity of nitrate reductase was equivalent in *D. desulfuricans* grown in the presence or in the absence of Autocolor paint. In the literature, there is little agreement concerning how nitrate reduction is regulated in sulphate–reducing bacteria, even in the well–studied *Desulfovibrio* genus (Marietou et al. 2009; Tarasova et al. 2009). Nitrate reductase genes are found only in some sulphate-reducing bacteria and various pathways of nitrate reduction regulation have been described (Marietou et al. 2009; Tarasova et al. 2009). In our experiments, we used a modified DSMZ 63 medium containing only half the normal concentration of sulphate, because four *Desulfovibrio* strains were reported to have constitutive nitrate reductase activity in media containing low concentrations of sulphate (Marietou et al. 2009; Tarasova et al. 2009). Other researchers (Keith and Herbert 1983; Seitz and

Cypionka 1986) reported that for *D. desulfuricans* Essex strain the presence of sulphate is essential for nitrate reduction to occur. In contrast, the study on *D. desulfuricans* FBA 20 by Mitchell et al. (1986) reported that nitrate reduction occurs only in the absence of sulphate. This diversity within one genus probably results from differences in the structure of nitrate reductases or from subunit combinations in different bacteria (Tarasova et al. 2009); moreover, growth conditions may affect the activity of this enzyme (Marietou et al. 2009). Our experiments showed higher activity of nitrite reductase in *D. desulfuricans* grown in the presence of Autocolor paints rather than in *D. desulfuricans* grown without paint. Nitrite reductase has been reported to be expressed constitutively and involved in respiratory nitrate ammonification in *Desulfovibrio* spp. that are able to grow using nitrate as terminal electron acceptor, e.g. *D. desulfuricans* (Simon 2002), and in detoxifying reactions in those species incapable of using nitrite as a terminal electron acceptor, e.g. *D. vulgaris* Hildenborough (Haveman et al. 2004; Pereira et al. 2000). More recently nitrite reductase was considered responsible for metal reductase activity (Barton et al. 2007), and this is an important feature for paint degradation as paint formulations include components such as pigments, additives and impurities, which may contain metals (Stoye and Freitag 1998).

In conclusion our findings proved that *D. desulfuricans* ATCC 13541 is able to degrade nitrocellulose as binder in paint and likely performs this degradation by the ammonification pathway.

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