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Reduction of pigment dispersions by *Shewanella* strain J18 143

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Abstract

In order to develop a biotechnological solution to the disposal of waste pigment dispersions, the ability of azo dye-reducing *Shewanella* strain J18 143 cells to reduce model pigment dispersions was assessed. Resting cells were able to couple the oxidation of formate to the reduction of the azo/ketohydrazone chromophore in virtually insoluble pigmentary species. Pigments from the azoacetoacetanilide range and the azonaphthol range were used to investigate the effects of process parameters such as dispersion quality, addition of biocides, temperature and use of exogenous extracellular redox mediators. The physical impact of bioreduction on the pigment structure was also assessed. Pigments present in industrially manufactured dispersions were more readily reduced than those in powder form and the presence of a biocide in the dispersion did not affect the activity of cells of *Shewanella* strain J18 143. The initial pigment reduction rate increased with temperature up to 50 °C, but the higher temperatures had a detrimental effect on the long term activity of *Shewanella* strain J18 143. The reduction of the pigment dispersions was stimulated by the addition of the soluble electron shuttle anthraquinone-2,6-disulphonate. Particle sizing and environmental scanning electron microscopy showed that *Shewanella* strain J18 143 was able to degrade large pigment aggregates to produce individual pigment particles. GemSperse Orange EX5 was the pigment dispersion reduced to the corresponding amines most efficiently by *Shewanella* strain J18 143.

Keywords: Bioreduction; Biodegradation; Bioremediation; Coloured waste

1. Introduction

Considerable attention has been given to issues associated with the presence of coloured compounds in aqueous wastewater emanating from colouration processes. Currently, the major methods of colour removal from the wastewaters involve physico-chemical process that can be costly and usually involve the formation of a concentrated sludge that creates a secondary, highly significant disposal problem [1]. Alternative, biodegradative systems based on colour removal through the use of whole bacterial cells have been shown to be highly effective [2]. Here, research activities have generally focused on redox transformations of dyes in solution. Under aerobic conditions, azo dyes are not easily metabolized [3]. However, under anaerobic conditions, several bacterial strains, including *Shewanella* strain J18 143, can enzymatically reduce the azo bond in the dye molecule to produce colourless, aromatic by-products [4,5]. Research into the biotransformation of coloured species, reported in the literature, has generally involved the reduction/oxidation of dyes in solution [2]. Some researchers have argued that the coloured species are required to pass through the cellular membrane, before the transformation can take place within the cytoplasm [6–8]. This intracellular mechanism would only be suitable for low molecular weight dyes with relatively few sulphonic acid substituents, whereas most commercial dyes are large, highly sulphonated molecules. An intracellular mechanism would also rule out

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any possibility of the direct biotransformation of solid azo bond containing species.

An alternative extracellular mechanism for the microbial reduction of coloured species has been reported in the literature [4,5,9]. This mechanism does not require the coloured species to be inside the cell, but instead involves the transport of electrons to the coloured species that are outside the cell, using a mechanism analogous to that required to reduce extracellular metals in other *Shewanella* strains [10]. Thus, *Shewanella* strain J18 143 cells have the potential to use the azo/ketohydrazone chromophore in solid coloured species, such as pigments, as an electron acceptor for anaerobic respiration. By definition, pigments will have (usually extremely) limited solubility in the medium in which they are applied. This has a marked influence on the strategies for colour removal from waste products and the current technology involves the use of a chemical coagulant which, with the addition of a polyelectrolyte, causes the coloured particles to form larger flocs. Settling tanks allow for the accumulation of these coloured flocs and the solid waste is then disposed of at landfill. In this research, a destructive treatment technology involving the use of *Shewanella* strain J18 143 cells to remove colour from wastewaters arising from pigment manufacture, and potentially from pigmented products (formulations, plastics, inks, prints, textiles, etc.) has been investigated. Model compounds from the azonaphthol range and the azoacetoacetanilide range have been used as these dominate the orange, yellow and red shade areas in commercial organic pigment manufacture [11]. The nitrogen–nitrogen bonds in these pigments are considered to exist almost exclusively in the ketohydrazone form (–N–NH–). Resting cell experiments were conducted to determine whether the pigment particles were reduced, and the physical impact of bioreduction on the pigment molecular structure. Particular emphasis was placed on the impact of the initial pigment size on bioreduction, as well as process parameters including temperature and the addition of exogenous extracellular redox mediators.

2. Materials and methods

All of the chemicals used were of analytical grade and obtained from Sigma–Aldrich (Dorset, UK), unless otherwise stated. Pigment powders and dispersions were obtained from Gemini Dispersions Ltd. (Lancashire, UK). The structures of the pigments, C.I. Pigment Orange 5, C.I. Pigment Yellow 74 and C.I. Pigment Red 112, which are used in the respective dispersions GemSperse Orange EX5, GemSperse Yellow EX74 and GemSperse Red EX112 are shown in Fig. 1. The pigment dispersions contained anionic surfactants such as a naphthalene sulfonic acid/formaldehyde condensate, along with the biocides Acticide® OTW and Acticide® AFM, obtained from the Thor Group Ltd. (Kent, UK).

2.1. Organism

Strain J18 143 is a facultative anaerobic bacterium that was originally isolated from soil contaminated with textile wastewater. A preliminary identification of the culture as a *Shewanella* species was based upon biochemical tests performed by the National Collections of Industrial and Marine Bacteria (NCIMB) Ltd. (Aberdeen, UK), and confirmed by 16S rRNA sequencing [5]. It was deposited at the ECACC on 19 August 1996 under the provisions of the Budapest Treaty; accession number 96081914 [12].

2.2. Culture conditions

Aerated starter cultures were grown overnight in 250 cm^3 Erlenmeyer flasks that were fitted with foam bungs, containing 100 cm^3 of sterile tryptone soy broth (TSB, 30 g dm^{-3}; Lab M, International Diagnostics Group plc). Flasks were incubated overnight at 30 °C, with shaking at 200 rpm. A 9% (vol/vol) inoculum from the starter culture was used in all experiments. Oxygen-limited growth cultures were grown in 100 cm^3 bottles (Adelphi (Tubes) Ltd.), sealed with 20 mm butyl rubber injection stoppers, containing 90 cm^3 of sterile TSB (30 g dm^{-3}). The bottles were incubated for 4 h at 30 °C. The growth of the *Shewanella* strain J18 143 cultures was determined by measurements of the absorbance at 600 nm using a Specord S100 spectrophotometer. The anaerobic cells were harvested by centrifugation (1771 × g for 10 min at ambient temperature using an MSE Centaur 2 centrifuge) and washed twice in 10 mM phosphate buffer supplemented with 0.1 M NaCl (pH 7.0, phosphate buffered saline; PBS). The washed anaerobic cells were suspended in the same buffer to an absorbance (at 600 nm) of 5.0.

2.3. Pigment reduction using resting cells

To determine the ability of the *Shewanella* strain J18 143 cells to reduce the azo/ketohydrazone bonds that were present in virtually insoluble pigment molecules, C.I. Pigment Orange 5, C.I. Pigment Yellow 74 and C.I. Pigment Red 112, powders were dispersed in PBS (10 mM, pH 7) using an ultrasonic bath, without the addition of surfactant. The industrially manufactured pigment dispersions, GemSperse Orange EX5, GemSperse Yellow EX74 and GemSperse Red EX112, both with and without biocide (Acticide® OTW and Acticide® AFM), were also dispersed in PBS (10 mM, pH 7). The respective pigment dispersions were added to a series of anaerobic cuvettes, to a final concentration of 50 μM. A sodium formate solution was added to the cuvettes as the electron donor (final concentration, 21 mM). To determine the effect of an exogenous redox mediator on dye reduction, the humic analogue anthraquinone-2,6-disulphonate (disodium salt) was added to a final concentration of 100 μM. The cuvettes were sealed and the headspace was filled with nitrogen. Anaerobic cell suspensions (5% of assay volume) were injected, by use of a syringe that was fitted with a hypodermic needle, into the cuvettes. The cuvettes were incubated at 30 °C, without shaking, but were agitated immediately prior to sampling to re-suspend the pigment particles. When determining the effect of temperature on GemSperse Orange EX5 reduction in the presence of AQDS, cuvettes were incubated at 30 °C, 40 °C

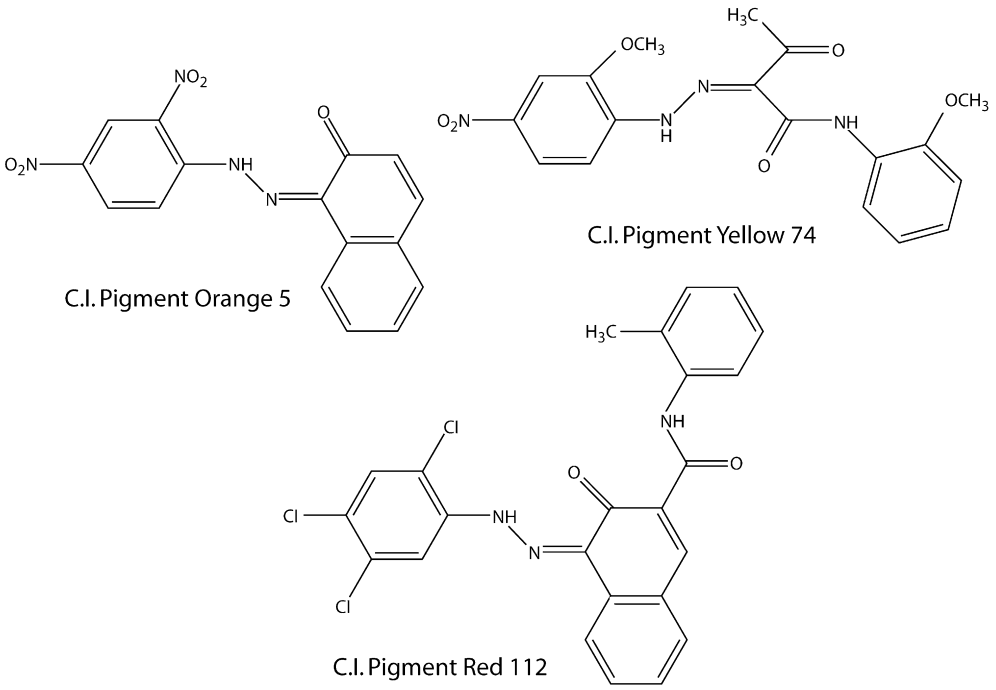

Fig. 1. Structures of C.I. Pigment Yellow 74, C.I. Pigment Red 112 and C.I. Pigment Orange 5.

and 50 °C. The absorbance of the dispersions was measured periodically at the λ_{max} for C.I. Pigment Orange 5 (500 nm) using a Specord S100 spectrophotometer. Calibration curves for dispersions of GemSperse Yellow EX74, GemSperse Red EX112 and GemSperse Orange EX5 were constructed by measuring the absorbance, at the λ_{max} for each pigment, of a series of pigment dispersions containing known concentrations of the pigment. The straight line graphs produced indicated that any possible molecular aggregation effects in the pigment dispersions did not result in a deviation from the Lambert–Beer law over the range of concentrations used. The calibration curves were used to ascertain the actual amount of each pigment, in micromoles, that was reduced by the *Shewanella* strain J18 143 cells.

2.4. Particle size analysis during pigment reduction using resting cells

To analyse the size of the pigment particles, dispersions of GemSperse Orange EX5 were added to a series of autosampler vials (size 10 cm^3) to a final concentration of 100 μM. Sodium formate was added to the vials as the electron donor (final concentration, 21 mM). The reaction mix was diluted to 9.0 cm^3 using PBS, the vials sealed and the headspace filled with nitrogen. The anaerobic cell suspension (5% of assay volume) was injected, by use of a syringe that was fitted with a hypodermic needle, into the sealed anaerobic vials. The vials were incubated at 30 °C. Aliquots were taken at 0 h, 2 h, 4 h and 20 h and the absorbance of the dispersions was measured. To determine the size of the pigment particles, the dispersions were measured using a Coulter model N4 sub-micron particle size analyser. The pigment dispersions were also analysed using a Phillips XL30 ESEM ODP scanning electron microscope. Sample preparation, involving centrifugation and re-suspension in deionised water, was carried out prior to ESEM analysis to remove salt crystals formed from the evaporation of the buffer solution.

All experiments were done in triplicate, and representative data are shown throughout. Standard errors were within ±10% of the mean.

3. Results

3.1. Reduction of C.I. Pigment Orange 5, C.I. Pigment Yellow 74 and C.I. Pigment Red 112 using Shewanella strain J18 143

The ketohydrazone bonds in the 'raw' pigment powder would be expected to be the least available to the *Shewanella* strain J18 143 cells due to the relatively large particle size of the pigment particles. The quality of the C.I. Pigment Orange 5, C.I. Pigment Yellow 74 and C.I. Pigment Red 112 dispersions prepared in the laboratory was relatively poor, owing to the limitations of the dispersion equipment, relative to industrial dispersion units. The insoluble nature of the pigment particles resulted in a relatively low absorbance value, even before exposure to the cells and only a small reduction in the visible absorbance of the pigment dispersions occurred over the period of measurement. However, a substantial change was observed in the absorbance in the UV region of the spectra, as can be seen in Fig. 2 for C.I. Pigment Red 112. This can be explained in terms of the difference in solubility of the original pigments and the products of reduction. The pigments were virtually insoluble making the absorbance

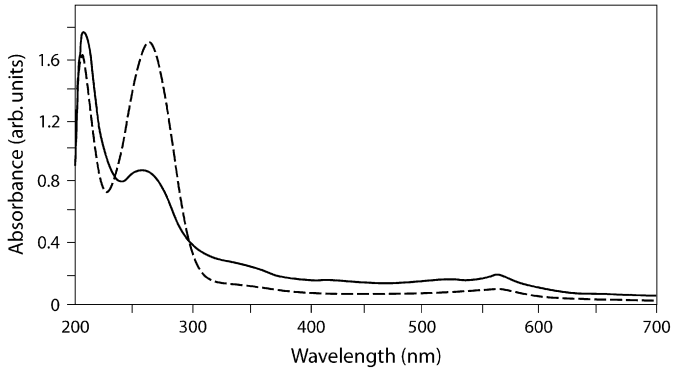

Fig. 2. UV–visible spectra for a dispersion of C.I. Pigment Red 112 after 0 h (—) and 162 h (– – –) incubation at 30 °C with *Shewanella* strain J18 143.

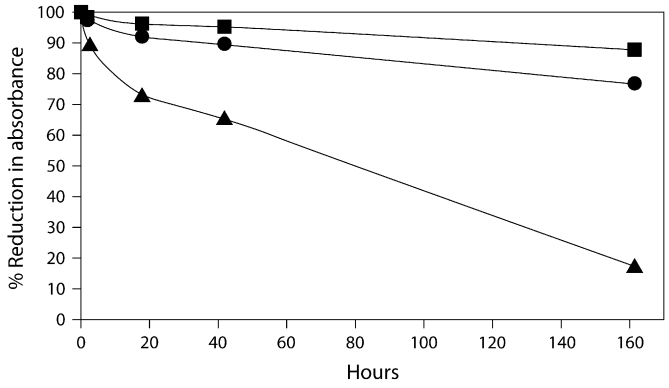

Fig. 3. Reduction of GemSperse Yellow EX74 (■), GemSperse Red EX112 (●) and GemSperse Orange EX5 (▲) pigment dispersions using anaerobic resting cell suspensions of *Shewanella* strain J18 143 in PBS at 30 °C, supplied with sodium formate. Absorbance data were normalized using data from a standard that contained no pigment.

difficult to measure, but the reduction products were smaller, more soluble molecules with a strong absorbance at 200–300 nm, which is consistent for aromatic amines [13]. These results suggest that the *Shewanella* strain J18 143 cells can remove the source of the colour produced by pigment molecules in which the nitrogen–nitrogen bonds are considered to exist almost exclusively in the ketohydrazone form (–N–NH–), implying either that the cells can reduce the ketohydrazone bond as well as the azo bond, or that an equilibrium exists between the azo form and the ketohydrazone form of each bond within the pigment molecules.

3.2. Reduction of GemSperse Orange EX5, GemSperse Yellow EX74 and GemSperse Red EX112 pigment dispersions using Shewanella strain J18 143

In the industrial manufacture of dispersions such as GemSperse Orange EX5, GemSperse Yellow EX74 and GemSperse Red EX112, the size of the pigment particles is reduced in an industrial ball mill, thereby maximizing colour strength and resulting in an increase in the initial absorbance at the λ_{max} for the pigment dispersions. Surface-active agents (surfactants) and biocides are also added during the milling process to improve the quality of the dispersions, and to protect the pigment dispersion from bacterial attack, respectively. The bioavailability of the azo/ketohydrazone bonds in these pigment dispersions should be increased compared with that of the pigment agglomerates developed in the laboratory-prepared dispersions, resulting in a faster rate of reduction, provided that the additives do not have a detrimental effect on the viability of the *Shewanella* cells. There was a small decrease in the absorbance of both the GemSperse Yellow EX74 dispersion and the GemSperse Red EX112 dispersion over the 162 h period, but a more significant decrease was observed with the GemSperse Orange EX5 dispersion, suggesting that the orange pigment molecules in the industrial dispersion have an increased level of bioavailability (Fig. 3). These results can be explained in terms of the structure of the yellow, red and orange pigment molecules, respectively. C.I. Pigment Orange 5, the pigment in GemSperse Orange EX5, is a member of the monoazonaphthol series, existing in the ketohydra zone form. Azonaphthols show good light fastness due to the presence of a nitro group, ortho to the hydrazone function that is capable of participating in an intramolecular hydrogen bond. However, it has been observed that, with the azonaphthol series, the intermolecular interactions involve only van der Waals' forces, which result in poor resistance to organic solvents and may also influence the susceptibility of the pigments to microbial degradation [11]. Therefore, the improved rate of bioreduction with GemSperse Orange EX45 may be a consequence of the relatively poor intermolecular interactions in the dispersion. Also, it must be considered that, in contrast to the other pigments in the experiment, the C.I. Pigment Orange 5 molecule is relatively small and has a simple molecular structure that is similar to that of many azo dyes.

C.I. Pigment Yellow 74, the pigment in GemSperse Yellow EX74, is a member of the Hansa Yellow monoazoacetoacetanilide series, also existing in the ketohydrazone tautomeric form, with a planar configuration. C.I. Pigment Yellow 74 has high colour intensity. This enhanced colour strength is due to the presence of a nitro group para to the hydrazone function, rather than in the ortho position. Azoacetoacetanilides show good light fastness due to the high stability that results from the extensive intramolecular hydrogen bonding, in which the nitro and amide groups participate. In the case of C.I. Pigment Yellow EX74, the intermolecular interactions involve weak hydrogen bonding as well as van der Waals' forces, which result in improved solvent resistance and potentially reduced susceptibility to microbial degradation.

C.I. Pigment Red 112, the pigment in GemSperse Red EX112, is a member of the Naphthol Red azonaphthol series and has also been shown to exist in the ketohydrazone form. C.I. Pigment Red 112 shows extensive intramolecular hydrogen bonding, in which the chloro substituent that is ortho to the hydrazone function may play some part. The Naphthol Red series exhibits increased molecular size and an amide functionality, which results in improved solvent resistance and possibly improved resistance to microbial degradation. The three chloro substituents in the molecule, incorporated

to enhance fastness properties, which are not present in other pigments, may be toxic to the *Shewanella* strain J18 143 cells. Indeed, Hu [14] noted that chlorine substituents had an inhibitory effect on microbial respiration.

These results are in agreement with those of Rau et al. [15] who found that differences in the degree of degradability of certain azo compounds were due to the different structures of the molecules containing the azo group, or to the reduced availability of fewer azo groups within larger, more complex azo compounds. As the pigment in GemSperse Orange EX5 exhibited the fastest rate of biodegradation, it was used in subsequent assays to optimise microbial pigment reduction.

3.3. Optimisation of pigment reduction in GemSperse Orange EX5 pigment dispersions using anaerobic resting cell suspensions of Shewanella strain J18 143

Pigmented systems are placed in demanding environments that often involve exposure to microorganisms. Under these circumstances, pigment–binder surfaces are particularly susceptible to fungal growth. The incorporation of the biocides Acticide® OTW and Acticide® AFM, which are based on isothiazolinones, in the pigmented binder system decreases the susceptibility of the pigment–binder combination to attack by microorganisms. The biocides are designed to counteract microbial activity and, as a consequence, may be detrimental to the viability of the *Shewanella* strain J18 143 bacterial cells in the reduction experiments. The rate of pigment reduction in GemSperse Orange EX5 was 11 μmol pigment g^{-1} (biomass) h^{-1} for the dispersion with biocides versus 16 μmol pigment g^{-1} (biomass) h^{-1} for the dispersion without biocides. Therefore, the biocides did not completely inhibit the activity of the *Shewanella* strain J18 143 cells, suggesting that bioreduction through this route is feasible even in the presence of potent inhibitors of microbial metabolism. The industrial pigment dispersions, including the biocides, were used in subsequent assays. Acticide® OTW is specifically targeted at protection from fungal growth and would not have a detrimental effect on the activity of the *Shewanella* strain J18 143 bacterial cells. Acticide® AFM is an anti-microbial agent and will be active at the concentration present in the original pigment dispersion but will be rendered less effective when it is diluted in wastewater streams and in the reduction assays described here, especially when considering the high concentration of cells present. Also, it is known that Gram-negative, rod-shaped bacteria are generally more resilient to these biocides than are other microbes.

The insoluble pigment particles cannot be transported into the microbial cell and are not free to move around in the solvent matrix. Therefore, the addition of a soluble extracellular electron shuttle should facilitate microbial pigment reduction by providing an efficient mechanism to transfer the reducing power generated by the internal metabolism to the external, insoluble pigment.

Fig. 4 shows the change in absorbance of GemSperse Orange EX5 pigment dispersion with AQDS and without AQDS, at a range of temperatures. The rate of reduction of GemSperse Orange EX5 was four times faster in the presence of the soluble electron shuttle AQDS (51 μM pigment g^{-1} (biomass) h^{-1} versus 14 μM pigment g^{-1} (biomass) h^{-1}). The results support the

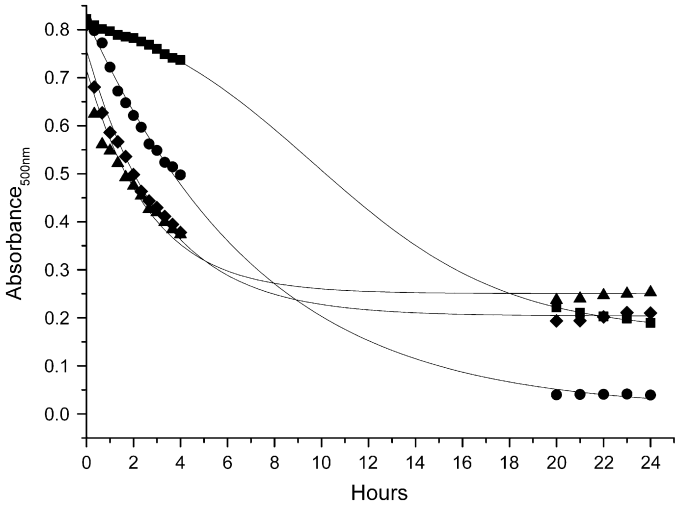

Fig. 4. Reduction of GemSperse Orange EX5 using anaerobic resting cell suspensions of *Shewanella* strain J18 143, in PBS, supplied with sodium formate, at 30 °C without AQDS (■), and at 30 °C (●), 40 °C (◆) and 50 °C (▲) with AQDS. Absorbance data were normalized using data from a standard that contained no pigment.

hypothesis that the transfer of reducing equivalents from the reductase enzyme to the pigment molecule, a rate-limiting step, can be made more efficient by the addition of AQDS. The rate of reduction of GemSperse Orange EX5 was more than twice as fast when the temperature was increased from 30 °C to 40 °C (117 μM pigment g^{-1} (biomass) h^{-1} versus 51 μM pigment g^{-1} (biomass) h^{-1}). Therefore, increasing the temperature increased the initial reducing activity of the *Shewanella* strain J18 143 and increased the rate of recycling of the electron shuttle. However, this level of enhancement in the reduction rate was not maintained at higher temperatures than 40 °C and the reduction rate at 50 °C (138 μM pigment g^{-1} (biomass) h^{-1}) was quite similar to the reduction rate at 40 °C (117 μM pigment g^{-1} (biomass) h^{-1}). Furthermore, after the 24 h incubation period, the absorbance of the reduced pigment dispersion incubated at 50 °C remained relatively high (0.25). The absorbance at 40 °C was slightly lower (0.21) and the absorbance at 30 °C was lower still (0.039). This suggests that the higher temperatures had a detrimental effect on the long term activity of the *Shewanella* strain J18 143 cells. These results are in agreement with those obtained for the microbial reduction of Remazol Black B [5], which suggested that the viability of the cells was not maintained over the time period at the higher temperatures. Therefore, the cells remained viable for longer at 30 °C and were able to degrade the GemSperse Orange EX5 dispersion further, resulting in a lower final absorbance.

3.4. Analysis of products obtained from the reduction of GemSperse Orange EX5 pigment dispersion, using anaerobic resting cell suspensions of Shewanella strain J18 143

The products obtained from the microbial reduction of a GemSperse Orange EX5 pigment dispersion using *Shewanella*

strain J18 143 cells were analysed and compared with the products obtained from the chemical reduction of the pigment dispersions using sodium dithionite. After the 7 day incubation period, the pigment dispersion that was treated with sodium dithionite remained noticeably orange coloured, whereas the pigment dispersion that was treated with the cells was relatively colourless. The difference in the visual appearance of the two dispersions indicates that the chemical reduction of the orange pigment in GemSperse Orange EX5 was only marginally successful, whereas the *Shewanella* strain J18 143 cells were able to successfully reduce the orange pigment and remove the colour.

The UV–visible spectra for the pigment dispersion that was treated with sodium dithionite and the pigment dispersion treated with *Shewanella* strain J18 143 cells are shown in Fig. 5. Despite the high absorbance in the UV region after the 7 day incubation period, suggesting the formation of some colourless reduction products, there was still a substantial visible absorbance at the λ_{max} for GemSperse Orange EX5 (500 nm) in the dispersions that were treated with sodium dithionite (A). In contrast, after incubation with the *Shewanella* strain J18 143 cells, the visible absorbance was almost zero (B). The high absorbance in the UV region was consistent with the formation of a quantity of aromatic amine reduction products. However, apart from the differences in the intensity of the peaks, the peaks in the UV region in Fig. 5(A and B) are at the same wavelengths (190 nm, 300 nm and 329 nm). Chemical reduction using sodium dithionite involves the destruction of the ketohydrazone chromophore in the pigment molecule by reductive cleavage of the nitrogen–nitrogen bond [16]. From the similar shape of the UV–visible spectra (Fig. 5) it can be suggested that *Shewanella* strain J18 143 cells also remove the colour from the pigment dispersions by this reductive cleavage mechanism, but to a greater extent than the chemical agent. A possible explanation for the increased effectiveness of the biological mechanism is provided in Fig. 6. If the *Shewanella* strain J18 143 cells were also capable of degrading the organic matrix of the orange pigment particles, reducing their size, prior to the actual degradation of the chromophore, it would increase the availability of the ketohydrazone bonds and improve the extent of biological reduction, compared with the chemical reduction process, which involved reductive cleavage alone.

The aromatic amines, given in Fig. 6, 2,4-dinitro aniline and 1-amino-2 naphthol, are the expected products of the reduction of the pigment in GemSperse Orange EX5, using anaerobic resting cell suspensions of *Shewanella* strain J18 143 as a result of previous work involving microbial reduction of azo dyes [5]. A sample of GemSperse Orange EX5 that had been biologically reduced over a period of 7 days at 30 °C, and centrifuged to remove the cells, was exposed to air, to give an indication of the presence of these aromatic amines. The effect of biological reduction, followed by oxidation, on the visual appearance of a sample of GemSperse Orange EX5 dispersion can be seen in Fig. 7. Aromatic amines, particularly naphthol amines, are known to be spontaneously unstable in the presence of oxygen and will rapidly form brown polymerisation products [17]. Even the pure chemicals deteriorate and become coloured upon storage. Therefore, when the reduced GemSperse Orange EX5 dispersion was allowed to become aerobic, it would be expected for the hydroxyl groups and the amino groups in the reduction products to oxidize to quinone and quinone imine moieties, respectively [18]. These compounds are capable of dimerisation or polymerisation to give dark coloured reaction products. Indeed, it was noted that, over a relatively short period of time, a very dark, murky brown colour was obtained, presumably due to the development of a variety of new chromophores (Fig. 7). The new chromophores were not the result of the reformation of the azo bond as the colour was quite different from that of the original

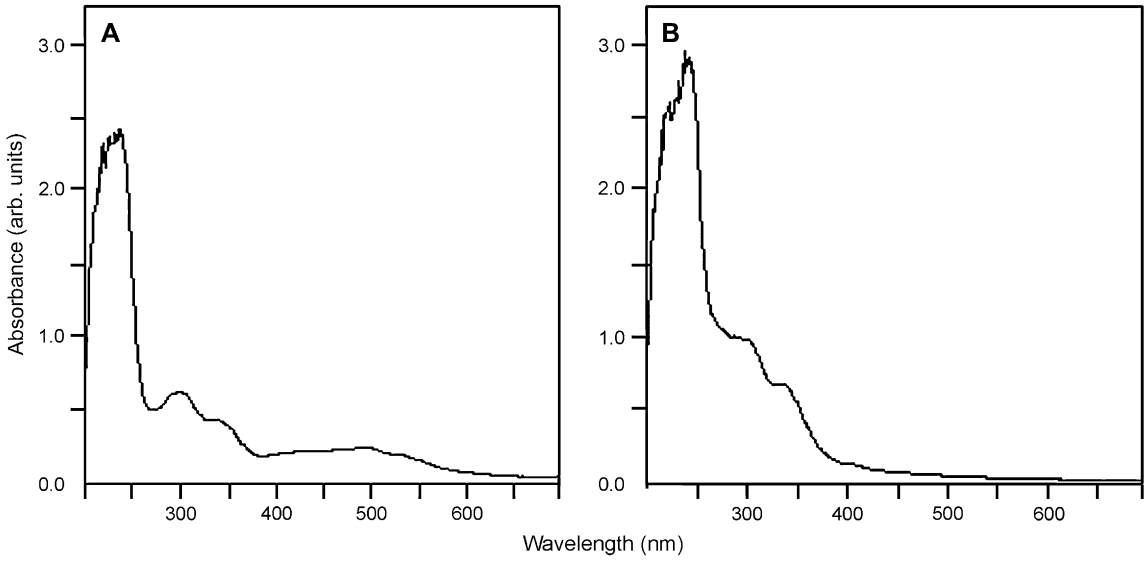

Fig. 5. UV–visible spectra for dispersions of GemSperse Orange EX5 after 7 days incubation at 30 °C with sodium dithionite (A) and *Shewanella* strain J18 143 (B).

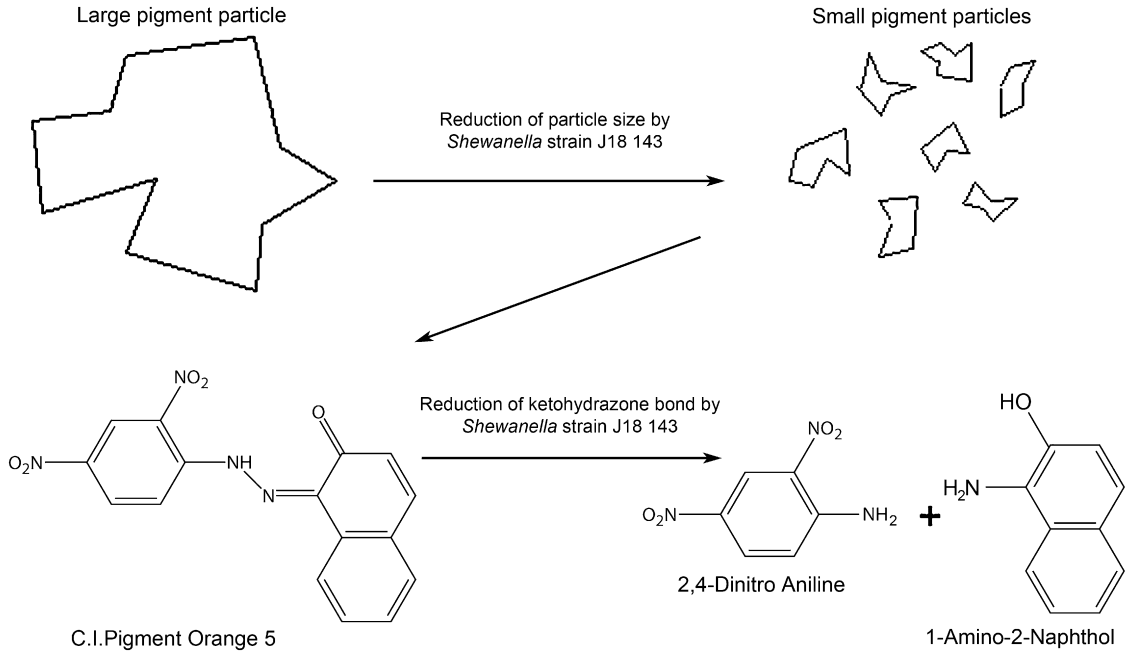

Fig. 6. Suggested possible mechanism for the reduction of the orange pigment in GemSperse Orange EX5 pigment dispersion using resting cell suspensions of *Shewanella* strain J18 143.

pigment dispersion. Knapp and Newby [19] also observed this darkening phenomenon, with samples of decolourised wastewater that had contained a diazo-linked chromophore. The production of dark colours occurred in response to the presence of oxygen and was due to the spontaneous oxidation of some of the reduction products in the wastewater. These auto-oxidation reactions would not present a significant problem in an industrial treatment process, as the reduction products can be biodegraded under aerobic conditions in the presence of activated sludge, for example at a municipal sewage treatment works [20].

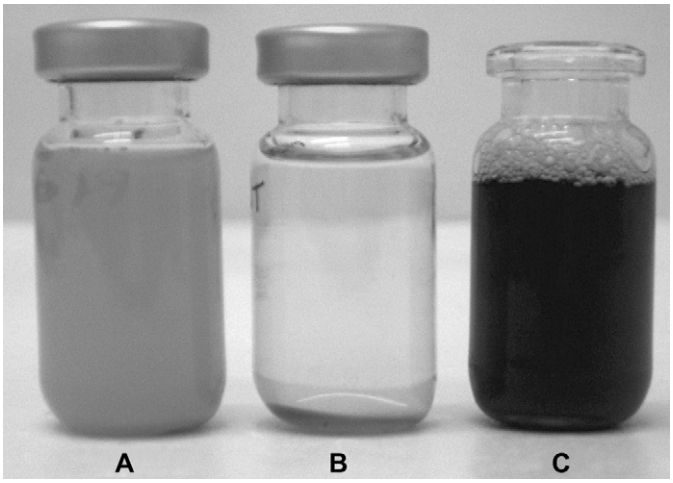

Fig. 7. Photographs of original GemSperse Orange EX5 dispersion (A), reduced pigment dispersion after incubation with cells (B) and reduced pigment dispersion after centrifugation and oxidation (C).

3.5. Effect of anaerobic resting cell suspensions of Shewanella strain J18 143 on the particle size distribution of GemSperse Orange EX5 pigment dispersions

The results from the Size Distribution Processor (SDP) particle size analysis, which provides a histogram of the sample particle size distribution, calculated in terms of an intensity distribution, showed that the particle size distribution of the anaerobic resting cell suspension of *Shewanella* strain J18 143 with AQDS, but no added pigment dispersion, did not change significantly over the 20 h incubation period (Fig. 8A and B). The size of the majority of the particles in this sample corresponded to the size range of the *Shewanella* strain J18 143 cells, which are approximately 1 μm wide and 2–5 μm in length. These results indicate that any change in the particle size distribution of the sample of GemSperse Orange EX5 with *Shewanella* strain J18 143 cells and AQDS was not a measurement of changes in the size of the cells themselves. The size distribution of the pigment particles in the GemSperse Orange EX5 pigment dispersion with AQDS before incubation with cells was quite broad, ranging from 0.1 μm to 1 μm (Fig. 8C). The smaller fraction of this size distribution is consistent with the target primary mean particle size of pigment particles in industrially manufactured dispersions. However, the presence of larger particles in the dispersion indicated some degree of aggregation. There was a shift towards the larger particle size during the incubation period, suggesting the occurrence of further aggregation of the pigment particles with time (Fig. 8D). There was also a significant change in the particle size distribution of the GemSperse Orange EX5 pigment dispersion with *Shewanella*

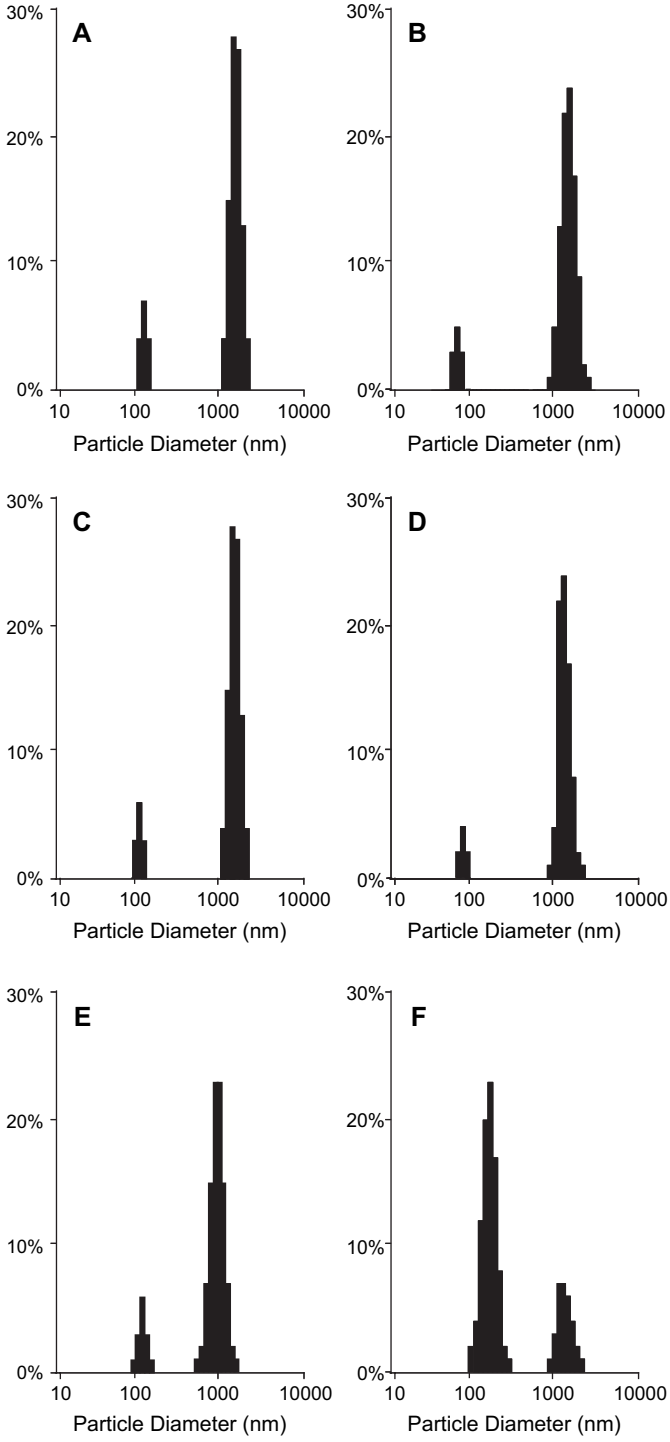

Fig. 8. Particle size analysis of *Shewanella* strain J18 143 cells with AQDS, before (A) and after (B) 20 h incubation at 30 °C, GemSperse Orange EX5 with AQDS before (C) and after (D) 20 h incubation at 30 °C and GemSperse Orange EX5 with *Shewanella* strain J18 143 cells and AQDS before (E) and after (F) 20 h incubation at 30 °C.

strain J18 143 cells and AQDS, over the 20 h incubation period. Before incubation, the size of a substantial proportion of the particles in the dispersion was 1–5 μM (Fig. 8E). These larger particles consisted of the *Shewanella* strain J18 143 cells and the pigment aggregates. After 20 h of incubation, this peak remained due to the presence of the cells in the dispersion (Fig. 8F). However, the size of this peak was much less than that was obtained before the incubation period, and the number of particles in the size range of 100–500 nm had substantially increased. From these results, it can be suggested that the *Shewanella* strain J18 143 cells were able to degrade the large pigment aggregates to produce smaller aggregates and, perhaps, individual pigment particles. This enabled the cells to reduce the azo/ketohydrazone bonds

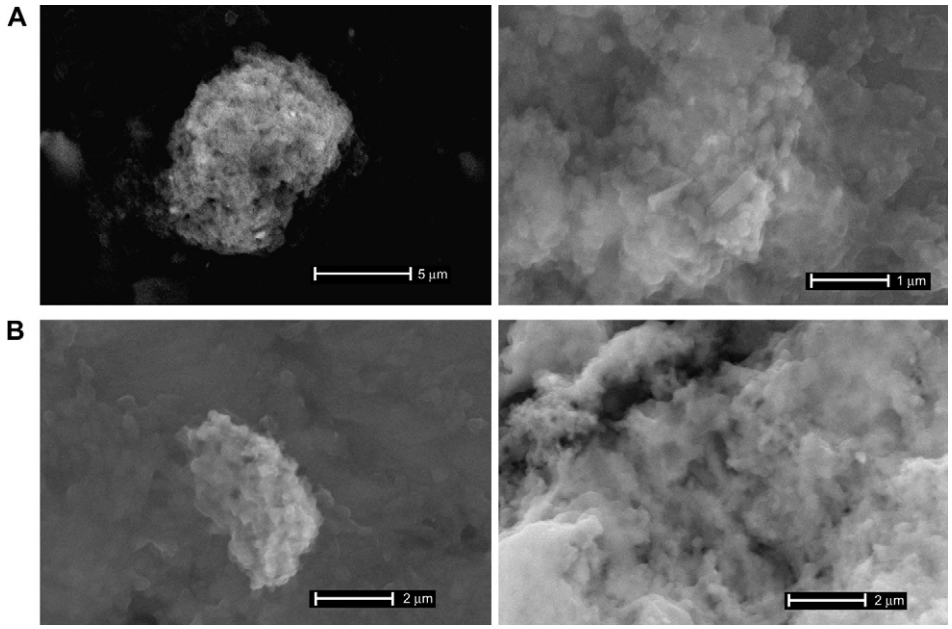

Fig. 9. Environmental scanning electron micrographs of pigment particles from GemSperse Orange EX5 before (A) and after (B) 20 h incubation at 30 °C with *Shewanella* strain J18 143 cells.

in the pigment particles to produce soluble, colourless amines.

Further evidence for this reduction in particle size was provided by analysis of the GemSperse Orange EX5 pigment dispersions using environmental scanning electron microscopy. Fig. 9(A) shows a representative range of pigment aggregates and individual pigment particles in the dispersion before incubation with cells of *Shewanella* strain J18 143, with a number of larger particles at around 1 μM. After exposure to active cells of *Shewanella* strain J18 143, the pigment aggregates were smaller with individual pigment particles that were less than 1 μM and not well defined (Fig. 9B).

4. Discussion

The nature of the electron acceptor is immensely important to microbial redox cycling and energy generation [21], with a range of soluble and insoluble substrates utilized by anaerobic microbial communities. *Shewanella* strains are particularly adept at exploiting a wide range of terminal electron acceptors present at redox boundaries, including solid-phase minerals. In addition to synthesising a range of potential reductases, *Shewanella* species also contain a large number of regulatory proteins that allow rapid detection of environmental changes, providing the ability to adapt to these changing conditions [22]. This study shows for the first time that *Shewanella* species have the potential to reduce extracellular insoluble azo/ketohydrazone pigments. This has obvious biotechnological potential for the treatment of problematic pigment-containing wastes. It also suggests the intriguing potential for anaerobic respiration supported by these compounds in contaminated environments.

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