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Dyes and Pigments 58 (2003) 179–196
DYES and PIGMENTS
www.elsevier.com/locate/dyepig

Review

The removal of colour from textile wastewater using whole bacterial cells: a review

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Received 24 February 2003; accepted 31 March 2003

Abstract

The delivery of colour in the form of dyes onto textile fibres is not an efficient process. The degree of efficiency varies, depending on the method of delivery. As a result, most of the wastewater produced by the textile industry is coloured. It is likely that coloured wastewater was a feature of the first practices of textile dyeing. However, treatment to remove this colour was not considered until the early natural dyestuffs were replaced by synthetic dyes, and the persistence of such synthetic dyes in the environment was recognised (Willmott NJ. The use of bacteria–polymer composites for the removal of colour from reactive dye effluents. PhD thesis, UK: University of Leeds; 1997.). Colour pollution in aquatic environments is an escalating problem, despite the fact that there has been substantial research into the modification of the dyeing process to improve the level of affinity/fixation of the dyestuffs onto the substrate. The recalcitrant nature of modern synthetic dyes has led to the imposition of strict environmental regulations. The need for a cost-effective process to remove the colour from wastewater produced by the textile industry has been recognised (Willmott NJ, Guthrie JT, Nelson G. The biotechnology approach to colour removal from textile effluent. JSDC 1998;114(February):38–41.). Several strategies have been investigated. However, the review presented here concerns the use of whole bacterial cells for the reduction of water-soluble dyes present in textile dyeing wastewater.

Keywords: Colour removal; Textile wastewaters; Azo dyes; Whole bacterial cells

Contents

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doi:10.1016/S0143-7208(03)00064-0

1. Introduction

Coloured wastewater is a consequence of batch processes both in the dye manufacturing industries and in the dye-consuming industries. Two per cent of dyes that are produced are discharged directly in aqueous effluent, and 10% are subsequently lost during the textile coloration process [3]. An indication of the scale of the problem is given by the observation that the annual market for dyes is more than 7×10^5 tonnes per year [4]. The main reason for dye loss is the incomplete exhaustion of dyes on to the fibre. The amount of dye lost is dependent upon dyestuff type, the application route and the depth of shade required [1]. Coloured wastewater is particularly associated with those reactive azo dyes that are used for dyeing cellulose fibres. These dyes make up approximately 30% of the total dye market [5].

The appealing properties of these dyes include the fact that they provide a wide range of brilliant shades, they can be applied using a number of application methods and they provide high wet fastness. Reactive azo dyes were initially made available to the industry in the 1930s. Therefore their persistence in treated wastewater has been recognised for a considerable period of time. Remazol Black B, shown in Fig. 1, is characteristic

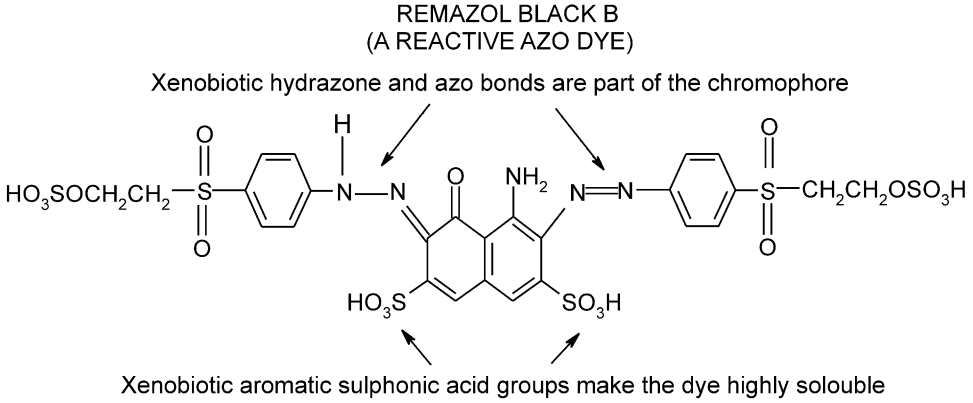

Fig. 1. The structure of Remazol Black B.

of reactive azo dyes, with its chromophore, containing azo/keto-hydrazone groups, the reactive centres and its solubilising components. Residual colour is a problem with reactive dyes because, in current dyeing processes, as much as 50% of the dye is lost in the wastewater. These losses are due to the relatively low levels of dye–fibre fixation and to the presence of unreactive hydrolysed dye in the dyebath. Dye hydrolysis occurs when the dye molecule reacts with water rather than with the hydroxyl groups of the cellulose. These problems are compounded by the high water solubility and characteristic brightness of the dyes.

Due to their stability and to their xenobiotic nature, reactive azo dyes are not totally degraded by conventional wastewater treatment processes that involve light, chemicals or activated sludge [6]. The dyes are therefore released into the environment, in the form of coloured wastewater. This can lead to acute effects on exposed organisms due to the toxicity of the dyes, abnormal coloration and reduction in photosynthesis because of the absorbance of light that enters the water [7,8]. Also, public perception of water quality is greatly influenced by the colour. The presence of unnatural colours is aesthetically unpleasant and tends to be associated with contamination [9]. Willmott has reported that up to 1.56 mg dm^{-3} dye can be detected in receiving watercourses, although dye concentrations as low as 0.005 mg dm^{-3} are visible in clear river water [10].

Fig. 2 shows the wastewater pipe at a company that bleaches, dyes and finishes material for the clothing market. It can be seen from Fig. 2 that a significant depth of colour is still present in the wastewater, even after it has been treated.

Fig. 2. Wastewater pipe at a textile dying and finishing company.

There is increasing pressure from the UK Government for an improvement in water quality. Environmental policy in the UK, since September 1997, has stated that zero synthetic chemicals should be released into the marine environment [4]. The current regulator to enforce Government standards, to monitor pollution in rivers and to manage water resources is the Environment Agency. The agency assesses the need for colour standards at a number of wavelengths, and consents are imposed where necessary. Standards are set using a range of coloured sewage effluent samples, taking into account the fact that the human eye has the capacity to detect 1ppm of colour [5].

As the combined effect of sewage treatment and dilution are not sufficient to remove the residual dye from process wastewater, an alternative industrial-scale decoloration system is needed to achieve the necessary colour consent limits. Banat et al. [10] make the point that many textile plants have rural locations and municipal treatment costs are increasing. Therefore the colour creating industries and the colour using industries are compelled to search for innovative novel treatments and technologies that are directed particularly towards the decoloration of dyes in effluents [10]. The majority of colour removal techniques work either by concentrating the colour into sludge, or by the complete destruction of the coloured molecule. It is likely that, as a consequence of Integrated Pollution Control (IPC) regulations, decoloration systems involving destruction technologies will prevail, as the transferral of pollution from one part of the environment to another is prevented [2].

2. Technologies available for colour removal—the advantages and the disadvantages

There are two possible locations for any technology that could be used to treat the colour that is present in the wastewater:

1. At the dyehouse, to remove the colour and allow partial or full re-use of the water.
2. At the sewage works, to treat the colour before the current biological/chemical processing, or as a final polishing step [11].

If the wastewater is treated at a sewage works, the cost of sewage treatment will increase the charges being laid on the dyehouse. These cost implications mean that, for most textile industries, developing dedicated on-site or in-plant facilities to treat their own wastewater before discharge is the preferred option. However, a single, universally applicable end-of-pipe solution is unrealistic because the colour and the chemical composition of textile wastewater is usually subject to daily and seasonal variations that are dictated by production routines and by fashion cycles [2]. It is likely that a combination of techniques will be used to provide effective treatment of coloured wastewater at a cost that enables textile coloration processors to invest in appropriate technologies.

Currently, the major methods of textile wastewater treatment involve physical and/or chemical processes. Some examples are summarised in Table 1. Such methods are often very costly and, although the dyes are removed, accumulation of concentrated sludge creates a disposal problem. There is also the possibility that a secondary pollution problem will arise because of excessive chemical use. Other emerging techniques, such as ozonation, treatment using Fenton's reagent, electrochemical destruction and photocatalysis may have potential for decoloration. However, such technologies usually involve complicated procedures or are economically unfeasible [12]. Kamilaki has found that biological and/or combination treatment systems that can effectively remove dyes from large volumes of wastewater at a low cost are considered to offer a preferable alternative [4].

3. Colour removal using whole bacterial cells

Alternative approaches to colour removal, utilising microbial biocatalysts to reduce the dyes that are present in the effluent, offer potential advantages over physio-chemical processes. Such systems are the focus of recent research. In particular, the ability of whole bacterial cells to metabolise azo dyes has been extensively investigated. Under aerobic conditions, azo dyes are not readily metabolised [4]. However, under anaerobic conditions, many bacteria reduce the highly electrophilic azo bond in the dye molecule, reportedly by the activity of low specificity cytoplasmic azo reductases, to produce colourless aromatic amines. These amines are resistant to further anaerobic mineralisation and can be toxic or mutagenic to animals. Fortunately, once the xenobiotic azo component of the dye molecule has been removed, the resultant amino compounds are good substrates for aerobic biodegradation. Lourenço et al. suggest that if a sequential anaerobic–aerobic system is employed for wastewater treatment, the amines can be mineralised under aerobic conditions by a hydroxylation pathway involving a ring opening mechanism [13].

For the degradation of dyes in coloured wastewater, the use of whole cells rather than isolated enzymes is advantageous, because costs associated with enzyme purification are negated and the cell can also offer protection from the harsh process environment to the enzymes. Also, degradation is often carried out by a number of enzymes working sequentially. Cheetham and Bucket [14] have found that this mechanism would be difficult to reconstruct using individually extracted enzymes [14].

Table 1
Current treatment technologies for colour removal involving physical and/or chemical processes

Physical and/or chemical methods	Advantages	Disadvantages
Oxidation	Rapid process	High energy costs and formation of by-products
Adsorption	Good removal of a wide range of dyes	Absorbent requires regeneration or disposal
Membrane technologies	Removes all dye types	Concentrated sludge production
Coagulation/flocculation	Economically feasible	High sludge production

4. Mixed bacterial cultures

Degradation of xenobiotics such as azo dyes is often carried out by mixed cultures. Many researchers have reported that a higher degree of biodegradation and mineralisation can be expected when co-metabolic activities within a microbial community complement each other. Knackmuss gives an example of this using the degradation of naphthalene sulphonates by a two-species culture [15]. *Sphingomonas* strain BN6 is able to degrade naphthalene-2-sulphonate, a building block of azo dyes, into salicylate ion equivalents. The salicylate ion cannot be further degraded and is toxic to strain BN6. Therefore, naphthalene-2-sulphonate can only be degraded completely in the presence of a complementary organism that is capable of degrading the salicylate ion [15]. Also, it can be difficult to isolate a single bacterial strain from dye-containing wastewater samples and, in some instances, long term adaptation procedures are necessary before the isolate is capable of using the azo dye as a respiratory substrate. Several studies reported in the literature, concerning the biodegradation of coloured wastewater using mixed bacterial cultures, are given in Table 2.

5. Single bacterial cultures

The advantages of mixed cultures are apparent as some microbial consortia can collectively carry out biodegradation tasks that no individual pure strain can undertake successfully [23]. Also, mixed culture studies may be more comparable to practical situations. However, mixed cultures only provide an average macroscopic view of what is happening in the system and results are not easily reproduced, making thorough, effective interpretation difficult. For these reasons, a substantial amount of research on the subject of colour removal has been carried out using single bacterial cultures. The use of a pure culture system ensures that the data are reproducible and that the interpretation of experimental observations is easier. The detailed mechanisms of biodegradation can be determined using the tools of biochemistry and molecular biology, and these tools may also be used to upregulate the enzyme system to give modified strains with enhanced activities. The quantitative analysis of the kinetics of azo-dye decoloration by a particular bacterial culture can be undertaken meaningfully. Also, the response of the system to changes in operational parameters can be studied [12]. Summaries of some of the studies that have been carried out are given in Table 3.

6. Mechanism of colour removal

The simplest mechanism of colour removal by whole bacterial cells is that of the adsorption of the dye onto the biomass [32]. However, this mechanism is similar to many other physical adsorption mechanisms for the removal of colour and is not suitable for long term treatment. This is because, during adsorption, the dye is concentrated onto the biomass, which will become saturated with time, and the dye-adsorbent composition must also be disposed of. Bio-association between the dye and the bacterial cells tends to be the first step in the biological reduction of azo dyes, which is a destructive treatment technology [11].

Biodegradation processes may be anaerobic, aerobic or involve a combination of the two. When considering the reaction between bacterial cells and azo dyes, it must be noted that there are significant differences between the physiology of microorganisms grown under aerobic and anaerobic conditions [61]. For aerobic bacteria to be significant in the reductive process the bacteria must be specifically adapted. This adaptation involves long-term aerobic growth in continuous culture in the presence of a very simple azo compound. The bacteria synthesise an azoreductase specific for this compound which, under controlled conditions, can reductively cleave the azo group in the presence of oxygen [61]. In contrast, bacterial reduction under anaerobic conditions is relatively unspecific with regard to the azo compounds involved, and is, therefore, of more use for the removal of colour in azo dye wastewater [61].

It is generally thought that, as most azo dyes have sulphonate substituent groups and a high molecular

Table 2
Biodegradation of dyes in coloured wastewater using mixed bacterial cultures: review of studies reported in the literature

Dyes/coloured wastewater studied	Organisms used	Comments	Reference number and year
Azo dyes	*Bacillus cereus, Sphaerotilus natans, Arthrobacter* sp., activated sludge	Dye reduction under anoxic conditions involving non-enzymatic intracellular reduced flavin nucleotides	[16] (1980)
C.I Acid Orange 6 C.I.Basic Violet 1 C.I.Basic Violet 3	Field-collected and laboratory cultures	Basic dye transformed under aerobic conditions	[17] (1986)
87 different acid, basic, direct, mordant and reactive dyes	Activated sludge	Short term aerobic biodegradation tests. Around 13% biodegraded and 23% absorbed	[18] (1986)
C.I.Mordant Yellow 3, C.I.Acid Red 27, C.I.Acid Yellow 23 and 21	Mixed bacterial consortium	Reduction of dye under anaerobic conditions followed by oxidation of amine metabolites after re-aeration. Aerobic desulphonation of sulphonated naphthalene	[19] (1991)
Reactive dyes	Anaerobic sludge	Reactive dyes decolorised via reduction	[20] (1994)
C.I.Acid Orange 10, C.I.Acid Red 14 and 18	Anaerobic digester sludge and aeration tank mixed liquor	2-stage anaerobic-aerobic (fixed film fluidised bed/suspended growth activated sludge) reactor. 65–90% decoloration in first stage	[21] (1995)
Diazo-linked chromophores	Mixed anaerobic culture	85% colour removal in 2 days (10% (v/v) dilutions of the effluent). Reduction of azo bond	[22] (1995)
Reactive dyes, diazo dyes, azo dyes, disperse dyes and phthalocyanine dyes	*Alcaligenes faecalis, Commomonas acidovorans*	100% decolorization under anaerobic conditions of 5 out of 9 component dyes within 48 h	[23] (1996)
Cationic, chromium-containing azomethine dye	Aerobic mixed bacterial culture	Partial degradation, releasing chromium, and partial dye adsorption	[24] (1996)
Reactive dyes	*Alcaligenes faecalis, Commomonas acidovorans*	67–89% decoloration of reactive dyes under anaerobic conditions	[25] (1996)
Remazol Black B	*Alcaligenes faecalis, Commomonas acidovorans*	Microbial consortium immobilised on gravel Over 95% decoloration within 48 h	[26] (1996)
Various azo and diazo reactive dyes	Thermophilic anaerobic bacterial culture	68–84% colour removal in 48 h	[10] (1996)
Simulated textile wastewater containing Procion Red H-E7B	Sludge from textile wastewater treatment plant (Inclined Tubular Digester) granules from paper pulp processing plant (Upflow Anaerobic Sludge Blanket)	78% colour removal by anaerobic treatment. Upflow Anaerobic Sludge blanket gave better colour removal than Inclined Tubular Digester	[27] (1999)
Orange G, Amido Black 10B, Direct Red 4BS and Congo Red	Four bacterial strains (*pseudomonads*) isolated from dyeing effluent-contaminated soils	The maximum degradation rate observed in the treatment system for Orange G was 60.9 mg/l per day, for Amido Black 10B 571.3 mn/l per day, for Direct Red 4BS 112.5 mg/l per day and for Congo Red 134.9 mg/l per day	[28] (2000)

(*continued on next page*)

Table 2 (*continued*)

Dyes/coloured wastewater studied	Organisms used	Comments	Reference number and year
Reactive Red 3.1	Activated sludge obtained from domestic and industrial effluent treatment plants	Decolorization rates of up to 20– 30 mg l^{-1} h^{-1} were given by activated sludge under anaerobic conditions. In an anaerobic packed bed reactor followed by an aerobic stirred tank reactor 90–93% dye removal occurred after 51 h	[29] (2000)
C.I.Acid Red 42, C.I.Acid Red 73, C.I.Direct Red 80, C.I.Disperse Blue 56	Original seed sludge collected from a municipal wastewater treatment plant	Average removal efficiency for acid dyes was between 80 and 90%. The removal efficiency for C.I.Direct Red 80 was 81%. Removal of C.I.Disperse Blue 56 was unsuccessful	[30] (2000)
Azo dyes (Sirusgelb and Siruslichtbraun)	Mesophilic sludge from a lab-scale UASB reactor	56% removal of azo dye COD was achieved at volumetric load of 0.3 g azo dye COD/l/day using an anaerobic–aerobic hybrid reactor	[31] (2000)
C.I.Acid Orange 7	Mixed and methanogenic cultures	94% colour removal. Colour removal faster in mixed cultures than in methanogenic culture. Addition of electron donor stimulated reductive cleavage of azo bond. Anaerobic degradation of dye metabolites did not occur	[32] (2001)
Industrial wastewater containing precursors and final synthesis products of up to 15 different sulphonated azo dyes and other sulphonated synthetic colorants	An anaerobic baffled reactor containing a mixed bacterial population, with sulphate-reducing bacteria, and a methanogenic population	On day 100 of operation of the anaerobic baffled reactor, a profile of colour removal in the reactor shows that colour removal was almost complete by compartment 2 out of 8	[33] (2001)
Hydrolysed C.I.Reactive Orange 96	Sulphate-reducing bacteria, methane producing bacteria and fermentative bacteria in an anaerobic mixed culture	Sulphate-reducing bacteria can remove 95% of the dye in 40 h. Methane producing bacteria take no part in dye removal. Fermentative bacteria can remove 30% of the dye in 90 h.	[34] (2001)
Hydrolysed Remazol Brilliant Violet 5R and Remazol Black B	Sequencing Batch Reactor inoculated with sludge collected in a full-scale, continuous activated sludge plant	90% colour removal was obtained for Remazol Brilliant Violet 5R and 75% colour removal was obtained for Remazol Black B in a 24-h cycle with a sludge retention time of 15 days and an aerated reaction phase of 10 h	[13] (2000) [35] (2001)
Acid Orange 7	An uncharacterised aerobic biofilm. Strain 1CX (*Sphinogomonas* sp.) and Strain SAD4I (Gram-negative bacterium)	Acid Orange 7 was completely degraded within 1 h in a rotating drum bioreactor containing the biofilm. The two bacterial strains were able to mineralise, in co-culture, up to 90% of the Acid Orange 7	[36] (2002)

Table 3
Biodegradation of dyes in coloured wastewater using single bacterial strains—studies reported

Dyes/coloured wastewater studied	Organism used	Comments	Reference number and year
Red 2G azo dye	*Streptococcus faeclis*	Reduced soluble flavins acting as electron shuttles between NAD(P)H-dependent flavoproteins and azo dye	[37] (1971)
Azo food dyes	*Proteus vulgaris*	Zero order decoloration reaction under anaerobic conditions. Increase in reduction rate after onset of cell mortality. Rate depends on redox potential of dye	[38] (1975)
p-Aminoazobenzene (PAAB)	*Bacillus subtilis*	Reduction of PAAB under aerobic conditions.	[39] (1977)
Aminoazobenzene compounds, Dimethyl Yellow, *p*-hydroxyazobenzene and azoresorcinol compounds	*Aeromonas hydrophilia* var. 24B	Colour removal 54–90% depending on dye structure. Lag phase is longer and colour removal is greater in static cultures compared with shaking cultures. Hydroxyl group inhibited degradation	[40] (1978)
p-Aminoazobenzene (PAAB)	*Pseudomonas pseudomallei* 13NA	The use of continuous culture enabled removal of ~80% of 10 p.p.m. PAAB.PAAB metabolism favoured in poor nutrient conditions	[41] (1981)
Acid, direct and basic dyes	*Pseudomonas pseudomallei*	Half decolorisation time correlated with azo dye molecular weight. Azo dyes decolorised more easily than triphenylmethanes	[42] (1981)
Orange I, Orange II	*Pseudomonas* sp.	After 7 generations of adaptation time to Orange I, ~35% was degraded. After 10 generations more than 90% of Orange II was degraded. Reduction of dyes by azoreductase enzymes	[43] (1983)
Orange I, Orange II	*Pseudomonas* sp.	Comparison of bacterial azoreductases for Orange I and Orange II, differing in their specificity with regard to position of the hydroxy group	[44] (1984)
C.I.Acid Orange 12 C.I.Acid Orange 20 C.I.Acid Red 88	*Pseudomonas cepacia* 13NA	Multi-stage continuous aerobic cultivation using buffered, low nutrient media. Microbial growth inhibited at high dye concentrations. 90% decoloration in 68 h	[45] (1986)
39 reactive dyes with different structures	Immobilised *Pseudomonas* sp.	22 dyes decolorised by >90%. Anthraquinone and metal-complex structures recalcitrant to biodegradation. Residence time of 1–2 h	[46] (1990)
9 different azo dyes based on amino-azobenzene and naphthalene-azobenzene	*Pseudomonas stutzeri* (cell free extract)	80–90% colour removal was obtained for all the dyes after a 20-min period. Specificity shown for lowest redox potential dyes	[47] (1990)
4 reactive azo dyes	*Pseudomonas luteola*	37–93% colour removal was obtained after 42 h. Colour removal was greatest under static conditions	[48] (1994)
p-Aminoazobenzene (PAAB)	*Bacillus subtilis*	PAAB concentration decreased from 10.2 to 7.5 mg/l in 25 h, in the presence of glucose and nitrate, under anoxic conditions. The stoichiometric products of PAAB degradation were aniline and *p*-phenylenediamine	[49] (1996)
Amaranth (sulphonated azo dye)	*Sphingomonas* sp. Strain BN6	Aerobic conversion of 2-naphathlenesulphonate by Strain BN6 stimulated the subsequent anaerobic reduction of amaranth at least 10-fold. The presence of both cytoplasmic and membrane-bound azo reductase activities was shown	[50,51] (1997)

(*continued on next page*)

Table 3 (*continued*)

Dyes/coloured wastewater studied	Organism used	Comments	Reference number and year
Amaranth, Orange II, tartrazine,	*Bacteroides fragilis*	80% aramanth, 50% Orange II and 20% tartrazine were removed in 8 h, under anaerobic conditions. These different rates of reduction could be correlated with the half-wave (redox) potential of the azo dye	[52] (1997)
Remazol Black B, 8 other reactive dyes, anthraquinone dye, industrial wastewater	*Shewanella putrefaciens*	95% Remazol Black B and other reactive dyes removed. Anthraquinone dye adsorbed. 84% colour removed from industrial wastewater. Colour removal greatest at pH8 and 35 °C. Decoloration inhibited by agitation and high salt concentrations	[1] (1997)
Amaranth, Orange II, 4-hydroxy-azobenzene, tartrazine, methyl orange, 4-amino-azobenzene	*Clostridium perfrigens*	All dyes were reduced to below the level of detection in 50 min, under anaerobic conditions. Dyes with smaller negative redox potentials were reduced before dyes with larger negative redox potentials	[53] (1998)
4-Carboxy-4′-sulphoazobenzene (CSAB)	*Hydrogenophaga palleronii*, Strain 5	CSAB (0.35 mM) was completely reduced in 40 min, under aerobic conditions. The products of the reductive cleavage, sulphanilate and 4-sulphocatechol, were mineralised by established degradation pathways	[54] (1998)
Triphenylmethane dyes, textile and dye-stuff wastewater	*Kurthia* sp.	Under aerobic conditions, 98% colour removal was obtained for the triphenylmethane dyes in 30 min, and 56% colour removal was obtained for the textile and dye-stuff wastewater in 90 h	[55] (1999)
Remazol Black B, textile wastewater	*Shewanella putrefaciens*	85% Remazol Black B removed in 6.5 h in a suspended growth reactor with continuous flow. 80% colour removed from textile wastewater in 24 h	[5] (2000)
C.I.Reactive Orange 96	*Desulfovibrio desulfuricans*	Under anaerobic conditions and in the presence of sulphide, over 95% colour removal was obtained in 2 h. The reduction products were 2 aromatic amines and elemental sulphur.	[56] (2000)
C.I.Reactive Red 22	*Pseudomonas luteola* Strain	A specific decolourisation rate of 113.7 mg dye g cell^{-1} h^{-1} and an overall decolourisation efficiency of 86.3 mg dye l^{-1} h^{-1} were obtained with gentle agitation, without aeration and with a constant dye loading rate of 200 mg/h	[12] (2000)
Acid azo dyes, Direct azo dyes and amaranth	*Sphingomonas* sp. Strain BN6	Whole cells showed less than 2% of the specific azo compound reduction activities found with cell extracts. This suggests that the cytoplasmic anaerobic "azo reductases" are presumably flavin reductases and that they are not important in the reduction of sulphonated azo compounds in vivo	[57] (2000)
Remazol Black B	*Paenibacillus azoreducens* sp. nov.	98% colour removal within 24 h, at a dye concentration of 100 mg dm^{-3} and a temperature of 37° C	[58] (2001)
Reactive azo dyes, Direct azo dyes and leather dyes	*Pseudomonas luteola*	59–99% colour removal after 2–6 days in a static incubator (28° C), at a dye concentration of 100 mg l^{-1}. Extent of colour removal depended on dye structure, with monoazo dyes showing fastest rate of decoloration	[59] (2001)
C.I.Reactive Red 22	*Pseudomonas luteola*	Specific decoloration rates of 18.6 mg dye/g cell/h for free cells and ~3.5 mg dye/g cell/h for immobilised cells were obtained. Differing rates attributed to mass transfer restriction arising from cell entrapment. Immobilised cells less sensitive to agitation rates than free cells. After 4 repeated experiments, decoloration rates of free cells deceased by 45% while immobilised cells retained over 75% of their original activity	[60] (2001)

weight, they are unlikely to pass through cell membranes. Therefore, the reducing activity referred to above is not dependant on the intracellular uptake of the dye [4]. This has been shown by Russ et al. who also suggest that bacterial membranes are almost impermeable to flavin-containing cofactors and, therefore, restrict the transfer of reduction equivalents by flavins from the cytoplasm to the sulphonated azo dyes. Thus, a mechanism other than reduction by reduced flavins formed by cytoplasmic flavin-dependent azoreductases must be responsible for sulphonated azo dye reduction in bacterial cells with intact cell membranes [57].

One such mechanism involves the electron transport-linked reduction of azo dyes in the extra-cellular environment. To achieve this, the bacteria must establish a link between their intracellular electron transport systems and the high molecular weight, azo dye molecules. For such a link to be established, the electron transport components must be localised in the outer membrane of the bacterial cells (in the case of gram-negative bacteria), where they can make direct contact with either the azo dye substrate or a redox mediator at the cell surface [62]. In addition, Gingell and Walker have shown that low molecular weight redox mediator compounds can act as electron shuttles between the azo dye and an NADH (nicotinamide adenine dinucleotide)-dependent azo reductase that is situated in the outer membrane [37]. These mediator compounds are either formed during the metabolism of certain substrates by the bacteria or they may be added externally [57]. The addition of synthetic redox mediators such as anthraquinone sulphonates, even at very low concentrations, will facilitate the non-enzymatic reduction of the azo dyes in the extra-cellular environment [33,34]. However, if the extra-cellular environment is aerobic, this reduction mechanism will be inhibited by oxygen, due to the preferential oxidation of the reduced redox mediator by oxygen rather than by the azo dye.

Kudlich et al. support the suggestion that the membrane-bound azo reductase activity, mediated by redox compounds, is different from the soluble cytoplasmic azo reductase that is responsible for the reduction of non-sulphonated dyes that permeate through the cell membrane. Their results show that a thiol-specific inhibitor almost completely inactivated, the membrane-bound azo reductase in *Sphingomonas* sp. but had no effect on the cytoplasmic azo reductase. Therefore, the membrane-bound and the cytoplasmic azo reductases are two different enzyme systems [51].

Fig. 3 shows a proposed mechanism for the redox-mediator-dependent reduction of azo dyes using whole bacterial cells, under anaerobic conditions. Although the final reduction of the azo dyes in the cell supernatants is a dominantly chemical redox reaction, the redox mediators depend on cytoplasmic reducing enzymes to supply electrons [34]. It is also possible that this chemical redox reaction works in conjunction with a direct enzymatic reaction involving an azo reductase, which may be a dehydrogenase enzyme that is synthesized throughout the cytoplasm and secreted without accumulation inside the cell [52].

A study by Van der Zee et al. into the kinetics of anaerobic colour removal indicated that the transfer, rather than the production of reduced redox mediators was the rate-limiting step in the reduction of azo dyes [63]. The reduction rate was also governed by the redox potential of the dyes and the redox mediators. In the same study, it was found that amino quinone compounds that are formed during the reduction of certain azo dyes were involved in autocatalysis and contributed substantially to the reduction process [63]. Another possible mechanism for colour removal involves the reduction of the azo bond by reduced inorganic compounds, such as Fe^{2+} or H_2S that are formed as the end-product of certain anaerobic bacterial metabolic reactions [51].

In summary, it is probable that there are at least two mechanisms for the decoloration of azo dyes in bacterial systems:

1. Direct electron transfer to azo dyes as terminal electron acceptors via enzymes during bacterial catabolism, connected to ATP-generation (energy conservation).
2. A gratuitous reduction of azo dyes by the end products of bacterial catabolism, not linked to ATP-generation.

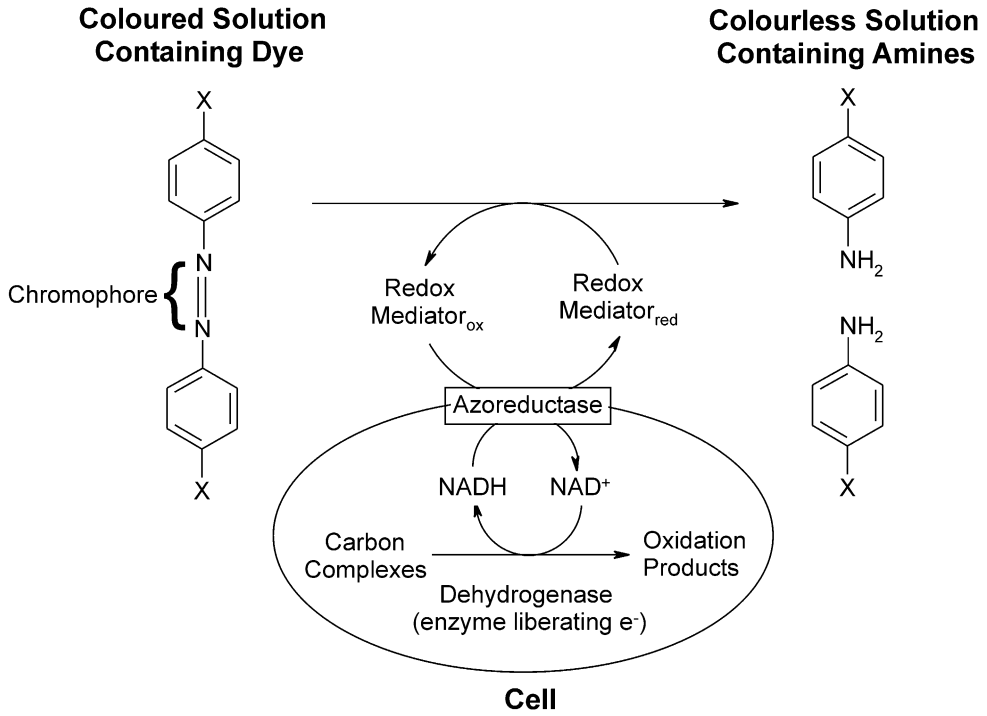

Fig. 3. Proposed mechanism for reduction of azo dyes by whole bacterial cells (modification of Keck et al. [50].

Organics or inorganics may be involved in both mechanisms by acting as electron shuttles between the reducting equivalents and the azo dyes [56].

7. Factors affecting colour removal

Research has shown that the efficiency of biological treatment systems is greatly influenced by the operational parameters. The level of aeration, temperature, pH and redox potential of the system must be optimised to produce the maximum rate of dye reduction. The concentrations of the electron donor and the redox mediator must be balanced with the amount of biomass in the system and the quantity of dye present in the wastewater. The ability of the bacterial cells to reduce dyes from a range of dye classes (acid, basic, direct, disperse, metal-complex, reactive, sulphur and vat) must be tested to determine the types of wastewater that can be treated by the system. The composition of textile wastewater is varied and can include organics, nutrients, salts, sulphur compounds and toxicants as well as the colour [5]. Any of these compounds may have an inhibitory effect on the dye reduction process.

The effect of each of the factors on the colour removal process must be investigated before the biological system can be used to treat industrial wastewater.

7.1. Oxygen

The most important factor to consider is the effect of oxygen on cell growth and dye reduction. As mentioned previously, during the cell growth stage, oxygen will have a significant effect on the physiological characteristics of the cells. During the dye reduction stage, if the extra cellular environment is aerobic, the high-redox-potential electron acceptor, oxygen, may inhibit the dye reduction mechanism. This is because the electrons liberated from the oxidation of electron donors by the cells are preferentially used to reduce oxygen rather than the azo dye, and the reduction product, water, is not a reductant [34]. Also, the postulated intermediates of the dye reduction

reaction, which include the hydrazine form of the dye and the azo anion free radical form of the dye, tend to be reoxidised by molecular oxygen [64].

It can be concluded from these observations that, for efficient colour removal aeration and agitation, which increases the concentration of oxygen in solution, should be avoided [12]. However, the inhibition of azo dye reduction under aerobic conditions tends only to be a temporary effect rather than an irreversible effect. If the air is replaced with oxygen free nitrogen, the reducing activity is restored and occurs at a similar rate to that which was observed under continuous anaerobic conditions [52]. The inhibitory effects of oxygen on bacterial azo reduction can be summarised as resulting from its toxicity for anaerobic bacteria, from a direct inhibition of the azo reductase enzyme or from the preferential reduction of oxygen rather than the azo derivatives [53].

Under anaerobic conditions, no further degradation of the dye molecule is observed after the reduction of the azo bond. Aerobic conditions are required for the complete mineralisation of the reactive azo dye molecule, as the simple aromatic compounds produced by the initial reduction are degraded via hydroxylation and ring-opening in the presence of oxygen [65]. Thus, for the most effective wastewater treatment, a two-stage process is necessary in which oxygen is introduced after the initial anaerobic reduction of the azo bond has taken place. The balance between the anaerobic and aerobic stages in this treatment system must be carefully controlled because it is possible for the re-aeration of a reduced dye solution to cause the colour of the solutions to darken. This is to be expected, as aromatic amines, produced when azo dyes are reduced under anaerobic conditions, are spontaneously unstable in the presence of oxygen. This results in the oxidation of the hydroxyl groups and of the amino groups to quinines and quinine imines. Compounds such as these can undergo dimerisation or polymerisation, leading to the development of new, darkly coloured chromophores, which are clearly unwanted by-products [29]. However, when the correct operating conditions have been established, many strains of bacteria are capable of achieving high levels of decoloration when used in a sequential anaerobic/aerobic treatment process.

7.2. Temperature

In many systems, the rate of colour removal increases with increasing temperature, within a defined range that depends on the system. The temperature required to produce the maximum rate of colour removal tends to correspond with the optimum cell culture growth temperature of 35–45 °C. The decline in colour removal activity at higher temperatures can be attributed to the loss of cell viability or to the denaturation of the azo reductase enzyme [66]. However, it has been shown that with certain whole bacterial cell preparations, the azo reductase enzyme is relatively thermostable and can remain active up to temperatures of 60 °C, over short periods of time [67]. Immobilisation of the cell culture in a support medium results in a shift in the optimum colour removal temperature towards high values because the microenvironment inside the support offers protection for the cells.

7.3. pH

The optimum pH for colour removal is often at a neutral pH value or a slightly alkaline pH value and the rate of colour removal tends to decrease rapidly at strongly acid or strongly alkaline pH values. As a result, the coloured wastewater is often buffered to enhance the colour removal performance of the cell culture. Biological reduction of the azo bond can result in an increase in the pH due to the formation of aromatic amine metabolites, which are more basic then the original azo compound [1]. Altering the pH within a range of ~7.0 to 9.5 has very little effect on the dye reduction process. Chang et al. found that the dye reduction rate increased nearly 2.5-fold as the pH was raised from 5.0 to 7.0, while the rate became insensitive to pH in the range of 7.0–9.5 [66].

7.4. Dye concentration

The concentration of dye substrate can influence the efficiency of dye removal through a combination of factors including the toxicity of the dye

(and co-contaminants) at higher concentrations, and the ability of the enzyme to recognise the substrate efficiently at the very low concentrations that may be present in some wastewaters, Indeed, the kinetic constants that govern process efficiency, in common with other enzyme catalysed processes, can be described using Michaelis–Menten kinetics:

$$v = V_{max}[S]/K_m + [S]$$

where; v is the observed velocity of the reaction at a given substrate concentration [S], V_{max} is the maximum velocity at a saturating concentration of substrate and K_m is the Michaelis constant. The application of Michaelis–Menten kinetics can, for example, allow predictions to be made on process efficiency including the degree of biomass loading or the operational temperature needed to maintain dye removal at a given efficiency within the constraints set by the reactor volume available, the background solution composition and flow-rates.

Wuhrmann et al. observed that, after an initially rapid reduction of colour, the rate of colour removal decreased more rapidly than would be predicted by a first order reaction [16]. This effect was attributed to the toxicity of the metabolites that were formed during dye reduction. The higher the dye concentration, the longer the time required to remove the colour. Sani et al found that dyes with concentrations of 1–10 μM were easily decolorised, but when the dye concentration was increased to 30 μM, colour removal was reduced [55]. However, Dubin and Wright [68] reported the absence of any effect of dye concentration on the reduction rate. This observation is compatible with a non-enzymatic reduction mechanism that is controlled by processes that are independent of the dye concentration [68].

7.5. Dye structure

Some azo dyes are more resistant to removal by bacterial cells [32]. Dyes with simple structures and low molecular weights exhibit higher rates of colour removal, whereas colour removal is more difficult with highly substituted, high molecular weight dyes [55]. In the case of the terminal non-enzymatic reduction mechanism, reduction rates are influenced by changes in electron density in the region of the azo group. The substitution of electron withdrawing groups ($–SO_3H$, $–SO_2NH_2$) in the para position of the phenyl ring, relative to the azo bond, causes an increase in the reduction rate [69].

Nigam et al. established that azo compounds with a hydroxyl group or with an amino group are more likely to be degraded than are those with a methyl, methoxy, sulpho or nitro groups [23]. Colour removal is also related to the number of azo bonds in the dye molecule. The colour of monoazo dyes is removed faster than is the colour of diazo or triazo dyes. Hu showed that the turnover rate of monoazo dyes increased with increasing dye concentration, whereas the turnover rate of the diazo dyes and of the triazo dyes remained constant as the dye concentration increased [59]. A number a researchers have correlated the level of colour removal with the dye class rather than with the molecular features [70]. Hitz et al. concluded that; (a) acid dyes exhibit low colour removal due to the number of sulphonate groups in the dye, (b) direct dyes exhibit high levels of colour removal that is independent of the number of sulphonate groups in the dye and (c) reactive dyes exhibit low levels of colour removal [71]. The effect of the sulphonate groups on colour removal is related to the mechanism by which the colour is removed. If the dye reduction takes place inside the cell, the presence of sulphonate groups will hinder the transfer of the dye molecule through the cell membrane. Therefore the rate of dye reduction will decrease as the number of sulphonate groups increases. However, if the dye reduction takes place outside the cell, the presence of sulphonate groups will have little effect on the rate of dye reduction.

Kulla found that cultures could be adapted to produce azoreductase enzymes that had very high specificity towards particular dye structures. One such enzyme, Orange I azoreductase, exclusively reduced the azo groups of Orange I and its derivatives with their hydroxy group in the para position. Another enzyme, Orange II azoreductase, was specific for Orange II-type compounds with their hydroxy group in the ortho position [72]. It was also shown that sulphonated dyes were reduced faster than carboxylated dyes due to the

higher electronegativity of the sulpho group, which renders the azo group more accessible to electrons [72].

Hydrogen bonding, in addition to the electron density in the region of the azo bond, has a significant effect on the rate of reduction [73]. The position and the nature of substituents on the dye molecule influence the azo-hydrazone tautomerism of hydroxyazo compounds. The hydroxy proton of phenylazo-naphthol derivatives is labile and can bond with a nitrogen atom of the azo group, causing a rapidly formed tautomeric equilibrium between the azo and hydrozone forms. This equilibrium is influenced by both structural factors within the molecule and by the nature of the medium surrounding the molecule. Zimmerman et al. found that, with certain azoreductases, a decreased rate of reduction was observed when the enzyme system was run with a substrate that was stabilised in the hydrazone form via hydrogen bonding, suggesting that the azo configuration of the substrate molecule was important for the enzymatic reaction. However, the degree of interference caused by the methyl group could not be appraised [44]. Zimmerman et al. made some generalisations with respect to the structural features that are required of the substrates for reduction by bacteria exhibiting the Orange II azoreductase:

(a) a hydroxy group in the ortho position of the naphthol ring is a prerequisite for the reaction;
(b) charged groups in the proximity of the azo group will hinder the reaction;
(c) a second polar substituent on the dye molecule lowers its affinity to the enzyme and inhibits the reaction; and
(d) electron withdrawing substituents on the phenyl ring will increase the rate of the reaction [64].

7.6. Electron donor

The oxidation of organic electron donors and/or hydrogen is coupled to the colour removal process. Bras et al. showed that the addition of electron donors such as glucose or acetate ions apparently stimulates the reduction cleavage of azo bonds [32]. The thermodynamics of the different electron-donating half-reactions are different. Therefore, the reaction rate is likely to be influenced by the type of electron donor [74]. It is important to determine the physiological electron donor for each biological colour removal process, as this will not only increase the rate of dye reduction, but it will also give an indication of the enzyme pathway responsible for the reduction reaction. For example, if formate is the most effective electron donor for the anaerobically induced electron transfer pathway to the dye molecule, then it can be concluded that the pathway must involve a formate dehydrogenase enzyme [75].

The concentration of auxiliary substrate (original electron donor) controls the rate of formation of reduction equivalents (intermediate electron donors). Yoo et al. found that the products of cell lysis can function as electron donors for anaerobic azo dye reduction with the active cells metabolising the lysis products [34]. Coenzyme-reducing equivalents that are involved in normal electron transport by the oxidation of organic substances may act as the electron donors for azo dye reduction [33]. Certain chemicals, such as thiomersal and p-chloromercuibenzoate, inhibit the alcohol dehydrogenase of NADH-generating systems required to generate reducing equivalents for dye reduction. Therefore, the rate of formation of NADH would be rate-limiting, causing inhibition of azo dye reduction [37].

7.7. Redox potential

Colour removal depends on the redox potential of the electron donors and acceptors, because the rate-controlling step involves a redox equilibrium between the dye and the extracellular reducing agent. The redox potential is a measure of the ease with which a molecule will accept electrons and can be reduced. Therefore, the more positive the redox potential, the more readily the molecule is reduced [52]. It follows that the rate of colour removal will increase with increasing (more positive) half-wave potential of the azo substrate. Bragger et al. showed that there is a linear relationship between the logarithm of colour removal

rate and the half-wave potential of the substrate [52]. This correlation between the reduction rate and the electrochemical property of the dye substrate suggests that the rate determining step in bacterial dye reduction does not involve a structure-specific phenomenon such as selective membrane permeation or enzyme binding [68].

The establishment of low oxidation–reduction potentials (<-400 mV) for the system, under anaerobic conditions, is required for high colour removal rates and has an effect on the profile of metabolites that are generated during the reduction process [35]. The colour removal rate is highest when the redox potential of the system is at its most negative and the rate falls as the redox potential of the system rises [29].

7.8. Redox mediator

As it is unlikely that highly charged sulphonated azo dyes will pass through the cell membrane, the dye reduction reaction must involve extra cellular reducing activity [50]. This reducing activity is achieved by using redox mediator compounds, such as flavins, to shuttle reduction equivalents from the cells to facilitate the non-enzymatic reduction of the extracellular azo dye [33]. A very small concentration of the redox mediator is sufficient for this electron transfer to take place. Redox mediators are characterised by a redox potential ranging from -200 to -350 mV [53]. The addition of synthetic electron carriers enhances the rate of reduction of azo dyes by bacterial cells. Quinone–hydroquinone redox couples are known to act as redox mediators [50]. Quinones undergo one-electron reductions to hydroquinone radicals or two-electron reductions to hydraquinones. For the reduction of azo dyes, reduction to the anion radical occurs by a fast one-electron transfer reaction, followed by a second, slower electron transfer event to produce the stable dianion [76]. The hydraquinone is then oxidised by the dye in a direct chemical reaction [74]. The application of natural, biodegradable quinones, such as lawsone, has technical potential for colour removal treatment systems because the reduction rate is increased without adding any environmentally problematic substances [76]. Lourenco et al. observed some colour removal in the presence of autoclaved cells, suggesting the existence of an active reducing factor that is capable of dye reduction in the absence of microbial activity [35]. Rate determining factors for the dye reduction reaction, involving the redox mediator, include the redox potential of the mediator in relation to the azo dye and the specificity of the reducing enzymes with respect to the mediator.

8. Conclusions

This review demonstrates the great potential of treatment systems incorporating whole bacterial cells to metabolise the azo dyes, present in coloured, aqueous wastewater from the textile coloration industry. Under anaerobic conditions, these systems can achieve total colour removal with short exposure times.

The application of treatment systems involving bacterial cells, on an industrial scale, is being encouraged by BIO-WISE, a major UK Government Programme funded by the DTI. The aim of BIO-WISE is to promote the commercial application of novel biotechnology solutions. A project in Northern Ireland to construct a pilot plant which uses a novel bioadsorption process, 'BIOCOL', to treat coloured textile wastewater, has already been funded [77]. The BIOCOL process uses whole *Shewanella putrefaciens* cells, which are grown and immobilised on an activated carbon support material. This carbon–biocatalyst amalgamation is contained in adsorption cartridges, through which the coloured wastewater is pumped for treatment. The carbon adsorbs the dyes, providing a large surface area on which bioreduction of the dyes by the biocatalyst can take place. The results obtained have been promising with the successful removal of colour from the wastewater stream.

It is important to understand the biochemical basis of the dye reduction so that biotreatment processes can be optimised fully, either through physiological manipulations or by genetic engineering. The techniques of molecular biology and biochemistry coupled with the latest advances in genomics and proteomics are revolutionising various aspects of fundamental biological sciences,

and will surely have an impact on the bioprocess industries. Indeed the availability of genome sequences for several potential important bacteria, including *Shewanella oneidensis* MR-1 (previously *S. putrefaciens* MR-1), will help facilitate a better understanding of the genetic basis of dye reduction by anaerobic bacteria including *Shewanella* species.

At present, BIOCOL is the only commercially available process in the UK that incorporates whole bacterial cells for the removal of colour from textile wastewater. However, as the knowledge base and the funding in this area of research increases, perhaps the use of bioprocessing will become the predominant solution to the problem of coloured wastewater in the textile coloration industry.

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