THE MICROBIAL DECOLOURISATION OF TEXTILE DYES

By

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ABSTRACT

Bacterial decolourisation of textile dyes, primarily Reactive Black 5, was studied using *Enterococcus faecalis* and a range of environmental isolates. Textile dye decolourising strains of *Shewanella*, *Pseudomonas* and *Clostridium* were isolated from a textile company effluent. The environmental isolates decolourised a wider range of dyes (17/18) than *E. faecalis* (8/18).

Dye decolourisation rates were determined using *Clostridium butyricum* and *E. faecalis* with the four reactive dyes: Reactive Black, Procion Navy, Procion Crimson and Procion Yellow. Glucose was supplied as electron donor and the dyes were tested in their parent and hydrolysed forms. The azoreductase activity ranged from 0.8 to 51.7mg dye/h/g dry cell weight for *E. faecalis* and 2.3 to 102.7mg dye/h/g dry cell weight for *C. butyricum.* The rates decreased in the order Reactive Black > Procion Yellow > Procion Navy > Procion Crimson, in both parent and hydrolysed forms. The initial decolourisation rate for hydrolysed Reactive Black was higher than for the parent dye (*E faecalis*: 51.7 and 21.2 mg dye/h/g dry cell wt, *C. butyricum*: 102.7 and 59.0 mg dye/h/g dry cell wt for hydrolysed and parent dye respectively). This was probably due to differences in degree of sulphonation. Bacterial decolourisation of Reactive Black proceeded via an intermediate, most likely the hydrazo, to produce di-amino H-acid and a vinyl sulphone side chain.

Azoreductase was detected in cell free extracts of *E. faecalis* using non-denaturing gel electrophoresis. Two bands of azo reduction were observed on Reactive Black stained PAGE gels. One band had a molecular weight of 114.4 kDa, whereas the second band was probably chemical in origin. Both decolourisation bands were oxygen insensitive and required the addition of NADH for visualisation.

Toxic effects were apparent in the *Vibrio fischeri* Microtox® test for decolourised Reactive Black (parent EC_{50} 1.7-4.9ppm and hydrolysed EC_{50} 0.15-0.25ppm). HPLC fractionation of decolourised hydrolysed Reactive Black samples demonstrated that the toxicity was due to the hydrolysed vinyl sulphone side chain which had an EC_{50} of 0.34ppm.

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1. INTRODUCTION.

1.1. General Introduction.

Colour is unique amongst pollutants in being highly visible and readily traceable (Churchley, 1995a). A survey by the Environment Agency (formerly the National Rivers Authority) in 1992 highlighted that colour was the subject of 700 complaints a year, nationally affecting over 600km of river. Most of the complaints were in the Severn Trent region (Waters, 1995). In particular it is deemed a serious problem in the East Midlands where dyehouse effluent is subject to very low dilution rates, in some cases as low as 1:2 (Pierce, 1994).

Of all the classes of dyestuffs azo dyes have attained the widest range of usage and are the most common type of dyes used for the colouration of textiles. Azo dyes are characterised by the presence of one or more azo groups (-N=N-) attached to two aromatic nuclei and can be classified as acid, basic, vat, sulphur, disperse, pigment, solvent or reactive. Increasing consumer demands for easy care fabric properties such as high fastness to washing, light and perspiration require dyes designed to be recalcitrant. At the present time only the reactive dyes are capable of producing the depth of shade and fastness properties demanded by both retailers and consumers (Easton, 1995).

Not all dyes give the same level of exhaustion in the dyeing process and the type of dye used has an important influence on the quantity that is eventually discharged to rivers. Many of the dye types (for example, basic, direct and disperse) are removed in conventional biological treatment processes by adsorption to biomass. The problem of coloured effluent is mainly associated with the reactive dyes which adsorb to a maximum of 30% and an average of only 10% (Pierce, 1994). The problem is exacerbated by the relatively poor fixation of the reactive dyes to substrates. The degree of fixation for the reactive dyeing of cellulose is 50 to 90%. As much as 50% of the reactive dye used may be lost to the effluent in a highly soluble hydrolysed form (Easton, 1995). Pierce (1994) estimated that the human eye can detect dye concentrations in the region of 0.005ppm whereas the discharge from a model dyehouse dyeing with reactive dyes will be on average 60ppm. Particular

problems encountered are the red and purple dyes as the eye is most sensitive to these colours and these are perceived as the most unnatural.

In February 1990 Severn Trent Water announced the Environment Agency's intentions to introduce standards for colour in rivers. The standards are absorbance values at a series of wavelengths (50nm intervals) based on a subjective study of what is and what is not acceptable in terms of coloured rivers. Conversion of the river standards into consents to discharge essentially left Severn Trent Water liable to prosecution for non-compliance and the problem was passed onto the dye effluent dischargers themselves. The Knitting Industries Federation (KIF) represents around 60 companies in the Nottingham, Leicester and Derby areas. An environmental committee formed by KIF established that there was no economically viable end of pipe treatment solution with current technology for the majority of dyers that discharge direct into sewers (ENDS report, 1993 and Harrison, 1995). If no solution could be found it was estimated that 4000 people employed directly in affected dyehouses would have their livelihoods threatened. Also to consider was the risk to jobs associated with the manufacture of fabrics and garments. In total 40,000 jobs could be in danger. This equates to 1 in 4 manufacturing jobs and 1 in 2 jobs in the inner city of Leicester. KIF's member companies had invested an estimated £300,000 by the end of 1992 in research to alleviate the colour problem. After considerable debate between KIF, Severn Trent Water and the Environment Agency the colour standards have been applied from January 1st 1996.

1.2. Textile colouration and resultant effluents.

Azo dyes are the largest class of dyes, more than 2000 of which are known and are used for dyeing every type of fibre. They have attained the widest usage due to variations in chemical structure being readily synthesised and methods of application being non-complex. A dual classification system is employed in the *Colour Index* (1971) according to usage and chemical constitution. Classification by chemical constitution yields four subdivisions: *monoazo, diazo, triazo* and *polyazo*. Classification by usage gives a better understanding of their practical applications. Azo dyes can be divided into various classes:

- *Acid dyes* are water-soluble and contain one or more sulphonate groups. The term "acid" refers to the acidic conditions used for dyeing. They are used to dye wool, silk, nylon and polyacrylic fibres.
- *Direct dyes* are those which can dye cotton directly without the use of a mordant. They are also used for dyeing paper and nylon.
- *Disperse dyes* are substantially water insoluble but can be dispersed in water as a fine suspension. They are used for dyeing cellulose acetate, nylon, polyacrylic and polyester fibres. Solvent dyes are also included in this class.
- *Mordant dyes* are metallised on the fibre after application and are used to dye wool, silk and nylon.
- *Sulphur dyes* are chemically complex and are used for dyeing cellulose fibres.
- *Vat dyes* are water insoluble dyes that are converted during the dyeing process into a water-soluble ionised form. They are principally used for dyeing cellulose fibres.
- *Reactive dyes* are a class capable of covalently bonding with fibres and are used mainly to dye cellulose fibres but also can be used to dye silk and nylon. They are characterised by a high degree of chemical and photolytic stability.

The extent of dye release into an effluent stream will depend on the dye used and the degree of fixation to the fibre in the dyebath, the lower the degree of fixation the greater the amount of dye will be lost to effluent. Table 1 shows a comparison of fixation for different dye/fibre combinations with the different dye classes. Sulphur dyes are known to have a low degree of fixation but are not considered problematic due to their low solubility and subsequent removal in conventional biological treatment processes. The reactive dyes are of more concern as up to 50% of dye used can be lost to the waste stream in a highly soluble hydrolysed form.

Reactive dyes are unique in that they contain reactive groups capable of covalently bonding with fibres. Many different reactive groups have been used for commercial dyes with the vinyl sulphone type being used in the highest volume (Locke, 1990). For the vinyl sulphone type of reactive dyes the reactive form of the dye is generated in the dye bath under alkaline conditions. The dye-fibre adduct is formed in a

subsequent reaction by 1,4 nucleophilic addition. Competing with this reaction is hydrolysis to give the hydroxyethyl sulphone (Weber and Stickney, 1993). Hydrolysis of the vinyl sulphone before formation of a covalent bond between dye and fibre will result in the hydrolysed dye having no affinity for the fibre and thus will be lost to effluent. An example of the formation of a vinyl sulphone and subsequent reaction with cellulose fibres or hydroxide ion (hydrolysis) is given in Figure 1.

Table 1. Estimated degree of fixation for different dye/fibre combinations.

Dyeclass	Fibre	Degree of fixation (%)	Loss to effluent (%)
Acid	Polyamide	80-95	5-20
Basic	Acrylic	95-100	0-5
Direct	Cellulose	70-95	5-30
Disperse	Polyester	90-100	0-10
Metal complex	Wool	90-98	2-10
Reactive	Cellulose	50-90	10-50
Sulphur	Cellulose	60-90	10-40
Vat	Cellulose	80-95	5-20

Reproduced from Easton (1995).

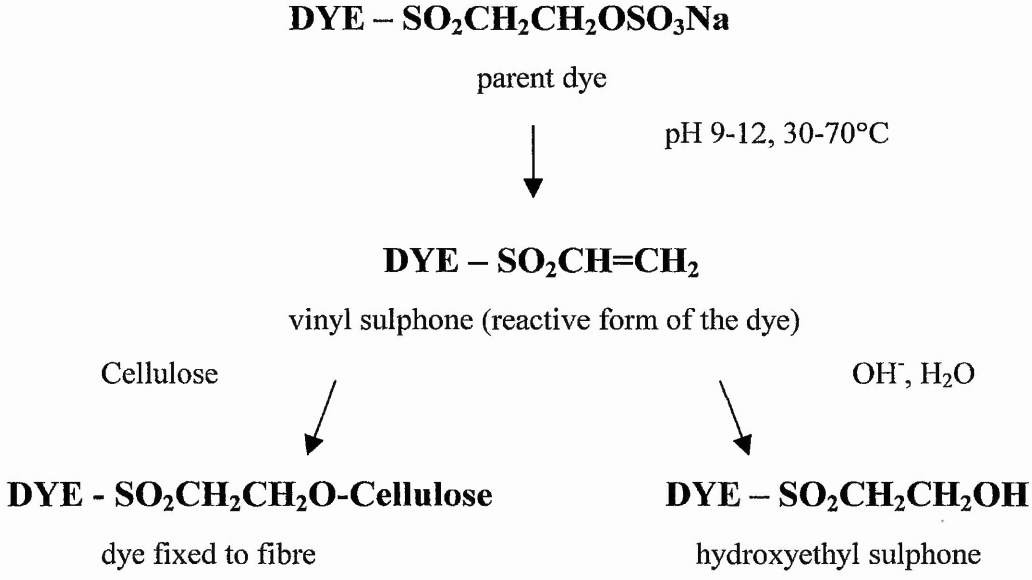


Figure 1. Pathway for the formation of a reactive dye vinyl sulphone and subsequent competing reactions: reaction with cellulose fibres and hydrolysis.

Reactive dyes have been much improved in recent years due to incorporation of multiple reactive groups on the dye molecule such that if one group is hydrolysed another is available for reaction with the fibre (Chakraborty, 1990). A range of high fixation reactive dyes have been developed but are of limited colour range (Bradbury *et al.*, 1992). A major change in technology would be needed to develop reactive dyes with >95% fixation across the full shade range under all application conditions. Dyestuff manufacturers, via the Chemical Industries Association, have stated categorically that this will not happen in the near future if at all (Pierce, 1994). If new dye structures are introduced they must be capable of application to substrates using current methods and equipment. A great deal of data is required under the New Substances Regulations 1982 covering areas such as identity of substance, information on use, physico-chemical properties and ecotoxicological studies (Hobbs, 1989). This would require a considerable investment of both time and money. Changing the structures of dyes to give more biodegradable alternatives would lead to less colour choice and poorer performance (Cooper, 1993).

Recovery and reuse of chemicals is another improvement option but is considered to be non-applicable to reactive dyes as any dye that does not react with the fibre is hydrolysed and exists in the effluent in an unrecoverable form (Cooper, 1993). Burkinshaw and Graham (1995) have investigated the recycling of exhausted dyebaths. The dyeing behaviour of the residual dyes bore little similarity to that of fresh reactive dyes with 50% lower exhaustion achieved with a reused dyebath than could be obtained with a fresh dyebath of the same concentration. For the reactive dyeing of cotton with four reactive dyes (3 reactive reds and 1 reactive green) the exhausted dyebath was found to contain both hydrolysed and reactive dye with the four dyes used undergoing differing degrees of hydrolysis during dyeing. Yang and Haryslak (1997) also stated that exhausted reactive dyes do not behave in a similar manner to the original dyes with the hydrolysed form of the dyes having low affinity for cotton fibres. In an investigation into the reuse of hydrolysed dyes for dyeing nylon and wool fibres hydrolysed Reactive Black 5 exhausted to 95.5% and 72.0% respectively. It was noted however that the hydrolysed dye did not show the same light fastness as Acid Black 1 used for comparison and the reuse of hydrolysed dyes for dyeing non-cotton fibres would need to be determined on an individual dye basis.

The structure of spent reactive dyes is generally considered to be the hydrolysed form. It has been proposed however that for Reactive Blue 19 the vinyl sulphone reactive form and the hydroxylate (hydrolysed) form of the dye would both be present in a wastewater from textile processing using this dye and that it is the vinyl sulphone form that predominates (Camp and Sturrock, 1990). The hydrolysis of the vinyl sulphone form of the dye was determined to be base mediated and that neutral hydrolysis by water was not a significant process. The half-life of the Reactive Blue vinyl sulphone form of the dye has been estimated to be 46 years at neutral pH and ambient temperature (Weber and Stickney, 1993). This would have serious environmental consequences if this form of the dye were to pass through treatment works and be discharged to surface waters. Under the alkaline conditions and higher temperatures encountered in a dyebath however it would seem reasonable to assume that the vinyl sulphone form of the dye would undergo hydrolysis. If this were not the case and the vinyl sulphone form of the dye was stable and did predominate then it would be expected that the degree of fixation of the reactive dyes would be higher. The well-documented hydrolysis reaction competing with the covalent bonding of dye to fibre would not be significant. It has not been determined in any other study to this author's knowledge whether significant quantities of the vinyl sulphone form of reactive dyes are reaching surface waters.

In general hydrolysed reactive dyes are not reused and are discharged to effluent for treatment. Due to the high solubility of spent reactive dyes they tend to pass through biological treatment works unchanged. Subsequent passage into receiving water can lead to the kind of complaints to the Environment Agency that have instigated the introduction of colour standards. Concentrations as low as 1ppm can cause complaints. Estimates of dye concentration in rivers due to dyehouse effluent are shown in Table 2. These values are clearly much higher than the minimum values detectable by the human eye (0.005ppm) and any such discharge would therefore be a matter of great public concern.

Table 2. Calculated estimates of dye concentration in river water as a result of dye release by the textile industry.

Scenario [a]	Weight of dye released (kg)	Concentration in receiving water (ppm)[b]	Number of days per year
1-average	0.8	5.3	50
1-worst case	56	1555	2
2-average	0.6	1.2	25
2-worst case	17.5	364	2

(a) 1-Batchwise dyeing of cotton with reactive dyes.
2-Batchwise dyeing of wool yarn with acid dyes.

(b) Assumes no adsorption of dye on the sludge at the sewage treatment works.

Reproduced from Hobbs (1989).

1.3. The Environment Agency colour standards.

As colour is an aesthetic problem the standards are based on a subjective panel assessment of what is and what is not acceptable in terms of colour in rivers. Waters (1995) gives an account of the approaches taken to define the standards. The standards are absorbance values at 50nm intervals across the visible spectrum. Examples of river standards are displayed in Table 3. Absorbance is measured at the specified wavelengths in a 1cm cell against distilled water after filtration through a 0.45μm filter.

Table 3. Standards for determining consents for discharge in three Severn Trent catchments.

River	Absorbance at wavelength (nm)						
	400	450	500	550	600	650	700
Churnet	0.025	0.015	0.012	0.010	0.008	0.005	0.003
Erewash	0.025	0.015	0.012	0.010	0.008	0.005	0.003
Bently Brook	0.027	0.016	0.014	0.012	0.010	0.008	0.004

Reproduced from Waters (1995).

Concerns expressed by industry in response to the consents were that as the eye is most sensitive at 555nm and approximately 45% of the total region requiring colour loss has no impact on the ability of the eye to detect colour, namely 400nm-475nm

and 650nm-700nm (Cooper, 1995). Despite this argument the river standards have been used to set consent limits across the whole visible spectrum.

Based on the river standards consents for discharge have been calculated as absolute limits and examples for the Severn Trent region are given in Table 4. These limits equate to concentrations in the region of 0.01 to 2ppm. If, for example, 1ppm were the potential limit a model dyehouse dyeing with reactive dyes can be considered to have a fully balanced effluent of 60ppm. This would require a dilution of at least 60:1 in order to comply with the potential consent. If the effluent were treated with an "end of pipe" decolourisation technique then a colour removal efficiency of 98% would be necessary for compliance (Pierce, 1994). This represents a theoretical situation but the model agrees with a study commissioned by KIF at Nottingham Trent University into colour samples taken from a representative selection of dyehouses (Harrison, 1995).

Table 4. Colour consent limits on selected sewage treatment works.

Location	Consent limit (absorbance) at wavelength (nm)						
	400	**450**	**500**	**550**	**600**	**650**	**700**
Loughborough	0.031	0.019	0.013	0.012	0.012	0.011	0.003
Wigston	0.034	0.029	0.017	0.016	0.014	0.014	0.007
Wanlip	0.035	0.023	0.020	0.021	0.012	0.012	0.008
Pinxton	0.047	0.027	0.023	0.019	0.016	0.008	0.004
Leek	0.060	0.040	0.035	0.025	0.025	0.015	N/A

Reproduced from Collins (1994).

In the East Midlands dilution rates in rivers may be as low as 1:2 and due to a large concentration of dyehouses coloured effluent is deemed a serious problem. An area of particular concern is the Erewash Valley. Maps of the region are given in Appendix 1. Upper reaches of the River Erewash are highly coloured with a pinkish or blackish appearance due to discharges from Pinxton and Pye Bridge sewage treatment works receiving coloured trade effluents. A similar problem is evident at Langley Mill due to coloured discharges from Miln Hay sewage treatment works. A detailed discussion of Pye Bridge and Miln Hay sewage treatment works and colour removal within the plants is given in Section 1.5. There are a total of 8 sewage

treatment works discharging to the River Erewash and during dry weather conditions it is estimated that the river is composed of 70% treated sewage effluent.

Responsibility for alleviation of the colour problem has been the subject of considerable debate. Colour limits have been defined for sewage treatment works (STW) and examples of colour measurements for two such works in the Severn Trent region are given in Table 5 (Environment Agency, 1996). The measurements were made during the period August 1994 to July 1996. The treatment works listed have consents imposed for the 500nm, 550nm, 600nm, and 650nm wavelengths. Comparison of the field data for Pinxton and Wanlip given in Table 5 with the consent limits detailed in Table 4 show that 100% compliance demanded for standards is not achieved.

Failure to comply with the colour limits has essentially left Severn Trent Water liable to prosecution by the Environment Agency and the costs of this are being passed down the chain to the dischargers themselves. The argument as to who has the responsibility of treating the effluent (i.e. STW or the dischargers) is ongoing.

Table 5. Measured absorbances at consent wavelengths for Wanlip and Pinxton sewage treatment works.

	Absorbance at wavelength (nm)			
	500	**550**	**600**	**650**
Wanlip [1]				
Min	0.003	0.001	0.001	0.001
Max	0.055	0.051	0.051	0.026
Mean	0.0183	0.0155	0.0152	0.0076
Pinxton [2]				
Min	0.010	0.005	0.004	0.002
Max	0.149	0.126	0.106	0.0790
Mean	0.0560	0.0462	0.0350	0.0243

1 Wanlip STW discharges to the river Soar. Data based on 91 samples.
2 Pinxton STW discharges to river Erewash. Data based on 84 samples.

From an industry point of view it has been estimated that the capital cost of an end of pipe treatment process would be in the region of £250,000 to £500,000 (Cooper, 1995). Considering an average of £375,000 for an estimated 75 dyehouses affected this cost would be in the region of £28 million with running costs of £0.72 - £1.17 m^{-3}. Land requirements could be up to 1 acre (ENDS report, 1993) and as many

companies in the East Midlands are located in cities they do not have the space required for installation of an end of pipe treatment plant. Location in city areas not close to rivers means that many companies have neither the space nor the substantial capital required to fund installation of on site treatment plants and therefore have no option but to discharge to the public foul sewer. As far as the industry is concerned the lowest cost option would be to treat the effluent at sewage treatment works where most of the infrastructure needed is already in place and maximum balancing effects and dilution benefits are apparent.

1.4. Environmental fate of dyestuffs and the potential for toxicity.

1.4.1. Dyes in the environment.

The predicted environmental concentration of a dye can be calculated from knowledge of the following: daily usage, degree of fixation, degree of removal in any treatment processes and dilution factor. Average volumes give concentrations in the range of 1ppm although concentrations can be much higher than this (Easton, 1995). Examples of worst case concentrations of dyes have been given in Table 2 (Section 1.2) and have been predicted to be as high as 1555ppm. In an assessment of the effect and fate of a dye in the environment comparison can be made of this predicted maximum concentration with the concentration at which the dye has demonstrated adverse effects. The dye could be considered unlikely to cause harm if there is a factor of at least 100 between the two concentrations (Hobbs, 1989).

Di Giano *et al.* (1992) used effluent from a textile dye manufacturing plant to assess the Environmental Protection Agency (EPA) Toxicity Identification Evaluation protocol (American standard). Identification of specific organic chemicals in the effluent was not performed but likely constituents were considered to be dyes (over 100 disperse, acid, basic, and azo dyes, 1 anthraquinone dye), dye intermediates, dye precursors and detergents. In addition the effluent had a high chloride loading. Toxicity testing was performed with *Ceriodaphnia dubia* with an LC_{50} value (concentration for 50% mortality in 48h) used as the end point for acute toxicity. Chloride was found to be the major source of toxicity accounting for approximately one third to one half of observed toxicity. The effluent was solid phase extracted (SPE) with various water/methanol ratios and the fractions re-tested for toxicity.

Fractions displaying toxicity were further High Performance Liquid Chromatography (HPLC) fractionated, re-submitted for toxicity testing and any toxic fractions were identified by GC-MS. The source of the remaining half to two thirds of observed toxicity not due to chloride was deemed difficult to determine. Approximately one third of all peaks detected by GC-MS were identified and among them 4-phenylazophenol (Solvent Yellow 7) and 4-phenylazoaniline (Solvent Yellow 1) were detected. Not all compounds present in the HPLC fraction were identified and it is not known whether the presence of other dyes, in particular the more polar dyes, contributed to the observed toxicity. It is doubtful that the GC-MS employed for identification would be suitable for dye determination.

Rutherford *et al.* (1993) have also used GC-MS to characterise organic compounds of a mill effluent and their contributions to toxicity. A range of toxicity tests were employed including the Ames test, Microtox® test and effects on rainbow trout (*Oncorhynchus mykiss*), *Daphnia magna*, *Selenastrum capricornutum* and *Ceriodaphnia dubia.* Measurement of accumulation of environmental contaminants at one of the textile mills studied was determined by deployment of caged fresh water clams in the effluent stream. The greatest number of organics identified in the effluent were auxiliary chemicals required for satisfactory dyeing with the largest portion due to dye carriers. Samples were collected (12 in total) from 3 mills and all samples were acutely toxic to all organisms with the exception of a single sample from one mill in the Microtox® test. All samples had sub-lethal effects on all species tested including reproductive impairment in *Ceriodaphnia dubia*, and growth impairment on the alga *Selenastrum capricornutum.* Untreated effluent was found to be moderately toxic to rainbow trout with LC_{50} values ranging from 11.5% to 35.4% v/v. All samples were mutagenic in the Ames test with different samples displaying different types of mutagenicity. Benthic macroinvertebrate surveys indicated that discharges had a biological impact on the receiving environment as species diversity was significantly lower at impacted sites compared with a control site that had not been in contact with any mill effluent. Aquatic insects, snails and leeches were negatively impacted. All caged clams sited within the effluent plume died and all but 4 died sited 120m from the effluent outfall. Dye carriers such as 1- and 2-methylnaphthalene and dimethylnaphthalene were detected in the clams sited

at 120m from the discharge. Recommendations made were that untreated textile effluent should not be discharged directly to watercourses. Given the complex nature of the effluent and that a number of components may be toxic to a variety of aquatic organisms a battery of toxicity tests should be employed when assessing the impact of textile wastewater.

Moran *et al.* (1997) investigated the toxicity of 3 textile effluent samples to *Daphnia magna* before and after sewage treatment. Two of the samples were from a cotton textile finisher and the third from a finisher working mainly with synthetic fabrics. The *Daphnia* were exposed to serial dilutions of effluent in artificial pond water and the concentration for 50% mortality (LC_{50}) determined. The effluent samples were then incubated with activated sludge from a major sewage treatment works for 10 days, filtered and concentrated back to their original volume. The toxicity assessment was performed again and the LC_{50} values before and after sewage treatment compared. Post aerobic digestion negligible toxicity reduction was observed. It was assumed that the non-biodegradable portions of the effluent such as metals, salts, complex organics and dyestuffs were responsible for the toxicity of the textile effluents to *Daphnia*. The toxic portion of the effluent would therefore pass through the treatment works and subsequently enter surface waters unchanged.

O'Neill *et al.* (2000) using a respiration inhibition test demonstrated an increase in toxicity following the anaerobic treatment of a simulated textile effluent containing Procion Red H-E7B. Elimination of toxicity was observed following subsequent aerobic treatment. This was thought to indicate that the products of dye reduction exiting the anaerobic stage were being aerobically degraded. The textile effluent and the final effluent (post aerobic treatment) had undetectable toxicity in the 3h respiration inhibition test whereas the effluent exiting the anaerobic stage showed inhibition in the same test at 17.9%.

Although there is a lack of research into concentration and identity of dyes in the environment several studies have been performed to assess the effects of textile effluents on a range of organisms including bacteria, fish and plants. A summary is given in Table 6.

Several authors have investigated the effect of textile effluents on fish and as illustrated by Table 6 many adverse effects have become apparent ranging from

damage to gill filaments, blood cells and ovaries to reduction in growth rate and behavioural changes. Selective accumulation of dyes in fish organs has also been observed (Riva *et al.*, 1992). In contrast Churchley (1995a) considered toxicity of dyes to fish to be negligible at visually noticeable concentrations. The role of dyestuffs in observed toxicity is difficult to determine as the majority of studies have used whole effluents containing numerous constituents. Murugesan *et al.* (1989), Murugesan and Haniffa (1992) and Chhaya *et al.* (1997) suggested that metal ions present in the effluents tested may be responsible for the toxic effects observed.

In cases where dye solutions have been assessed for toxic effects opposing views as to cause are apparent. Sujatha *et al.* (1992) suggested that zinc present as a contaminant in solutions of 2 dyes tested was responsible for adverse effects observed on Greengram seedlings. Wells *et al.* (1994) periodically tested the toxicity of textile effluents to *Daphnia pulex* over approximately 2 years and found the major contributor to be zinc entering the system as an impurity in other chemicals. Shukla and Gupta (1994) suggested that for the anionic dyes they tested complexes formed between the dyes and metal ions in test organisms with subsequent disruption to metabolic processes.

Fractionation of effluents and identification of toxic components as described by DiGiano *et al.* (1992) has had limited success due to the reliance on GC-MS as the identification technique. It has already been highlighted that this is not appropriate for identification of dyes due to their non-volatile nature. Toxicity directed fractionation of a textile effluent has also been described by Svenson *et al.* (1996). Dominant acute toxicants in a textile effluent were fractionated using solid phase extraction (SPE) and toxic contribution evaluated using the Microtox® test. The results indicated that for the effluent tested toxicity observed in the Microtox® test was due to 2 unsaturated fatty acids, 2 tridecanols and a mixture of aliphatic hydrocarbons. Identification was again achieved using GC-MS.

It is thought likely that variations in toxic loading both between and within dyehouses would occur due to the inconsistent nature of the textile business. Dyes used and hence dyeing processes will change from week to week and season to season depending on consumer demands and fashion trends.

Table 6. Research into the environmental effects of textile effluents.

Organism	Exposure	Effect	Reference
Allium roots	2 sewage plant and 12 industrial effluents including textile waste	Textile waste: no genotoxic effect	Nielsen and Rank (1994)
2 freshwater marsh plants	6 industrial effluents including textile mill	Textile waste: inhibited early growth in the light.	Walsh *et al.* (1991)
Greengram (*Vigna radiata*) and symbiotic bacterial population (*Rhizobium*)	Navy Blue M3R Direct Brown 2G	Decreased germination, root and shoot lengths, conc. proteins, amino acids, carbohydrates and chlorophyll. Decreased growth of *Rhizobium*.	Sujatha *et al.* (1992)
Greengram (*Vigna radiata*)	Textile mill effluent	Inhibited germination. >25% v/v decreased growth and dry weight.	Vijayarengan and Lakshmanachary (1993)
8 tree species	Textile effluent	No effect observed after 18 months irrigation.	Aggarwal *et al.* (1994)
Rainbow trout (*Oncorhynchus mykiss W.*)	Acid Violet 66 Acid Red 217	Selective accumulation in individual organs.	Riva *et al.* (1992)
Freshwater fish (Trewaves) *Oreochromis mossambicus*	Textile effluent	Elevation of blood cell count and haemoglobin, decrease in carbohydrate, protein, fat and ash contents.	Haniffa and Selvan (1991a)
Freshwater fish (Trewaves) *Oreochromis mossambicus*	Bleaching and dyeing effluents	Bleaching effluent LC_{50}(96h) 6.12% v/v, dyeing effluent LC_{50}(96h) 5.2% v/v	Haniffa and Selvan (1991b)
Carp (*Cyprinus carpio*)	Prussian Turquish Blue Prussian Red	Toxic to fish eggs and delayed development of dye exposed hatchlings behavioural changes in higher conc. dyes.	Rao *et al.* (1988)
Carp (*Cyprinus carpio*)	Textile effluent	LC_{50}(96h) 10% v/v, 15% v/v caused 100% mortality in 24h, sublethal conc. reduction in food intake and growth rate noted	Sakthivel and Sampath (1989)
Freshwater prawn (*Macrobrachium idea*)	Textile effluent	Damage to gill filaments and ovaries	Xavier *et al.* (1992)

Table 6. Continued.

Organism	Exposure	Effect	Reference
Nigerian catfish (*Clarias lazera*)	Textile effluent	Genotoxicity detected as increase in micronucleus frequency in erythrocytes	Odeigah and Osanyipeju (1995)
Indian catfish (*Heteropneustes fossilis*)	Partially treated textile effluent	Change in erythrocyte appearance cytoplasm of leucocytes displayed vacuolation	Murugesan *et al.* (1989)
Indian catfish (*Heteropneustes fossilis*)	Textile effluent	Adverse effects on ovaries	Murugesan and Haniffa (1992)
Nile Bulti (*Tilabia nilotica*)	Textile effluent	LC_{50}(96h) 14.8% v/v	Abo-Elela and Wahaab (1988)
Mudskipper (*Periophthalmus dipes*)	Dyeing and printing industry effluent	LC_{50}(96h) 1.72% v/v	Chhaya *et al.* (1997)
Diazotrophic cyanobacterium (*Nostoc muscorum*)	Omega Chrome Red ME Metomega Chrome Orange GL	Exposure to 20ppm dye decreased protein, chlorophyll *a*, cartenoid and phycocyanin contents and reduced photosynthetic oxygen evolution	Shukla and Gupta (1994) Shukla *et al.* (1994)
Daphnia pulex	Textile effluent	LC_{50}(48h) 74.14% v/v	Wells *et al.* (1994)
Daphnia magna	Textile effluent	LC_{50}(48h) 5-8% v/v	Moran *et al.* (1997)
Ceriodaphnia dubia	Textile effluent	LC_{50}(48) 6% v/v	DiGiano *et al.* (1992)
Bacillus subtilis	Aminoazobenzene, hydroxyazobenzene, Acid Orange 12, Acid Orange 10, Direct Red 28	All except Direct Red 28 inhibited cell division	Ogawa *et al.* (1981)
Vibrio fischeri	Textile effluent	EC_{50}(15min) 8.7%	Svenson *et al.* (1996)
Microtox® test, rainbow trout, *Daphnia magna*, *Selenastrum capricornutum*, *Ceriodaphnia dubia* and freshwater clams	Textile effluent	Acute toxicity to all organisms reproductive impairment in *C. dubia*, and growth impairment in *S. capricornutum* rainbow trout LC_{50} 11.5-35.4% v/v, mutagenic in the Ames test	Rutherford *et al.* (1993)

The chemical nature of an effluent will change with changes in production hence characterisation of effluent toxicity based on small numbers of samples from specific dyehouses will not provide a true representation of the environmental effects of a textile wastewater discharge. There is a lack of data with regards to dyestuff toxicity and its contribution to the overall toxicity of an effluent. A list of dyes classified as toxic based on their acute per-oral LD_{50} value in accordance with the EEC Council Directive 79/831 has been published by Anliker *et al.* (1988). The list constitutes 2 acid dyes, 6 basic dyes, 1 direct dye and 3 azoic diazo components. When considering the thousands of dyes and intermediates currently in use it is surprising that so few are classified as toxic under the directive. Categorisation of toxicity on this basis may be somewhat misleading as many dyes are known to be non-toxic but may yield toxic breakdown products. Dyes and dye breakdown products have been shown to be toxic/mutagenic and a discussion of this area is given in Section 1.4.2. Dyes are rarely produced with 100% purity, in particular commercial grades and potentially toxic intermediates used for their manufacture may be present in the final dyestuff. The toxic effects of dye intermediates and potential breakdown products must be taken into account in the development of decolourisation systems for dyehouse effluents.

1.4.2. The toxicity of dyestuffs.

As early as 1895 the German surgeon Rehn reported on what he considered to be an undue incidence of bladder tumours in a group of men employed in the manufacture of fuchsine (magenta). He concluded that aniline was the "most suspicious of the substances used in this process" (Case *et al.*, 1954). Case *et al.* (1954) studied tumour incidence in workmen engaged in the dyestuff manufacturing industry. Data available indicated that the overall risk of dying of bladder tumour in this industry was approximately 30 times that of the general population. Morris-Brown *et al.* (1995) investigated the relationship between high bladder cancer mortality and occupation and found elevated risks for men employed in the textile industry for more than 5 years. Risk was significantly increased for men who both worked at and lived within 1 mile of a textile factory. Exposure to aromatic amines was thought to be a major occupational contributing factor to bladder cancer.

The potential health risks associated with exposure to benzidine and benzidine based dyes has been the subject of a National Toxicology Program (America) research initiative undertaken by Morgan *et al.* (1994). The study investigated the metabolism, disposition, mutagenicity, toxicity and carcinogenicity of a select group of prototypical dyes (acid and direct classes) derived from two primary benzidine congeners known to increase tumour incidence in rats, namely 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine. The aims were to predict the carcinogenic potential of other dyes derived from benzidine. Conclusions drawn from the study were that following ingestion the azo linkages of most dyes tested were reduced to release free benzidine or the respective congeners that were subsequently excreted in the animal's urine. All soluble dyes tested (13 in total) with the exception of Direct Blue 218 were found to require reductive metabolism before they exhibited mutagenicity with only Direct Black 38 and Direct Blue 2 found to be mutagenic without activation. Consumption in drinking water of the benzidine congeners and the dyes Acid Red 114 and Direct Blue 15 resulted in high incidences of malignant tumours in experimental animals. From the study it was concluded that dyes based on benzidine or its congeners should be regulated as probable human carcinogens.

Mutagenicity and carcinogenicity testing has been mainly concerned with dyes based on benzidine although their use is restricted in some countries and benzidine manufacture prohibited in others (Notani *et al.*, 1993). Information regarding the safety of other textile dyes is limited.

1.5. Effluent treatment.

A great deal of research has been undertaken to find viable treatment options for coloured effluents. Biological techniques are in competition with chemical and physico-chemical processes. Chemical and physico-chemical processes are usually less specific and need less time to be developed. Some are readily available but few have been proven at full scale. Capital outlay and running costs can be high. Biological treatment needs investment in research and development but may prove to be a better long-term option due to considerably lower energy costs.

In general aromatic compounds can be readily degraded in conventional sewage treatment. The inability of conventional sewage plants to degrade azo dyes has been attributed to their xenobiotic nature. The inadequacy of conventional sewage treatment works with respect to dyehouse effluent has already been highlighted. It is not surprising that dyes, reactives in particular, are not susceptible to biodegradation as they have been specifically designed to be stable, robust compounds.

Collins (1994) has made a detailed study of colour removal in three sewage treatment works. The treatment works are located at Hinckley (Leicester District), Pye Bridge (Sherwood District) and Miln Hay (Nottinghamshire District). Each of the treatment works receives effluent from one or more dyehouses.

(a) Hinckley

Average daily flow was 24Ml with textile waste received from four dyehouses accounting for 16.5% of the flow (50% of which due to reactive dyes). The Treatment system consisted of primary sedimentation, biological filtration and humus tanks, with 40% of flow treated by grass plots prior to discharge at Sketchley Brook. Average colour removal achieved through the works was 53%, 25% due to primary settlement and 28% to conventional biological treatment and the tertiary treatment of 40% of the flow in grass plots. The final effluent discharged within the colour consent limits in 50% of samples taken. Failures were at 400nm and 450nm. Of the 7 samples that failed the consent limits 4 coincided with increased colour upstream of the treatment works.

(b) Pye Bridge.

Average daily flow was 1.3Ml, 19% of which was due to textile waste (disperse, sulphur and basic dyes). The treatment system comprised primary settlement, biological filtration and final settlement prior to discharge into the River Erewash. Average colour removal achieved was 66%, 44% due to primary settlement (thought to be a function of the dye types present) and 22% due to removal in the biological stage. Compliance with consents was achieved for 28% of samples taken. When influent colour was lower removal in the biological stage remained constant at 23% but a greater percentage of removal was achieved through primary settlement (56%). The overall increase was 81%, which would have met consent discharges. The

River Erewash is however highly coloured by the time Pye Bridge effluent is discharged due to effluent from Pinxton Sewage treatment works located up river.

(c) Miln Hay.

Average daily flow was 10.2Ml, 12.5% of the flow due to textile waste (acid, basic, reactive and premetallised dyes). The treatment system comprised primary settlement, biological filtration and humus tanks prior to discharge to the River Erewash. Average colour removal achieved was 80.3%, with 53% due to primary settlement and 26% due to the biological stage. The final effluent discharge was within consents at all wavelengths except 400nm. During the sampling period the dyehouse discharging to Miln Hay was closed for maintenance and no coloured effluent was received at the works during this time. Even during this time however the treatment works did not achieve the 100% compliance demanded for the colour consent limits.

Conclusions of this study were that the colour removal across the works was influenced by the dye types discharged and can be attributed to three main areas:

(a) Primary settlement where dyes were adsorbed onto suspended solids and settled out.

(b) Secondary biological treatment where there was an element of degradation and adsorption onto biomass.

(c) In the case of Hinckley, colour removal was observed across the grass plots (mechanism likely to be filtration and possibly some anaerobic degradation).

Compliance demanded by the new colour standards is 100%. This cannot be achieved by any of the sewage treatment works described here. It was noted that all three treatment works received a discontinuous inflow of effluent creating peaks of colour. It was considered likely that colour removal across the works would improve with suitably designed equalising systems to ease the load on the treatment works and avoid adverse effects caused by peaks and troughs of colour. It was also noted that although the final effluent from Hinckley treatment works was within the specified river quality objective (RQO), the colour upstream of the works exceeded the RQO causing non-compliance for downstream samples.

In an investigation into the degradation of dyes in aerobic systems Pagga and Brown (1986) tested 87 dyes in a short-term aerobic degradation test. This test was designed to simulate conditions of an adapted activated sludge plant. The dyestuffs selected for the test were readily water-soluble as it was assumed that they would be less likely to be adsorbed onto the activated sludge. For 44 of the 87 dyes tested decolourisation and simultaneous reduction in dissolved organic carbon (DOC) was achieved. Adsorption of the intact dye molecules was proposed as the prime removal mechanism. The authors considered it unlikely that any of the dyes tested would be degraded in such short term aerobic tests.

In a subsequent study Brown and Hamburger (1987) examined 14 azo, one anthraquinone and one phenoxazine dye in a two step procedure with anaerobic digestion followed by an aerobic phase for 9 of the dyes. The anaerobic phase produced removal efficiencies of 100% for 8 of the 16 dyes tested (initial concentration 100mg/l). Of the remaining 8 dyes, 6 were removed to >90% with Acid Yellow 25 and Basic Blue 3 having removal efficiencies of 57% and 44-73% respectively. The anaerobic phase gave rise to the expected aromatic amine metabolites predicted from the dye structure assuming reduction takes place via azo bond cleavage. For the example of Acid Yellow 36, equimolar amounts of 3-aminobenzenesulphonic acid and N-phenyl-1,4-diaminobenzene would be expected to be produced upon decolourisation of the dye. Both these metabolites were detected but at levels much lower than the theoretical yield (65% and 5% respectively). Less than quantitative yields of expected metabolites were also observed for some of the other dyes. It was thought that the amines would not be further degraded under anaerobic conditions and that the purity of the dyes or production of sparingly soluble or sorptive metabolites may be a more likely explanation for the lower quantities of amines detected. Aerobic degradation of the resultant aromatic amines from decolourisation was accompanied by monitoring dissolved organic carbon (DOC) or specific analysis of particular metabolites. Measurement of DOC indicated that for 3 of the 9 dyes tested metabolite degradation of >75% was achieved. Measurement by specific analysis indicated that metabolite degradation of >75% was achieved for 5 of the 9 dyes. The aerobic degradation of selected aromatic amines was demonstrated but believed to be

dependent on structure, as some of the sulphonated aromatic amines were not degraded.

The conclusions of Pagga and Brown (1986) with regards to the inability of aerobic systems to degrade dyes have been confirmed by a study into the fate of azo dyes in the activated sludge process by Shaul *et al.* (1991). Eighteen water-soluble acid and direct azo dyes were studied and 11 were found to pass through the activated sludge process untreated. Ten of the 11 had at least 2 sulphonic acid groups (the remaining dye had 1) and it was thought that due to the high water solubility conferred there would be little or no adsorption by the sludge thus limiting the possibility of biodegradation occurring. Of the other 7 dyes, 4 were found to be adsorbed onto the waste activated sludge whereas the other 3 were apparently degraded as little of these dyes could be recovered during sampling.

This series of studies by Brown and co-workers suggest that although dyestuffs generally do not appear to be susceptible to aerobic treatment, the decolourisation of dyes under anaerobic conditions can be readily achieved. An extensive review of the anaerobic treatment of textile effluents has been presented by Delee *et al.* (1998). An account is given of various factors affecting the application and performance of anaerobic digestion with regards to textile effluent treatment namely: organic load, colour, nutrients, pH, salt effects and the presence of inhibitory compounds. In the context of this thesis the following discussion is restricted mainly to the potential of anaerobic digestion for decolourisation of textile dyes.

Jiang and Bishop (1994) employed rotating drum biofilm reactors for the decolourisation of 3 acid dyes under various operating conditions. Acid Orange 10 and Acid Red 14 were decolourised only when the bulk liquid dissolved oxygen (DO) concentration in the reactor was less than 1mg/l. Under this condition dye decolourisation up to 60% for both dyes was achieved. Acid Orange 8 removals ranged from 20-90% depending on the reactor operating conditions. The 90% removal was achieved with a low chemical oxygen demand (COD, 70mg/l) and high DO (6mg/l). Under these conditions however the biofilm became thinner indicating the conditions applied were detrimental to the biofilm stability. At a concentration of 5mg/l the dyes inhibited biofilm growth.

FitzGerald and Bishop (1995) have studied a similar range of dyes in a two-stage reactor system. The reactor system consisted of an anaerobic fixed-film fluidised bed reactor followed by an aerobic suspended growth activated sludge reactor. The dyes studied were Acid Red 14, Acid Orange 10 and Acid Red 18 (10mg/l). In the anaerobic stage decolourisation >90% was achieved for the two red dyes and >65% for Acid Orange 10. Further decolourisation in the second aerobic stage was negligible. Analysis of dye degradation products was undertaken and it was observed that the azo cleavage products expected to exit the anaerobic first stage untreated were only detected at low levels. The actual concentration of the expected decolourisation products detected by Liquid Chromatography-Mass Spectrometry (LC-MS) inferred that mineralisation of the dyes (>99%) was occurring in the first anaerobic stage. Adsorption to biomass and low analytical recovery efficiencies were suggested as possible explanations but were thought unlikely to account for the total apparent loss of the dye metabolites. Low metabolite recoveries considerably less than expected yields were also observed by Brown and Hamburger (1987) as discussed above.

Carliell *et al.* (1994,1995) studied the decolourisation of selected reactive dyes in conventional anaerobic digestion. A total of 18 dyes were studied (100mg/l) using a serum bottle assay inoculated with digester sludge and 80% of the dyes used were decolourised to between 70% and 97% with the exception of Reactive Yellow 95 for which no decolourisation was observed. Reactive Yellow 95 was a printing dye and it was thought that the commercial preparation of the dye used might contain inhibitory or toxic components. The other dyes not significantly decolourised were of the anthraquinone and phthalocyanine type, which were assumed to be more stable and less susceptible to reduction.

In a more detailed study the metabolites of Reactive Red 141 were tentatively identified using proton Nuclear Magnetic Resonance Spectroscopy (NMR) analysis. Four products were obtained upon reduction of the dye and it was proposed that under anaerobic conditions decolourisation proceeded via azo bond cleavage and breakage of the amine linkages between the chromophore and the reactive group and within the reactive group itself. The proposed degradation products of Reactive Red 141 are given in Figure 2.

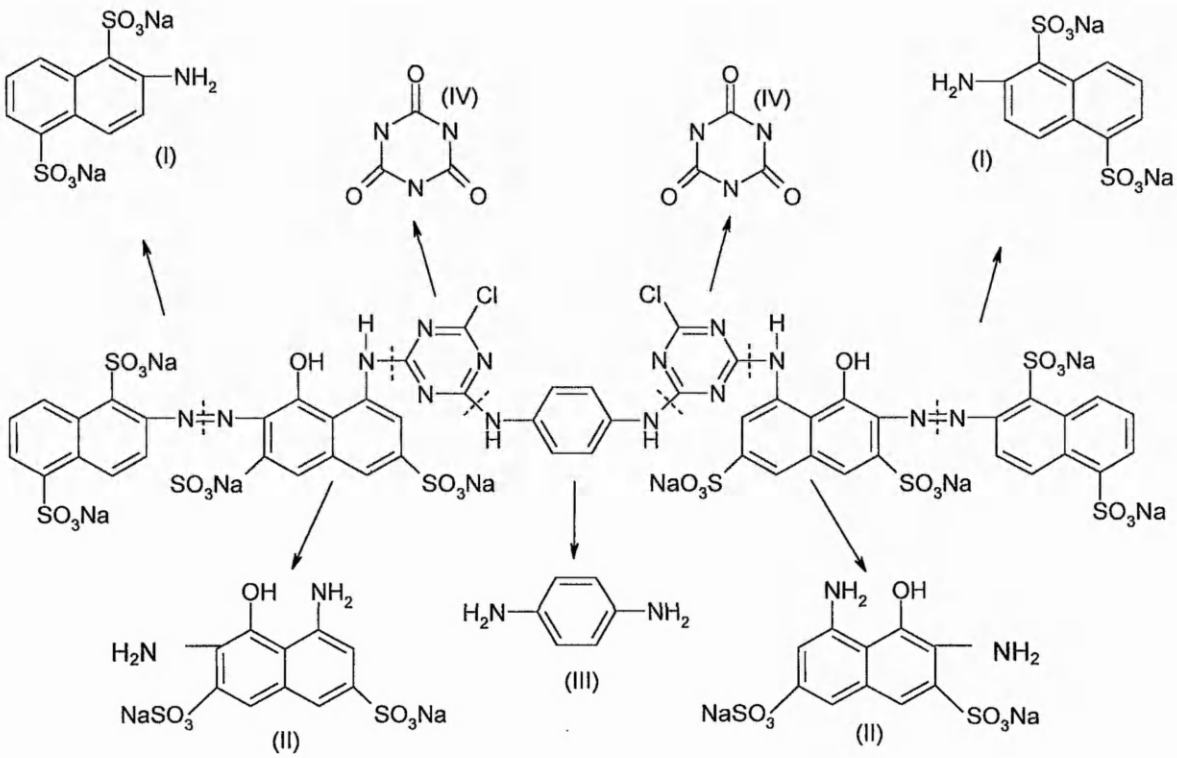


Figure 2. **Proposed degradation products of Reactive Red 141. Reproduced from Carliell *et al.* (1994).**

In a further study the fate of CI Reactive Red 141 in a Bardenspho nutrient removal reactor (1 anaerobic zone, 2 anoxic zones), was investigated and complete decolourisation was achieved within 30 minutes. Small-scale studies using biomass from the reactor implied that removal was due to breakage of azo bonds. Extension of the study to full scale trials indicated that addition of an exhausted reactive dyebath effluent did not adversely affect system performance of an experimental anaerobic digester as compared to a control digester that did not receive any dyebath effluent (Carliell *et al.*, 1996). The dyebath effluent, containing a mixture of reactive dyes and dyeing auxiliaries, was discharged (3kl/d on weekdays only) to a primary anaerobic digester (volume 1.34Ml, average flow 48kl/d) for a 151 day period. Comparison of system performance with the control digester was achieved via analysis of the following parameters: visual colour, pH, alkalinity, volatile acids, total/volatile solids and sodium/sulphide concentrations. No noticeable difference in the colour of samples from the 2 digesters was observed. Elevated levels of sodium and sulphide were detected in the experimental digester and thought to be due to the presence of sodium sulphate in the dyebath effluent. The concentrations were not increased to such a level as to cause instability. A laboratory digester was set up for comparison inoculated with digested sludge from the full-scale system. The amount of dye effluent added to the laboratory digester was equivalent to 10kl/d in a 1.34Ml volume digester and as such represented a more intense treatment system. The laboratory digester had increased levels of sodium and sulphide to the extent that digester instability occurred due to inhibition of the methanogenic bacteria. Increasing the loading of dye bath effluent did therefore result in deterioration of digester performance.

O'Neill *et al.* (2000) used the same dye, Reactive Red 141, in a two stage anaerobic-aerobic system. The dye was used in its hydrolysed form and colour removal was determined to be 63.9% in the anaerobic stage and 11.1% in the aerobic stage. An increase in toxicity following anaerobic treatment was observed as discussed in Section 1.4.1. Subsequent aerobic treatment eliminated the observed toxicity coinciding with a decrease in total organic nitrogen. This was thought to indicate aerobic degradation of nitrogen containing aromatic derivatives produced by the anaerobic decolourisation of the dye.

Beydilli *et al.* (1998) studied the anaerobic biodegradability and potential toxicity of 6 commercial reactive dyes using an anaerobic methanogenic culture from municipal sewage sludge. The 6 reactive dyes used were Black 5, Red 2, Red 120, Yellow 3, Yellow 15, and Yellow 17. All dyes were tested in their parent and hydrolysed forms. Initial dye concentrations were 300mg/l and colour removal efficiencies ranged from 65% (Red 2) to 97.3% (Yellow 17) after 83h incubation. In addition to determination of decolourisation rates for parent and hydrolysed dyes, total gas production of the dye-exposed cultures was monitored. Decolourisation was found to occur at a faster rate in the presence of an external carbon source and for Yellow 17 no significant difference in rate was observed for the parent and hydrolysed form of the dye. For Red 2 however the hydrolysed dye exhibited a faster decolourisation rate than the parent dye with a 20% lower gas production. This result was thought to indicate that the reduced environment created was sufficient for dye reduction to proceed irrespective of the culture activity. Red 2 (form of the dye used was not specified) was used for further investigations into toxicity and at concentrations up to 300mg/l the methane production in the Red 2 fed culture was 20% higher than a control culture. The increased gas production was attributed to dye degradation. At higher dye concentrations (500 to 2000mg/l) total gas and methane production dropped to approximately 60% of controls. The toxic effect at higher concentrations was thought to be due to accumulation of aromatic amines from the dye decolourisation.

Decolourisation of the hydrolysed form of selected reactive dyes has also been investigated by Ganesh *et al.* (1994). Batch sludge digestion studies were performed under anaerobic and aerobic conditions for Reactive Black 5 and Navy 106 wash water (a mixture of 3 dyes). For Reactive Black 5 under aerobic conditions it was found that the vinyl sulphone form of the dye was reduced from 32 to 14mg/l and 14 to 5mg/l in two digesters during the first day of operation. The residual sludge from both reactors was blue and sorption of the dye to the sludge was deemed to be the primary dye removal mechanism. For the hydrolysed Reactive Black 5 no colour removal was observed and no dye appeared to be sorbed to biomass. For the Navy 106 wash water no apparent colour removal was observed under aerobic conditions. At higher biomass concentrations the colour of the Navy 106 wash water was

observed to decrease steadily. This was thought to be due to microanaerobic zones within the aerobic system or to sorption of the dye to sludge due to increased solids. Under anaerobic conditions the colour of hydrolysed Reactive Black 5 decreased by approximately 60% during the first day of operation and the reactor filtrate changed visually from blue to greenish-yellow. No significant decrease in total organic carbon (TOC) and chemical oxygen demand (COD) indicated that under anaerobic conditions the dye was converted to aromatic amines with no net organic degradation. For the Navy 106 wash water a 50% reduction in colour was observed during day 1 under anaerobic conditions.

Razo-Flores *et al.* (1997a, 1997b) investigated the decolourisation of Mordant Orange I in a continuous upflow anaerobic sludge bioreactor. The products of decolourisation were identified as 5-aminosalicylate and 1,4-phenylenediamine. After a long adaptation period (189 days) the 5-aminosalicylate was detected at trace levels only suggesting further mineralisation of this compound. Following this adaptation period the reactor was supplied with the pharmaceutical dye Azodisalicylate (constructed from two 5-aminosalicylate molecules). The breakdown product 5-aminosalicylate was recovered at very low levels indicating extensive mineralisation. Transient accumulation of 5-aminosalicylate as the degradation intermediate and its subsequent anaerobic metabolism was shown to provide electrons for the initial reductive cleavage of the azo group. The authors suggest that design of dyes constructed from aromatic amines that can be anaerobically metabolised may avoid accumulation of potentially toxic intermediates in the environment. For reasons discussed previously (Section 1.2) this approach may not be a feasible option for textile dyes.

A more practical approach for treatment of coloured effluents may be the development of multistage treatment systems where a single process is not accountable for the complete mineralisation of a dye. The demonstration of azo dye decolourisation under anaerobic conditions and the knowledge that the resultant aromatic amines produced can be potentially degraded under aerobic conditions has led to the development of 2 stage anaerobic-aerobic systems for total dye degradation.

Zaoyan *et al.* (1992) proposed the use of fixed film suspended growth rotating biological contractors (RCB) for a 2 stage anaerobic-aerobic treatment of dye wastewater. The anaerobic step was used for decolourisation and the aerobic step for further decomposition of decolourisation products. The effluent treated in the system contained reactive, disperse, basic and naphthol dyes together with dyeing auxiliaries. The individual dyes present were not specified. The authors considered the efficiency of the system to be high and colour removal achieved was in the region of 72% to 78%.

A two-stage anaerobic-aerobic treatment system was evaluated by Seshadri *et al.* (1994) with regards to anaerobic fluidised bed hydraulic retention time and bed fluidisation rate and effects on dye/COD removals. Highest dye percentage removals for four acid dyes (Acid Orange 7,8 and 10 and Acid Red 14 at 5mg/l) were observed at 12 and 24 hours with removals being in the region of 81.5% to 98%. COD removals were also highest at these times. Initial results indicated that the aromatic amines from decolourisation were not further degraded. The authors state that further studies are underway to determine the fate of aromatic intermediates in the second stage.

Jian *et al.* (1994) have demonstrated decolourisation of a dye factory effluent in a 2-stage system employing an immobilised cell reactor followed by an activated sludge reactor. The immobilised cell reactor was inoculated with 8 bacterial strains known to decolourise triphenylmethane dyes and azo dyes, namely *Aeromonas* spp. (2 strains), *Alcaligenes* sp. and *Pseudomonas* spp. (5 strains). With the exception of Basic Violet 10 the dyes present in the effluent were not specified. The pilot scale plant was operated for a 7-month period during which decolourisation of the factory effluent was reported to be consistently good. No details were given however of the procedure for colour analysis, initial/final colour levels and measurement units employed.

A pilot scale plant for the treatment of textile wastewater employing strains chosen specifically for their decolourisation abilities has also been demonstrated by Yang *et al.* (1991). Two separate systems were established with facultative anaerobic and aerobic phases. The first system was inoculated with dye decolourising bacteria for the anaerobic phase and polyvinyl alcohol (PVA) degrading bacteria for the aerobic

phase. All bacterial strains employed were isolated from sludge/soil and included *Altermonas* spp., *Alcaligenes* sp., *Pseudomonas* spp. and *Paracoccus* sp. The second system was inoculated with acclimated sludge for both phases. The bacterial strains employed have been shown to decolourise 11 dyes including Acid Red B, Mordant Orange G and Cibacron Blue KGL. No details are given however of the dyes present in the textile wastewater treated. The pilot plants were run for 10 months and the colour removal in the plant containing the dye decolourising bacteria was approximately 20% higher than the acclimated sludge system. The average decolourisation efficiency was 80%. Influent/effluent colour levels were expressed as dilution ratios however the authors give no definition of this term.

The most promising way forward for development of biological systems to successfully treat dyehouse effluent is a combination of anaerobic and aerobic phases. Studies have shown that many dyes are reduced under anaerobic conditions and that resultant metabolites can be degraded in aerobic systems. In general the systems tested at pilot scale to date appear to be applicable to a range of dyes. Some success has been achieved with specific inoculation of conventional treatment systems with bacterial strains with known degradative abilities and in such systems decolourisation efficiency is higher compared to standard sewage sludge. A great deal of research has been undertaken to enrich for and isolate bacterial strains from several sources capable of degrading azo dyes. A detailed discussion of this area is given in Section 1.6. Many of the strains isolated are highly substrate specific and the subject, as a whole, needs further investment. There appears to be a somewhat random approach in terms of the dyes tested in the above decolourisation systems and no clear pattern exists with regards to the types of dyes studied. It is generally accepted that the problem of coloured effluent is associated with the reactive dyes due to their presence in textile wastewater in a highly soluble hydrolysed form and the relatively poor fixation of these dyes to substrates. With the exception of research by Carliell *et al.* (1994, 1995), Beydilli *et al.* (1998) and O'Neill *et al.* (2000) there appears to be a lack of data in the literature for decolourisation of this type of dye and treatment of the hydrolysed form of reactive dyes in particular.

1.6. Designed microbial approaches for azo dye degradation.

The variety of microbial strains reported to decolourise dyes and the great number of dyes available has resulted in a somewhat random approach in the search for decolourisation solutions with regards to the types of dyes used and initial approaches taken. A wide range of dyes has been studied including food colourants, textile dyes (from various classes), laboratory indicators and pure dyes synthesised specifically for decolourisation investigations. Previous research into the degradation of azo dyes can be divided into five main areas:

(a) Investigation of conventional treatment systems for the decolourisation of azo dyes.
(b) General screening of environmental isolates and culture collection strains for characterisation of decolourising ability.
(c) Specific isolation of bacterial strains for dye decolourisation.
(d) Development of specific bacterial systems for total degradation (decolourisation and mineralisation) of azo dyes.
(e) Adaptation of fungal strains to growth on dyestuffs.

Aspect (a) has already been discussed in Section 1.5. The remaining four areas will be discussed in the following sections.

1.6.1. General screening of environmental isolates and culture collection strains for characterisation of decolourising ability.

In a general screen Zhou and Zimmerman (1993) tested 159 actinomycete strains isolated from soil for the ability to decolourise 5 reactive dyes: Reactive Red 147, Reactive Red 171, Reactive Blue 114, Reactive Blue 209, and Reactive Blue 116. All dyes were in their hydrolysed form. Eighty-three of the 159 strains decolourised the dyes to varying extents. Of the 5 reactive dyes, only the 2 copper complex dyes were degraded (Reactive Red 171 and Reactive Blue 209) with the other dyes being removed by adsorption.

Knapp and Newby (1995) conducted a screen of bacterial cultures for decolourisation of a highly coloured stilbene effluent. Twenty different mixed cultures were isolated from various sources including human/animal faeces, mud from rivers/household drains and sludge from anaerobic digesters. All 20 cultures

were found to produce decolourisation during 15 days incubation with efficiencies of 77% to 85% observed. A further 35 days incubation resulted in a slight improvement with a maximum of 91% decolourisation produced. It was noted that decolourisation was more effective in strictly anaerobic conditions and with increased concentrations of peptone in the media. Higher concentrations of glucose however proved to be inhibitory. In a further study a mixed culture obtained from anaerobic digester sludge and thought to contain four distinct strains was found to decolourise the stilbene effluent at a faster rate than any of its isolated components suggesting a degree of synergism. Three of the 4 strains were facultative anaerobic spore forming Gram-positive rods thought to be *Bacillus* spp. The fourth strain was an obligate anaerobe thought to be a *Clostridium* sp. Attempts made to isolate further strains capable of mineralising the aromatic amines produced from the effluent reduction were unsuccessful.

Hu (1992) screened 47 strains of bacteria and yeasts isolated from soil samples and sludge from a textile wastewater screening plant for the ability to decolourise Red G. Fifteen of the 47 strains could reduce the dye within 8 days. One of the strains, identified as *Aeromonas* decolourised the Red G in 6 days. Small differences in BOD reduction led the author to the conclusion that the dye was most probably adsorbed rather than degraded. Adsorption capacity of the *Aeromonas* cells was tested for 11 reactive dyes (abbreviated names only are given for the dyes used: four blues including Reactive Blue 5, three reds including Red G and Reactive Red 22, two violets including Reactive Violet 815 and two yellows including Reactive Yellow 18972). Removal efficiencies ranged from 12.9% to 94.3% with 8 of the 11 dyes tested having removal less than 33%.

In a subsequent study 5 bacterial strains and activated sludge from a dyeing wastewater treatment plant were screened for adsorption capacity toward 6 reactive dyes including Reactive Blue 5, Reactive Red 22, Reactive Violet 2 and Reactive Yellow 2 (Hu, 1996). The Gram-negative strains (*Aeromonas* sp., *Pseudomonas luteola* and *Escherichia coli*) showed better adsorption than the Gram-positive strains (*Bacillus subtilis* and *Staphylococcus aureus*). This was thought to be due to higher lipid content in the cell walls of the Gram-negative strains.

Degradation of four reactive dyes by *P. luteola* has also been investigated by Hu (1994). Growth required 2 days shaking incubation and decolourisation only occurred during subsequent static incubation and was thought to indicate anaerobic transformation to the hydrazo form only for Red G occurring in microanaerobic zones within aggregates in the aerobic system.

1.6.2. Specific isolation of bacterial strains for dye decolourisation.

A mixed community capable of decolourising Reactive Red 120 (synthesised for the study) has been isolated from a mixture of garden soil, activated sludge and sludge from leather tanning wastewater by Sangaleti *et al.* (1995). Repeated cultivation of the consortium shortened the decolourisation time from 120h to 24h. Ten morphologically different species were present in the mixed community. Identification of the individual strains was not performed however 9 of the 10 were Gram-negative irregularly shaped bacillus. Decolourisation was only observed under static culture conditions and no single strain isolated could decolourise the dye in pure culture suggesting a mutualistic interaction between the strains in the mixed culture.

Nigam *et al.* (1996) isolated a microbial consortium from a mixture of garden soil and soil from a textile plant during a 12 month enrichment with a mixture of 9 commercial textile dyes. The consortium was found to be a mixture of two isolates identified as *Alcaligenes feacalis* and *Commamonas acidovorans*. The individual strains could not decolourise the dyes to the same extent as the original mixed culture and reconstitution of the two isolates also did not result in the same decolourisation ability as the original culture. The 9 dyes used were Cibacron Red C-2G, Cibacron Orange CG, Remazol Navy Blue GG, Remazol Red RB, Remazol Blue B, Remazol Black B, Remazol Golden Yellow RNL, Disperse Navy D2GR and Remazol Turquoise Blue G133. Eight of the 9 dyes were decolourised by the mixed culture by a minimum of 67% within 24h. No decolourisation was observed in the absence of an external carbon and energy source. The mixed culture did not decolourise Remazol Turquoise Blue G133 and this was thought to be due to this dye being a phthalocyanine dye containing copper and thus differing considerably from the other dyes in its structure.

1.6.3. Development of specific bacterial systems for total degradation (decolourisation and mineralisation) of azo dyes.

Coughlin *et al.* (1997) isolated culture TBX65, a mixture of strains including MC1 (identified as a *Sphingomonas*) and M12 (unidentified), from a wastewater treatment plant receiving azo dye discharges. Dye utilisation by MC1 and M12 was determined for 43 dyes (a total of 24 being purified). Decolourisation was observed for 4 dyes only: Acid Orange 7, Acid Orange 8, Acid Red 88 and Acid Red 151 (all purified). TBX65 was determined to be capable of growth on Acid Orange 7, Acid Orange 8 and Acid Red 88 as sole source of carbon, energy and nitrogen. The culture was also able to use Acid Red 151 as a source of nitrogen. Growth however was considered to be poor. Significant growth was observed only with the M12 strain growing on solid medium containing Acid Orange 7 or Acid Orange 8 at 25mg/l.

Strains capable of growth on azo dyes have also been isolated by Kulla (1981) and Kulla *et al.* (1983). Using long time adaptation a chemostat culture (the initial inoculum being soil) was adapted for growth on Carboxy Orange I and Carboxy Orange II (models for commercially used textile dyes) as sole carbon, nitrogen and energy source. The two strains isolated were identified as pseudomonads and designated *Pseudomonas* K22, Carboxy Orange I degrader and *Pseudomonas* KF46, Carboxy Orange II degrader. Growth was not observed *vice versa*. The two isolates were found to be highly substrate specific. Specificities of the two strains were tested with a range of azo compounds based on Orange I and Orange II. Strain K22 had an absolute requirement for a hydroxy group in the *para* position relative to the azo configuration group for decolourisation to occur. Strain KF44 exclusively reduced dyes with the hydroxy group in the *ortho* position relative to the azo configuration. The decolourisation ability of K22 was constitutive whereas KF46 had to be induced. The cultures were tested for the ability to degrade the corresponding sulphonated dyes. The two strains did not grow with or degrade the sulphonated dyes. The degradation pathways of the dyes were inhibited by the presence of sulphonate groups and a diffusion barrier for the highly polar sulphonated dyes was thought to exist. The enzymes initiating degradation, Orange I azoreductase and Orange II azoreductase however showed catalytic activity towards

both the carboxylated and sulphonated dyes. In a further study strain K22 was adapted to grow on Orange I (sulphonated) under non-sterile conditions in a chemostat fed on a mixture of Carboxy Orange I as limiting substrate and Orange I in excess. After 7 generations 35% of the Orange I was degraded. As growth did not occur with the sulphonated dye as sole substrate it was assumed that dye removal was due to cometabolism. Sulphanilic acid was not identified as an end product as expected and was thought to be channelled into a dead end pathway. The presence of sulphanilic acid and/or its metabolites interfered with the degradation of the aminonaphthol part of the dye molecule and complete mineralisation was not achieved. Strain KF46 capable of growing on Carboxy Orange II as the sole carbon source could degrade Orange II when other suitable carbon sources such as glycerol or acetate were present. As the sulphonated and carboxylated dyes should theoretically be cleaved at the same site several reasons were suggested to explain the problems encountered by the *Pseudomonas* strains when confronted with sulphonated dyes:

(a) Rate transport into the cell was low.
(b) Sulpho groups hindered attack for steric reasons or because of increased electronegativity.
(c) Sulpho groups destroyed the capacity of molecules to induce azoreductase.
(d) Sulphonated aromatics were attacked but channelled into dead end pathways.

This latter point was considered to be of prime importance and the need for further research into the degradation of sulphonated dyes was highlighted. The author regarded highly bred strains such as those described to be unlikely to survive in a wastewater treatment plant and that it is not feasible to breed or construct specific degraders for the many dyes currently in use.

Other *Pseudomonas* strains capable of degrading dyes have been described by Haug *et al.* (1991). Two strains, designated *Pseudomonas* BN6 and *Pseudomonas* BN9, were capable of total degradation of the dye Mordent Yellow 3 using an alternating anaerobic-aerobic treatment system. Strains BN6 and BN9 were isolated from a mixed bacterial culture capable of degrading 6-aminonaphthalene-2-sulphonic acid. The ability of the mixed culture to reduce sulphonated azo dyes was tested using Mordant Yellow 3 as the expected breakdown products of this dye were 6-

aminonaphthalene-2-sulphonic acid and 5-aminosalicylate. No decolourisation was observed when the dye was incubated aerobically with the mixed culture. When the culture was grown aerobically with 6-aminonaphthalene-2-sulphonic acid and then incubated anaerobically with the dye decolourisation was observed. Within 5 days 50% of the original dye present (1.5mM) was decolourised. The rate of decolourisation was greatly enhanced in the presence of glucose and total decolourisation was observed in 3 days. After complete reduction of dye aerobic degradation of the decolourisation products was initiated by the reintroduction of air. Degradation of 5-aminosalicylate was apparent immediately and the degradation of 6-aminonaphthalene-2-sulphonic acid was restored after a lag period. Oxygen starvation of >19h did not affect the ability of the culture to subsequently aerobically degrade the dye decolourisation products. The proposed degradation scheme is given in Figure 3.

Upon incubation of the individual strains, BN6 and BN9 with Mordant Yellow 3 it was found that only BN6 showed significant decolourisation of the dye. Pure cultures of strain BN6 showed higher decolourisation activity than the mixed culture. The same mixed community in the presence of glucose also metabolised Acid Yellow 21, Amaranth and 4 hydroxybenzene 4 sulphonic acid. The degradation of Amaranth by *Pseudomonas* BN6 was enhanced by aerobic conversion of 2-naphthalene sulphonate then subsequent anaerobic conversion of the dye (Keck *et al.*, 1997). This induction produced a 10 fold increase in reduction rate. This increase in rate however was not observed for assays with crude cell extracts. This aspect will be discussed in Section 1.7.

Azo dye decolourisation under anaerobic conditions has been demonstrated using a variety of bacteria giving rise to aromatic amines although work with textile dyes is limited. Under anaerobic conditions these amines are not degraded to any real extent. Under aerobic conditions however it has been shown that these compounds (including those that are sulphonated) can be degraded. A treatment system utilising an anaerobic phase for decolourisation and an aerobic phase for mineralisation may be a viable treatment option. Table 7 summarises research into degradation of aromatic compounds and isolated strains capable of performing these transformations.

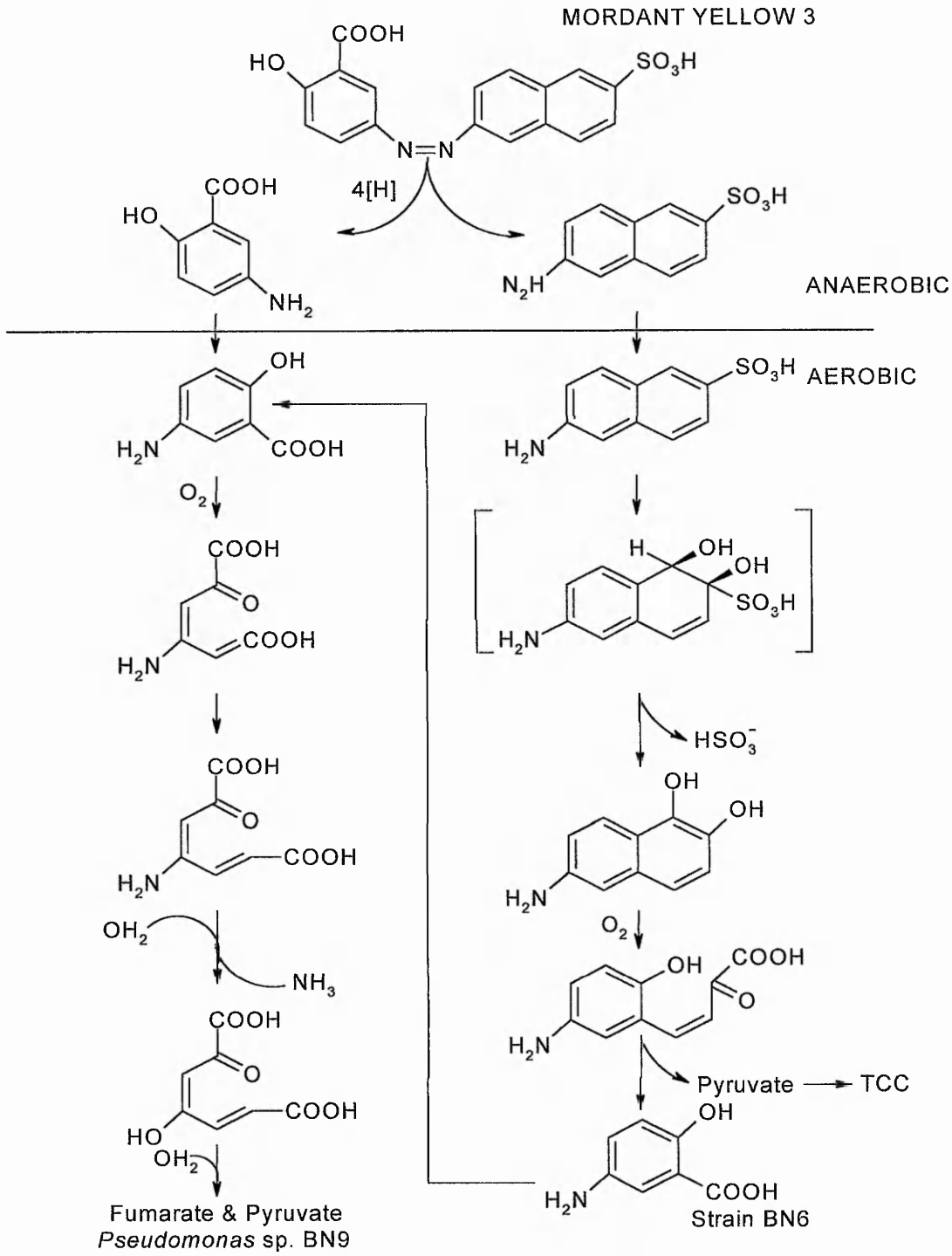


Figure 3. **Proposed pathway for degradation of the azo dye Mordant Yellow 3 by a mixed bacterial community. Reproduced from Haug *et al.* (1991).**

Table 7. Strains capable of mineralising aromatic compounds.

Strain	Substrate	Source	Reference
Mixed culture	Phenol	Soil/manure sludge	Bakker (1977)
Pseudomonas sp.	Naphthalene, 1- and 2-naphthalene sulphonic acid	Activated sludge	Brilon *et al.* (1981a,b)
Mixed culture	Aniline, o-toluidine p-phenetidine, o-dianisidine, 3,3' dichlorobenzidine	Sewage treatment works	Brown and Laboureur (1983)
Arthrobacter sp., *Pseudomonas* sp., Unidentified	18/19 as sulphur source, 17/19 as sulphur source, 18/19 as sulphur source	Municipal sewage	Zurrer *et al.* (1987)
Alcaligenes sp. Unidentified Strain M1 *Pseudomonas* spp. Unidentified S-1	Various benzene sulphonic acids	NS	Thurnheer *et al.* (1988)
Rhodococcus sp.	Naphthalene	PAH contaminated soil	Grund *et al.* (1992)
Pseudomonas sp.	2-naphthalene-1-sulphonate, 1-naphthalene sulphonate, 2-naphthol-1-sulphonate	Soil	Ohe *et al.* (1990)
Pseudomonas sp.	Naphthalene, Phenanthrene, Anthracene	PAH degrading sludge	Sanseverino *et al.* (1993)
Alcaligenes sp. *Pseudomonas* sp.	2,6-dimethylnaphthalene	Soil	Miyachi *et al.* (1993)
P. testeroni	1- and 2- naphthalene sulphonic acids	Activated sludge	Krull and Hempel (1994)
Mixed culture	Four naphthalene disulphonic acids	Soil	As above
Pseudomonas sp BN6	Various naphthalene sulphonic acids carrying substituents in the 4,5,6,7 or 8-position of naphthalene ring system	River water	Nortemann *et al.*, (1994)
P. alcaligenes *P. mendocina* *P. putida* *P. stutzeri*	Phenol	Cattle dung enrichments and soil	Sarnick and Kanekar (1995)

KEY
NS Not specified.

1.6.4. Adaptation of fungal strains for growth on dyestuffs.

Fungi have been investigated as potential degraders of dyes due to the suitability of dyestuffs to serve as substrates for the fungal lignin degradation system. Few microorganisms are capable of degrading complex lignin polymers and are exemplified by the white rot fungi. Most research to date has been conducted on *Phanerochaete chrysosporium*. An account of the range of dyes decolourised by *P. chrysosporium* and other decolourising fungi shown to be capable of dye decolourisation is given by Banat *et al.* (1996). The extracellular enzymes involved in lignin degradation by *P. chrysosporium* are composed of lignin peroxidases and manganese dependent peroxidases and H_2O_2 producing peroxidases. The random nature of lignin requires its degradation to proceed in a non-specific manner hence other compounds having an aromatic structure are susceptible to degradation by ligninolytic enzymes. Under culture conditions that favour ligninolysis some hydrocarbons undergo oxidation and are mineralised to varying extents. A summary of fungal cultures used and corresponding dyes is given in Table 8.

Glenn and Gold (1983) used dyes as indicators for the onset of secondary metabolism in *P. chrysosporium*. The same strain was used by Cripps *et al.* (1990) specifically for the biodegradation of azo and heterocyclic dyes. The dyes Orange II, Tropaeolin O, Congo Red and Azure B were added to nitrogen-limited and nitrogen-sufficient 6 day old cultures of the fungus. The rate of decolourisation was less for the nitrogen-sufficient cultures and complete decolourisation was not observed after 12 days incubation. With the nitrogen-limited cultures disappearance of dye was observed after 48 hours. Some of the dye was found bound to the mycelium, this being more pronounced in the nitrogen-sufficient cultures. Only 1 in 4 replicate cultures secreted the lignin peroxidase implying that more than one enzyme system may be responsible for the decolourisation. *P. chrysosporium* was also investigated by Pasczcynski *et al.* (1992) for its ability to degrade sulphonated dyes. Five dyes were used and *P. chrysosporium* mineralised all of them with no apparent effect on susceptibility to degradation from the sulphonate groups or substitution patterns. *Streptomyces* was also tested and found to be unable to mineralise aromatics with sulpho groups and both azo and sulpho groups but did mediate mineralisation of modified dyes containing lignin like substitution patterns. In an investigation of the

influence of substituents on degradability by fungi Pasti-Grigsby (1992, 1996) used 2 dyes with various substitution patterns and eight fungal strains. For monosulphonated azo dye derivatives of azo benzene, decolourisation was observed for those having a hydroxyl group in the para position relative to the azo linkage and at least one methoxy/alkyl group. Different patterns were observed for dyes with different substitution patterns.

Table 8. Fungal cultures used for decolourisation of dyes.

Fungi	Dye	Reference
P. chrysosporium	Poly B-411, Poly R-481, Poly Y-606	Glenn and Gold (1983)
P. chrysosporium	Orange II, Tropaeolin O, Congo Red, Azure B	Cripps *et al.* (1990)
P. chrysosporium	4 sulphonated azo dyes	Chivukula *et al.* (1995)
P. chrysosporium	10 dyes from groups: azo, triphenylmethane, heterocyclic, polymeric	Ollika *et al.* (1993)
P. chrysosporium	Cibacron Red C-29, Cibacron Orange C9, Remazol Navy Blue 99, Remazol Red RB, Remazol Blue B, Remazol Black B, Remazol Golden Yellow RNL, Disperse Navy D2GR, Remazol Turquoise Blue G133	Kirby *et al.* (1995)
P. chrysosporium	Acid Yellow 9, Orange I, Orange II, 2-benzene sulphonic acids	Paszczynski *et al.* (1992)
S. chromofuscus *Streptomyces spp* *S. rochei* *S. diastaticus*	22 dyes with various substitutions patterns of hydroxyl and methyl groups	Pasti-Grigsby *et al.* (1992,1996)
Myrothecium verrucaria	Orange II, RS(H/C)-benzidine based, 10B(H/C) sulphonated azo	Mou (1991)
20 unspecified strains	Poly R-478	De Jong *et al.*(1992)

1.7. The mechanism of bacterial decolourisation and factors affecting dye reduction.

Azo reduction is considered to be an anaerobic reaction. In the absence of oxygen the azo compound will be the terminal electron acceptor and the reduction rate will be governed by the rate of formation of the electron donor. Bragger *et al.* (1997) has suggested that in aerobic conditions oxygen would be reduced in preference to azo compounds due to its more positive redox potential. Exposure of *Bacteroides fragilis* to oxygen did not irreversibly inhibit its azoreductase activity as decolourisation ability of the culture was restored with a return to anaerobic conditions.

Gingell and Walker (1971) studied the mechanism of azo reduction by the gut bacterium *Enterococcus faecalis* (formerly *Streptococcus faecalis*). It was proposed that azo reduction proceeds via an indirect mechanism where no enzyme-substrate complex between azoreductase and dye exists. Flavin mononucleotide (FMN) was considered to be the true substrate of bacterial azoreductase and that it is the enzymatically reduced flavins that non-enzymatically reduced the dye. Although the reduced flavins can act as one or two electron donors a double electron transfer via the hydrazo intermediate was considered more likely. The reaction can be considered thus:

$FMNH_2$ + R-N=N-R' $\rightarrow$ FMN + R-NH-NH-R'

$FMNH_2$ + R-NH-NH-R' $\rightarrow$ FMN + R-NH_2 + R'-NH_2

The reduced flavin acts as an electron carrier from the NADH linked azoreductase to the azo dye electron acceptor. The protein responsible for flavin reduction (hence azo reduction) in *E. faecalis* was thought to be a flavo protein.

Keck *et al.* (1997) proposed the production of an extracellular factor formed during the degradation of 2-naphthalene sulphonate by *Pseudomonas* strain BN6 that acted as a redox mediator in the subsequent decolourisation of Amaranth. Aerobic conversion of 2-naphthalene sulphonate by strain BN6 was found to stimulate the subsequent anaerobic conversion of Amaranth by a factor of 10. An increase in reduction rate of Amaranth was also observed with a mutant of BN6 (unable to metabolise 2-naphthalene sulphonate) when re-suspended in the culture supernatant

of BN6 grown on the naphthalene sulphonate. The redox mediator was thought to shuttle reduction equivalents from the BN6 cells to the extracellular azo dye and reduce the dye non-enzymatically. The redox mediator did not stimulate reduction of the dye by crude cell free extracts. The cell debris was removed during preparation of the cell free extracts and it was thought that the mediator was associated with the cell wall material.

The site of azo reduction was also determined to be in the cell wall fraction by Kudlich *et al.* (1997) in an investigation of the effect of artificial redox mediators on *Pseudomonas* BN6 and activated sludge. The effects of different redox mediators and the location of the enzyme system responsible for reduction of dyes by whole cells were determined. The reduction rate of Amaranth in the presence of the redox mediator 2-anthraquinone sulphate (AQS) was found to increase with increasing concentration of AQS up to 0.6mM. It was thought likely that a highly polar compound such as AQS would not penetrate the cell membrane and that the azoreductase activity observed with whole cells was present in the cell membrane. Externally added FAD did not affect the reaction with whole cells but did significantly increase the activity of crude cell extracts (Haug *et al.*,1991).

The most generally accepted hypothesis is that bacterial cells possess an unspecific cytoplasmic azoreductase that transfers electrons under anaerobic conditions via soluble flavins to azo dyes. Kudlich *et al.* (1997) proposed two different azoreductase activities for strain BN6. Azoreductase activity was located in cell membranes and the soluble fraction of cell extracts. Different responses of the two preparations to potential inhibitors led to the conclusion that the membrane bound azoreductase and the cytoplasmic azoreductase are different enzyme systems.

Semde *et al.* (1998) noted faster reduction rates for azo dye solutions by *Clostridium perfringens* in the presence of redox mediators such as riboflavin or benzyviologen. Degradation was linear (zero order) and substrate redox potential did not significantly affect reduction rate. In a mixture of several dyes however the dyes were reduced preferentially in the order of smallest negative redox potential. No change in degradation rate was observed for Amaranth when the initial concentration of the dye was doubled. Correlation between redox potential and decolourisation rate was also observed by Bragger *et al.* (1997). In agreement with Semde *et al.*

(1998) the rate of reduction for 4 dyes was found to decrease with decrease in the redox potential of the dye under test.

Characterisation of the site of azo reduction has been investigated via specific detection of azoreductases from several strains. Employment of gel electrophoresis and specific activity staining of the gel has resulted in identification of several proteins capable of producing azo fission. A summary of azoreductase isolation is given in Table 9.

Rafii and Cerniglia (1990) described a non-denaturing gel activity assay for the detection of azoreductases from anaerobic bacteria. The experimentally determined azoreductase molecular weight was cited for *C. perfringens* only and found to be a dimer with a molecular weight of 181,000 (116,000 and 65,000). Using the same gel system and staining with Nitro Blue tetrazolium, dehydrogenase activity was also detected. For 3 of the 4 strains tested 1 formazan band indicating the presence of 1 dehydrogenase enzyme was observed. For *C. clostridiiforme* 2 formazan bands were detected (1 sharp slow moving band and 1 diffuse fast moving band). The azoreductase was found to co-migrate with the dehydrogenase suggesting that a single protein may be involved in the dye and tetrazolium reduction. For *C. clostridiiforme* the azoreductase co-migrated with the fast moving dehydrogenase band only indicating that some but not all anaerobic dehydrogenases have azoreductase activity.

Other strains analysed using the system were *Eubacterium hadrum* (2 strains), *Eubacterium* spp. (2 species), *Butyrivibrio* sp., *Bacteroides* sp. and *C. nexile* (Rafii *et al.*, 1990). All azoreductases isolated were produced constitutively and released extracellularly. Of the strains tested 3 different types of azoreductase were observed judging by molecular weight and/or charge. The activity of all isolates was irreversibly inactivated by exposure to oxygen.

Homology of azoreductases between strains was investigated by Rafii *et al.* (1992). Structural similarities of azoreductases from 4 *Clostridia* and 1 *Eubacterium* were evaluated. An antibody against *C. perfringens* azoreductase inhibited azoreductase activity in all 5 strains to varying extents. In a subsequent study Rafii and Cerniglia (1993a) determined that azo and nitro reduction were due to the same enzyme. Nitroreductase was assayed by detecting 4-aminobenzoic acid produced from the

reduction of 4-nitrobenzoic acid. Conclusions were based on response of the 2 enzymes to inhibitors (menadione and *o*-iodosobenzoic acid), electrophoretic mobilities, reaction of an antibody against *C. perfringens* azoreductase inhibiting nitroreductase indicating homology or substantial cross-reactivity. The presence of nitro compounds inhibited azo reduction with substrates for both activities competing for the same enzyme suggesting a steric similarity of the active site of the enzyme for both compounds.

Table 9. Characterisation of bacterial azoreductases from several strains.

Organism	Molecular weight	Reference
Clostridium perfringens	181,000	Rafii and Cerniglia (1990)
Klebsiella sp.	27,000	Dykes *et al.* (1994)
Pseudomonas sp.	29,000	Dykes *et al.* (1994)
Pseudomonas K24	21,000	Zimmerman *et al.* (1984)
Pseudomonas KF46	30,000	Zimmerman *et al.* (1984)
Shigella dysenteriae	55,000	Ghosh *et al.* (1992)
	11,000	
E. coli	53,000	Ghosh *et al.* (1993)
	12,000	

An account of the occurrence of 2 different azoreductases from a single biological strain has also been given by Kakuta *et al.* (1998). In an investigation into the purification and properties of azoreductase from *Candida curvata*, 2 azoreductases were identified having molecular weights of 47,000 and 56,000. The enzymes had different properties with respect to electron donor with azoreductase 1 (47,000) showing higher activity with NADPH than NADH and azoreductase 2 (56,000) showing higher activity with NADH rather than NADPH. The addition of FAD or FMN did not produce decolourisation.

In a study of factors affecting decolourisation rate using *Bacillus cereus* and *Sphaerotilus natans* and a series of structurally related dyes, cell permeability was found to be a pertinent factor as all dyes not measurably reduced by whole cells were decolourised by cell extracts (Wuhrmann *et al.*,1980). Comparable rates were obtained for Orange II and Lanasylviolet reduced by cell free extracts of *B. cereus* and *Sp. natans* with the absolute rates being faster than with whole cells of the 2

organisms. The adsorption of dyes by the cell walls of test cultures was found to vary between dyes with Gram-negative cells retaining smaller amounts of dyes than Gram-positive cells. No difference in reduction rate of Orange II was observed however between control cells and cells stained with a strongly adsorbing dye. The adsorption of one dye was therefore thought not to inhibit the reduction of another remaining in the solution. This was considered to be important in the event that transport of a dye from the external medium through the cell wall to the plasma membrane is governed by adsorption-desorption equilibria. In contrast Hu (1996) found that Gram-negative organisms displayed a greater adsorption capacity for reactive dyes than Gram-positive cells. The adsorption of dyes to cell wall surfaces may be organism and/or dye specific and as such no generalisations for this phenomenon can be made.

Wuhrmann *et al.* (1980) compared degradation rates for a series of sulphonated dyes with a series of similar compounds in which the sulphonic acid group was replaced by a carboxylate group. Decreased decolourisation rates were observed for the sulphonated dyes compared with the carboxylated analogues. Permeabilisation of *B. cereus* cells by toluene treatment was found to increase the passage of both carboxylated and sulphonated dyes into cells observed by increases in reduction rates (Mechsner and Wuhrmann, 1982). The reduction rate of sulphonated compounds was found to be consistently higher than that of the carboxylated analogues once the dyes had entered the cells suggesting it is transport into the cell alone that is inhibited by the presence of sulphonate groups. This effect may be explained by the observation of Walker and Ryan (1971) that reduction rate is determined by electron density in the region of the azo group. In a study of the relative rates of reduction of a series of dyes based on Red 10B by cell free extracts of *Enterococcus faecalis* they proposed that reduction rate depends on the molecular parameters influencing the ease with which electrons are accepted by the azo group. If azo reduction proceeds via a terminal non-enzymatic mechanism then reduction rates would be greatly influenced by changes in the electron density in the region of the azo group. Substitution of constituent aromatic rings within a dye with electron withdrawing groups such as sulphonate groups may lower the electron density of the azo region

and render the dye more susceptible to reduction and hence increase the reduction rate.

Higher rates of reduction were observed for dyes containing electron-withdrawing groups on the aromatic ring by Shargel *et al.* (1984). The relationship between azo dye structure and rat hepatic azoreductase activity was investigated for 7 dyes. Amaranth and azosulfamide had the highest rates of decolourisation for the dyes tested attributed to the presence of 3 sulphonate groups per molecule (more than any of the other dyes tested). A direct correlation between partition coefficient (1-octanol-0.05M phosphate buffer) and azoreductase activity was not observed. Comparison of activity with reciprocal partition coefficient produced a regression line with correlation coefficient of 0.74. It was proposed that there could be an increase in azoreductase activity with increase in aqueous solubility although the correlation was not very strong.

1.8. Aims and Objectives.

The prime objective of this study is to achieve decolourisation of a reactive textile dye to yield environmentally acceptable end products.

As a very large number of dyes are in use it is deemed necessary to nominate a model dye. The principal dye of concern in this study will be Reactive Black 5. This dye is chosen because of its class (a reactive dye being of the class most problematic to treat), the commercial availability of the intermediate 8-amino-naphthol 3,6 disulphonic acid (H-acid) and the popularity of the dye due to the sustained fashion for black clothing. Reactive Black 5 is mentioned by Pierce (1994) as being used almost exclusively for dyeing black shades. The dye will be used in both its parent and hydrolysed form as it is thought that it would be the hydrolysed form present in an effluent containing this dye. Other dyes will be included in some studies to ensure techniques employed are transferable.

Any decolourisation system must be applicable to a range of dyes, as must the analytical techniques used for monitoring decolourisation due to the large numbers of dyes from different dye classes in use. A decolourisation assay will be developed using Reactive Black and *Enterococcus faecalis* (a strain capable of producing azo fission used for the decolourisation of food dyes by Sweeney (1995), a previous

researcher in the Department of Life Sciences). Once a working decolourisation system is obtained comparisons will be made between the decolourisation abilities of *E. faecalis* and the specifically isolated decolourising strains (range of dyes decolourised and decolourisation rate for a selected few). The molecular weight of the azoreductase will be determined for both *E. faecalis* and a selected isolate.

Analytical techniques will be employed in order to achieve two main aims: routine analysis of decolourisation products and use as preparation scale techniques for preparing purified samples of decolourised products for structural determination. Products from chemical and biological decolourisation will be compared to determine if decolourisation proceeds via a common mechanism. Decolourisation products will also be compared between bacterial strains (*E. faecalis* and selected bacterial isolates) to determine if different microorganisms produce different end products.

Assessment of the toxic effects of textile dyes and effluents has concentrated mainly on fish toxicity studies. The Microtox® test (short-term bacterial test) has been used for the assessment of a variety of chemicals although only two accounts are given on its use in the assessment of textile effluents (Rutherford *et al.*, 1993 and Svenson *et al.*, 1996). In view of this, the toxicity of decolourisation products in this study will be assessed using the Microtox® acute toxicity test.

2. MATERIALS AND METHODS.

2.1. Chemicals and sources.

All chemicals were used and stored in compliance with C.O.S.H.H. regulations. Solvents and buffer components used for HPLC and TLC were of gradient analysis grade. All chemicals used for gel work were electrophoresis grade. With the exception of dyes all other chemicals used were of standard laboratory reagent grade. Dyestuffs were donated by two textile companies (Welbeck fabric dyers and Courtaulds Jersey Underwear), and Table 10 shows a list of dyes, related chemicals, grade and original source. Sunset Yellow (a food colourant) has been included in some studies as an example of an azo dye, of known structure, having a single azo bond. Solutions were prepared using distilled water and standard laboratory glassware. For HPLC and gel electrophoresis all solutions were prepared with distilled deionised water (Millipore 18MΩ water). All dye solutions and solutions of 8-amino-1-naphthol-3,6-disulphonic acid (H-acid) were prepared fresh in sterile distilled water and filter sterilised where possible using a Dynaguard 0.2μm hollow fibre syringe tip filter (New Brunswick). Where dye solutions could not be filtered they were steamed for 10 minutes in a boiling water bath immediately prior to use.

2.2. Preparation of hydrolysed dyes.

The method used was according to Weber and Stickney (1993). The hydrolysed form of the dye was prepared by adjusting the pH of a 1% (w/v) dye solution to pH 11 using sodium hydroxide (NaOH) and refluxing for 3 hours. After this time the dye solution was allowed to cool to room temperature and the pH was adjusted to pH 7 using hydrochloric acid (HCl).

2.3. Instrumentation and analytical techniques.

2.3.1. UV/VIS Spectrophotometry.

The instrument used was the Pye Unicam SP1800 UV/VIS dual beam scanning spectrophotometer. Data output devices were a chart recorder and a PC with the Labtech Aquire data acquisition package. Raw data from the Labtech Aquire programme was imported into Microsoft Excel for analysis.

Samples were measured at the wavelength required or scanned against a reference blank of sample matrix with all biological samples being centrifuged (1ml Eppendorf tubes, microfuge 11,600 x g for 5min) and supernatants decanted prior to scanning. Samples and reference blanks were diluted as necessary with sample matrix to ensure all readings were within the linear scale of the spectrophotometer.

Spectrophotometer parameters are given in Table 11. Labtech Aquire parameters are given in Table 12.

Table 10. Dyes, related chemicals, grades and sources.

Chemical	Grade	Source
Reactive Black 5	55%	Aldrich
Procion Crimson H-EXL	>75%	Zeneca Colours
Procion Navy H-EXL	33% liquid	Zeneca Colours
Procion Yellow H-EXL		ICI Organics Division
Serilene Rubine 2B-LS	150 powder	Yorkshire Chemicals
Serilene Yellow 4GN-LS		Yorkshire Chemicals
Serilene Yellow Brown 2RL	150 powder	Yorkshire Chemicals
Dispersol Black C-VS	300 grains	Zeneca
Dispersol Blue C-RN	200 grains	ICI
Dispersol Navy C-2G	150 grains	Zeneca
Foron Black RD-2GS	200 grains	Sandoz
Indosol Blue SF-2G	400%	Sandoz
Indosol Rubinole SF-RG		Sandoz
Indosol Yellow SF-2RL		Sandoz
Solophenyl Black FG	400%	Ciba Geigy
Solophenyl Navy BL	167%	Ciba Geigy
Erionyl Navy Blue R	180%	Ciba Geigy
Erionyl Yellow RXL		Ciba Geigy
Naphthol Blue Black	80%	Aldrich
Sunset Yellow	Microscopy	Gurr
H- Acid		Kodak

Table 11. Spectrophotometer parameters for the Pye Unicam SP1800.

Parameter	Setting
Scan speed[#]	4nm/s
Band width	1.6 mm
Slit width	0.5 mm
Total scan range [#]	200 – 700 nm
Path length	1cm

[#]Changed as necessary

Table 12. Parameters for the Labtech Aquire data acquisition package.

Parameter	Setting
N°. analogue channels	1
N°. digital channels	0
Time stamp data	Yes
Sampling rate (Hz)[#]	1
Run duration [#]	200.000
Starting method	Immediate
Analogue trigger channel	1
Analogue trigger threshold	0.000
Trigger polarity	Low
File name	
N° windows	1
Width of window in sec [#]	200.000
Channel N°	1
Channel name	Scan
Display in window N°	1
Scale factor	1.000
Offset constant	0
Min displayed value	0.000
Max displayed value	2.5000

[#]Parameters changed as necessary with change in spectrometer scan speed

2.3.2. High Performance Liquid Chromatography (HPLC).

The instrument used was a Perkin-Elmer Series 410 LC pump with GP100 UV diode array detector. The column used was a Hibar Lichrocart manu-fix 250-4 C_{18} reverse phase (Merck). The detection wavelength was 260nm and the sample volume applied was 20µl. Solvents were prepared using Millipore 18MΩ water. The method used was taken from Grossenbacher *et al.* (1986).

Solvent 1 100mM potassium phosphate buffer (pH 6.7)

Solvent 2 70% gradient analysis grade methanol in 10mM potassium phosphate buffer (pH 6.7)

Solvents were filtered through 0.2µm cellulose acetate filter (Sartorius) and degassed on line with helium prior to use. The column was equilibrated with solvent 1 for 10min prior to each injection. Biological samples were centrifuged (1ml Eppendorf tube, microfuge 11,600 x g for 5min) and the supernatants decanted. Sample supernatants and all non-biological samples were injected through a 0.45µm nylon

66 syringe tip filter (Gelman Sciences). The linear gradient was started 10min post injection (0-100% solvent 2, 30min duration). At the end of the gradient solvent 2 was pumped for 10min then the gradient was reversed (100-0% solvent 2, 10min duration). At the beginning of each analysis session the column was equilibrated with solvent 1 for 20min and a freshly prepared 0.1% (w/v) solution of Reactive Black was injected to ensure day to day reproducibility. At the end of each analysis session the column and pump were flushed with Millipore water for 20min and then a mixture of Millipore water (30%) and methanol (70%) for a further 20min prior to shutdown.

2.3.3. Thin Layer Chromatography (TLC).

The solvent system used for TLC was taken from Schweppe (1977). The solvent contained n-propanol, ethyl acetate and water in the proportions 6:1:3. Solvents used were freshly prepared for each analysis session. Development tanks were lined with Whatman No 1 chromatography paper soaked in solvent with a final solvent depth of 0.5cm. Tanks were equilibrated for 1h prior to plate insertion. The plates used were Merck silica gel 60 (BDH) and development times were varied according to plate size, 10 x 10cm plates being developed for 2 hours and 20 x 20cm plates developed for 4 hours. The plates were spotted with sample (1µl) using an SGE HPLC syringe 1cm from the bottom edge of the plates. The spots were allowed to air dry and overlaid with repeated spotting if necessary. Post development, plates were air dried in a fume cupboard then viewed in daylight and UV light (366nm model UVL/56 blak-ray lamp). Results were expressed as hR_f values where:

$$R_f = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by solvent}} \quad \text{and} \quad hR_f = R_f \times 100 \text{ (first 2 digits only used)}$$

For preparation scale chromatography samples were treated the same way as above but with 1 plate (20 x 20cm) dedicated per sample and spotted with repeated overlays across its width. Post development, plates were viewed as above and sample bands marked. Bands required were scraped from the surface into 1ml Eppendorf tubes and the component of interest extracted from the silica with 0.5ml aliquots of methanol (3 extractions per sample). The silica was concentrated by

centrifugation (microfuge 11,600 x g for 5min) and supernatants reserved for further analysis.

2.3.4. Fourier Transform Infra-Red Spectroscopy (FTIR).

The instrument used was the Perkin Elmer 1800 series Fourier Transform Infra-Red spectrometer (FTIR) with a Hewlett Packard plotter. Methanol extracted samples from TLC were evaporated to dryness and ground with approximately 200mg of dry potassium bromide (KBr) using a dry agate mortar and pestle. The KBr-compound mixtures were then pressed under vacuum and a pressure of 1.08×10^8 Nm^{-2} for 5min. The KBr sample discs were scanned against a KBr disc blank.

2.3.5. Fluorescence.

The instrument used was the Perkin Elmer LS30 scanning luminescence spectrometer. The sample compartment was flushed with methanol prior to use and the instrument zeroed. The sample compartment was then flushed with the sample with the excitation wavelength set at 366nm (wavelength of lamp used to view TLC plates) and the emission spectra scanned. Samples were diluted with methanol as necessary to bring measurements on scale.

2.4. Microbial cultures, media and storage.

2.4.1. Cultures.

Enterococcus faecalis, a laboratory strain from the culture collection of the Department of Life Sciences, The Nottingham Trent University was used for the majority of studies. Additional dye decolourising organisms were isolated from textile effluent samples as described in Section 2.5. Fungal cultures were also obtained from the Department of Life Sciences culture collection.

2.4.2. Routine culturing.

Growth media used were tryptone soya agar (TSA) and tryptone soya broth (TSB) for *E. faecalis* and for initial work with all aerobic isolates. Nutrient agar (NA) and nutrient broth (NB) were used for subsequent work on aerobic isolates. Schaedler agar (SA) and Schaedler broth (SB) were used for culturing of anaerobic isolates. Fungal cultures were grown on malt extract agar (MEA).

All media was obtained from Unipath Ltd. (Basingstoke) in dry powdered form and prepared according to the manufacturer's instructions.

2.4.3. Culture storage.

Cultures were stored mainly on agar slopes (at 4°C) of the corresponding media used for routine culturing. Other methods employed were storage in growth media-glycerol (7:3 mixture) and Lab M protect beads. The glycerol cultures and protect beads were inoculated from plates and stored at -20°C. All cultures were checked for viability (growth and decolourisation) regularly and stored by each system in 5 replicates. Fungal cultures were stored on MEA plates at 4°C. Slopes, glycerol cultures and protect beads were sub-cultured a maximum of 3 times with the initial sub-culture from each replicate being used to inoculate fresh culture stocks.

2.4.4. Dye plate preparation.

Dye plates were prepared by pipetting a specified volume of a freshly prepared pre-steamed 1% (w/v) dye solution into sterile plastic disposable petri dishes and agar medium poured as normal. The plates were swirled to mix the agar and dye, then left to set. The volume of dye added to a plate was varied such that a relatively strong colour was achieved when mixed with the agar medium, for example this was typically 200µl of a 1% (w/v) solution of dye per plate for Reactive Black. All plates were pre-incubated overnight at 30°C to dry the agar and to ensure sterility. Any plates showing contaminant growth were discarded.

2.5. Isolation of decolourising bacteria.

Textile effluent samples were obtained from the effluent holding tank at Welbeck fabric dyers, Somercotes, Derbyshire. Serial dilutions of the effluent samples were prepared in 0.9% saline and spread onto dye plates produced as in Section 2.4.4. Nine spread plates for each dilution were prepared of which 3 were incubated aerobically, 3 anaerobically and 3 microaerophilically (candle jar) at 30°C. Plates intended for aerobic and microaerophilic incubation were prepared from TSA initially, whereas later isolations used NA plates. Plates for anaerobic incubation were also prepared from NA as SA contained cysteine capable of decolourising the dye plates during the pre-incubation stage as detailed in Section 2.4.4.

Decolourising colonies (those surrounded by a zone of clearing in the dye) were picked off using a sterile loop and streaked to purity on identical dye plates using the corresponding incubation technique. Cultures were deemed to be pure when the following conditions had been satisfied:

(a) Single colonies on a plate had the same colour and morphology.

(b) Five single colonies chosen at random displayed identical Gram reactions.

(c) No contaminants were found on the prepared Gram stain slides when viewed at X100 magnification (oil immersion) under a light microscope.

Following purification isolates were again streaked onto dye plates to ensure no loss of decolourising ability. The cultures, once pure, were transferred to appropriate agar slopes (no dye) for storage at 4°C. Glycerol and bead cultures were also prepared for long term storage as detailed in Section 2.4.3.

2.6. Bacteria identification.

Identification of isolates was performed using standard microbiological techniques. The Gram stain, catalase test, oxidase test, spore staining and motility were performed in accordance with Sirokin and Cullimore (1969) and the API biochemical test systems from BioMerieux (Basingstoke, U.K. API 20e/API 20ne for aerobic isolates and the API 20a/Rapid ID 32A for anaerobic isolates), utilised according the manufacturer's instructions.

2.7. Decolourisation screening.

2.7.1. Bacterial screening.

Dye plates (NA for all textile effluent isolates and TSA for *E. faecalis*) were prepared according to Section 2.4.4. The plates were divided into four sectors and chosen isolates were streaked onto each sector. This was repeated until all isolates had been streaked onto all chosen dye plates. The plates were incubated for 48h aerobically for *E. faecalis* and the aerobic isolates and anaerobically for the anaerobic isolates. *E. faecalis* inoculated plates were incubated at 37°C, all other plates were incubated at 30°C.

2.7.2. Fungal screening.

Dye plates (NA) were prepared according to section 2.4.4. A sterile cork borer was used to remove a plug of culture from the colony edge of an established fungi plate. The plug was placed growth side downwards in the centre of a dye plate. The plates were incubated at 25°C and observations were made after 7 days.

2.8. Decolourisation enzyme assay with whole cells.

2.8.1. *E. faecalis.*

For *E. faecalis* the following method was used:

- A 10 ml volume of TSB was inoculated with a loop from a TSA slope of the culture. The broth was incubated statically for 6 hours at 37°C.
- A large volume of TSB was inoculated from the pre-culture (1% v/v inoculum) and incubated for 18h at 37°C. The volume inoculated was dependent on the final assay volume required but was typically 400-800ml.
- The overnight culture was centrifuged at 14,200 x g for 15min at 12°C.
- The supernatant was discarded and the cell pellet re-suspended in potassium phosphate buffer (100mM, pH7) using a sterile glass pipette gently to avoid excessive aeration to give a pre-determined increase in cell concentration. Typically 400ml of overnight culture would be re-suspended in 20ml buffer to give a 20 fold increase in cell concentration.

The assay was then set as follows:

- 8ml thick cell suspension
- 1ml glucose at required concentration
- 1ml dye at required concentration

A control assay was prepared in the same way but with 1ml of phosphate buffer (100mM, pH 7) substituting the dye. The assays were started by addition of the thick cell suspension. The volume, cell density, dye and glucose concentrations in the assay were varied according to the final levels required but the relative volumes of each component were always kept in the same ratio.

For routine preparation of decolourised material for chemical analysis and Microtox® (Section 2.13) tests the assay was prepared in universal bottles or test tubes and incubation was at 37°C in a thermostatically controlled water bath. Thick cell suspensions used were typically a 50 fold increase in cell density from an 18h culture. Dye concentration in assays for UV/VIS, HPLC, TLC and the Microtox® analysis was 0.05% w/v (500ppm). A glucose concentration of 1% was employed for all dyes except Procion Crimson where 0.1% was used. Control assays were set without the addition of dye. For UV/VIS, TLC and HPLC samples were removed as required by automatic pipette and immediately centrifuged (1ml Eppendorf tubes, microfuge 11,600 x g for 5mins). The supernatant was decanted and reserved for analysis. For the Microtox® test complete assay contents were centrifuged and supernatants reserved for analysis. All samples were analysed on the day of preparation.

To measure initial decolourisation rate, assays were set in 5ml glass tubes (1cm diameter) with incubation directly in the spectrophotometer (thermal circulator set at 37°C). Thick cell suspensions used were typically a 10 fold increase in density from an 18h culture. Glucose concentration was 1%. The dye concentration used was such that the absorbance at the start of the assay was in the region of 1AU at the λ_{max} of the dye under test, typically 0.0025% (w/v) for Reactive Black. Assays for rate measurements were started by the addition of dye and absorbance readings at λ_{max} for the dye of interest recorded automatically by the Labtech Aquire program. A control assay was set as a reference without the addition of dye. For determination of rate linearity with cell density, assays were set in the same way with variation in the cell density being achieved by a series of dilutions of the initial thick cell suspension prepared. To obtain a measure of cell numbers the optical density of the thick cell suspension was measured at 650nm against a distilled water blank, diluting with distilled water if necessary to bring the reading on scale. All cell suspensions were used within 90min of preparation.

2.8.2. Aerobic isolates.

The assay was essentially the same as for *E. faecalis* except that medium used was NB and the 10ml pre-culture was grown for 24h at 30°C. The overnight culture was

grown shaken (180rpm, 30°C). Decolourisation assays were incubated at 30°C. Measurement of cell numbers was as described for *E. faecalis* (Section 2.8.1). All cell suspensions were used within 90min of preparation.

2.8.3. Anaerobic isolates.

The assay was essentially the same as for *E. faecalis* except that medium used was SB. All media, buffers and glucose solutions were degassed in an anaerobic cabinet (Don Whitley compact M) for a minimum of 24 hours prior to use. Pre-cultures were inoculated anaerobically and incubated at 30°C for 24h. The large volumes of broth were autoclaved in 400ml centrifuge bottles and inoculated with the pre-culture anaerobically (1% v/v inoculum). For centrifugation the bottles were removed from the anaerobic cabinet and balanced against water weighted bottles. After centrifugation the bottles were then returned to the anaerobic cabinet, the supernatant decanted therein and discarded. All manipulations to prepare the assay were performed in the anaerobic cabinet and the assays were incubated inside at 30°C. Assays were sampled inside the cabinet to avoid oxygen exposure.

Assays for preparation of decolourised material for chemical analysis and Microtox® tests were as for *E. faecalis* except with anaerobic preparation and incubation at 30°C. All samples were analysed the same day.

To measure initial decolourisation rate, assays were set in 5ml glass tubes (1cm diameter) fitted with gas tight rubber seals before removal from the anaerobic cabinet. The assays were started by addition of the dye via a syringe through the seal thus avoiding oxygen exposure. The dye concentration used was such that the absorbance at the start of the assay was in the region of 1 AU at the λ_{max} of the dye under test, typically 0.0025% (w/v) for Reactive Black. Incubation was directly in the spectrophotometer (circulator set at 30°C) and absorbance readings at λ_{max} for the dye of interest recorded automatically by the Labtech Aquire program. A control assay was set as reference without the addition of dye. For determination of rate linearity with cell density, assays were set in the same way with variation in the cell density being achieved by a series of dilutions of the initial thick cell suspension prepared. Measurement of cell numbers was as for *E. faecalis* (Section 2.8.1). All cell suspensions were used within 90min of preparation.

2.8.4. Determination of cell numbers from optical density.

For each thick cell suspension prepared the optical density was measured at 650nm against a distilled water blank with dilution as necessary to bring the measurements on scale. Aliquots of a typical suspension (100µl) were pipetted into pre-weighed Eppendorf tubes and dried at 80°C under vacuum overnight. The tubes were cooled in a desiccator, weighed and returned to the vacuum oven for a further 4h. The tubes were cooled in a desiccator, weighed (to ensure no further loss in mass) and the dry cell weight content in mg of the 100µl aliquots calculated. The mg of dry cell mass for an optical density of 1AU was calculated assuming a linear relationship. Using this procedure conversion factors were obtained for *E. faecalis* and *C. butyricum.* All measurements were performed in triplicate. An example calculation is given in Appendix 3. An OD650nm of 1AU corresponded to 1.3mg dry cell weight for *E. faecalis* and 2.5mg dry cell weight for *C. butyricum.*

2.9. Preparation of cell free extracts.

2.9.1. *E. faecalis.*

A volume of overnight culture (typically 500ml) was prepared according to Section 2.8.1 and centrifuged at 14,200 x g for 15 min at 12°C. The supernatant was discarded and the cell pellet re-suspended in potassium phosphate buffer (100mM, pH7) using a sterile glass pipette avoiding excessive aeration. The centrifugation was repeated, supernatant discarded and the pellet re-suspended a second time in phosphate buffer. The centrifugation was repeated a third time, the supernatant discarded and the pellet re-suspended in a volume of distilled deionised water (Millipore 18MΩ water) such that the cell density was effectively increased 100 fold. For example a 500ml culture would be washed twice with phosphate buffer and re-suspended in 5ml of distilled deionised water. The thick cell suspension was then dispensed into 1ml volumes in Eppendorf tubes and frozen immediately at -20°C until required.

Cell breakage was achieved using a soniprobe electronic disintegrator (MSE, Loughborough, U.K.) with a frequency of 6Hz and maximum power output. An Eppendorf tube containing the 1ml volume of thick cell suspension was defrosted at

room temperature then placed in a beaker of closely packed crushed ice. The sonicator probe was then inserted into the suspension as far as possible without touching the sides or base of the Eppendorf tube. Sonication was applied for 30s followed by 30s cooling time. This cycle was repeated until the suspension had received a total of 20mins sonication. The suspension was cooled in the ice for 5 min then centrifuged (microfuge 11,600 x g) for 10min. The supernatant was withdrawn by pipette into a fresh Eppendorf tube and centrifuged again. This was repeated until no cell debris was visibly present. The supernatant was then kept on ice for use as a crude cell free extract (CFE). All cell extracts were prepared fresh immediately before use. A portion of each was withdrawn and frozen at -20°C for protein determination.

2.9.2. *C. butyricum.*

A volume of culture (typically 500ml) was prepared according to Section 2.8.3. The cells were centrifuged and washed twice as for *E. faecalis* (Section 2.9.1) except that all manipulations were performed in the anaerobic cabinet and centrifuge tubes were balanced against water weights as for the decolourisation enzyme assay for whole cells. The final re-suspension was in distilled deionised water to give a 100 fold increase in cell density. The buffer used for washing and the water for the final re-suspension were degassed in the anaerobic cabinet for 24h before use. The final thick cell suspension was dispensed into 1ml volumes in gas tight Eppendorf tubes inside the cabinet, sealed and then removed and frozen at -20°C until required.

Initial attempts to achieve cell breakage were as for *E. faecalis* using the soniprobe electronic disintegrator except that the cell suspension received a total sonication time of 3min. The extracts prepared in this manner however showed no decolourisation activity so the following method for cell breakage was devised which minimised exposure of the cell suspension to oxygen. Thick cell suspensions were prepared as described above except that following the final re-suspension in distilled deionised water the suspension was not dispensed but frozen as a whole in a tightly sealed bijou bottle of a size such that headspace was a minimum. When required the suspension was defrosted in the anaerobic cabinet then transferred to a 100ml centrifuge tube containing a pre-weighed quantity of acid washed glass beads (No 8, 452-520μm). The weight of beads was 5g to 10ml thick cell suspension. The

centrifuge tube was sealed then removed from the cabinet. The tube was then subjected to 30s of vortex mixing followed by 30s cooling in an ice-water mixture. This cycle was repeated until the suspension had received a total of 6min vortex mixing. The tube was then cooled for 5 min, balanced against a water-weighted tube and centrifuged at 9,000rpm at 4°C for 20min. Following centrifugation the tube was returned to the anaerobic cabinet and the supernatant withdrawn by pipette, taking care not to disturb the pellet, into a fresh bijou bottle. The bottle was then tightly sealed and removed from the cabinet and kept on ice for use as a crude cell free extract (CFE). The bijou bottle was returned to the cabinet for all subsequent manipulations to avoid oxygen exposure. All extracts were prepared fresh immediately before use. A portion of each preparation was withdrawn and frozen at -20°C for protein determination.

2.9.3. Determination of the protein content of cell free extracts.

The method used was a modification of that described by Lowry *et al.* (1951).

Reagents

Folin A	2% (w/v) Na_2CO_3 in 0.1N NaOH
Folin B	0.5% $CuSO_4.5H_2O$ in 1% (w/v) trisodium citrate
Folin C	49ml of Folin A and 1ml of Folin B mixed well before use

A standard curve was constructed using bovine serum albumin (BSA, Sigma) as standard protein. A 1mg/ml solution of BSA was prepared in distilled deionised water. To a series of test tubes pre-determined volumes of the standard were pipetted ranging from 0 to 500µl giving a range of standard protein concentrations from 0 to 0.5mg per tube. All standards were prepared in duplicate. Samples for analysis (previously frozen at -20°C) were defrosted at room temperature and mixed well by automatic pipette. Known volumes of the samples were pipetted into test tubes in duplicate as for the standards. To each tube (samples and standards) 3ml of Folin C was added and the tube vortex mixed. The tubes were then left to equilibrate at room temperature for 10min. A 50% (v/v) solution of Folin and Ciocalteu phenol reagent (Sigma) was prepared and 0.3ml added to each tube. The tubes were vortex mixed and after exactly 30min the absorbance at 660nm was read against a water blank. Addition of Folin reagent to the tubes was staggered over

time such that absorbances could be read after exactly 30min. All values were corrected for the blank readings obtained from the tubes with no protein present. A calibration curve was constructed with corrected absorbances for the standards plotted against mg protein present per tube. Protein content of the samples were then read directly from the calibration curve giving mg protein values for the known volume added to the tube for each sample analysed. A calibration curve was constructed each time the assay was performed.

2.10. Decolourisation enzyme assay with cell free extracts.

The method used for decolourisation with cell free extracts was based on that described by Zimmerman *et al.* (1982). Crude cell free extracts were prepared fresh immediately before use as in Section 2.9. Assays were followed in thermostatically controlled cuvettes at the growth temperature of the organism under test in a spectrophotometer at the λ_{max} of the dye being assayed.

The main components of the assay were cell free extract, potassium phosphate buffer (100mM, pH7), dye and NADH. Dye concentrations were chosen such that initial absorbances at λ_{max} were within the linear range of the spectrophotometer. For Reactive Black this was typically 0.002% (w/v). Zimmerman *et al.* (1982) used 1mM NADH for decolourisation of Orange I and Orange II both of which have single azo bonds. It was shown by Zimmerman *et al.* (1982) that 1mol Orange II was reduced by 2mol NADH which was consistent with the expected stoichiometry for reduction of a single azo linkage. In view of this it was decided to increase the NADH concentration to 2mM as the number of azo bonds in the model dye Reactive Black was 2 as opposed to 1 for Orange II. The number of azo bonds in the remainder of the dyes under test was unknown but thought unlikely to be more than 2. A 28mg/ml solution of NADH was prepared using distilled de-ionised water and 50µl added per ml of assay to give a final concentration of 2mM NADH (equivalent to a concentration of 0.14% (w/v)). The remainder of the assay volume was phosphate buffer. Total assay volumes were 1ml for *E. faecalis* and 3ml for *C. butyricum*. For each batch of cell free extract prepared decolourising activity was checked by measuring the decolourisation rate for Sunset Yellow (an example of a dye with a single azo bond). Rates for Sunset Yellow were compared between

batches to ensure consistency in decolourising activity between cell extract preparations. Examples of assays for *E. faecalis* and *C. butyricum* and Reactive Black are given below. The volume of cell free extract added to each assay was varied as necessary to obtain a measurable rate of decolourisation.

(a) *E. faecalis.*

To a 1ml (1cm path length) quartz cuvette the following was added.

- 20µl dye (0.1% w/v solution)
- 50µl NADH (28mg/ml solution)
- 100µl crude cell free extract
- 830µl potassium phosphate buffer (100mM, pH7)

A blank assay was also prepared for the reference cell as above but without the addition of dye. The volume of the assay was kept constant by increasing the volume of buffer added to 850µl. Cuvettes containing dye, cell free extract and buffer were pre-incubated in the spectrophotometer for 3min then the reaction started by addition of NADH. Decolourisation was followed at 600nm (λ_{max} for Reactive Black) at 37°C in a spectrophotometer with data collection using the Labtech Aquire package. The volume of dye, cell free extract and buffer were varied as required. The NADH concentration was kept constant for all assays.

(b) *C. butyricum.*

Quartz cuvettes (3ml) equipped with blown glass reagent side arms with ground glass fittings were used. All manipulations were performed in the anaerobic cabinet and the cuvettes were sealed using the reagent side arms before removal.

To a 3ml (1cm path length) quartz cuvette the following was added.

- 60µl dye (0.1% w/v solution)
- 1500µl crude cell free extract
- 1000µl potassium phosphate buffer (100mM, pH7)

To the reagent side arm the following was added.

- 150µl NADH (28mg/ml solution)
- 290µl potassium phosphate buffer (100mM, pH7)

A blank assay was also prepared for the reference cell as above but without the addition of dye. The volume of the assay was kept constant by increasing the total volume of buffer added to 1350µl. The side arms were fitted to the cuvettes and the whole assemblies removed from the anaerobic cabinet. The cuvette assemblies were pre-incubated in the spectrophotometer for 3min. The reaction was started by inverting the assembly such that the NADH-buffer mixture in the reagent side arm flowed into the cuvette. The contents were mixed by inversion and the cuvettes were replaced in the spectrophotometer. Decolourisation was followed at 600nm (λ_{max} for Reactive Black) at 30°C in a spectrophotometer with data collection using the Labtech Aquire package. The volume of dye, cell free extract and buffer were varied as required. The NADH concentration was kept constant for all assays.

2.11. Gel Electrophoresis.

2.11.1. Gel preparation.

The method for gel preparation was based on the Laemmli (1970) system and the Sigma technical bulletin No. MKR-137. All solutions were prepared with distilled de-ionised water (Millipore 18MΩ). Unless stated otherwise all chemicals were purchased from Sigma and were of electrophoresis grade. The gel system employed was a Mini-Protean II system (Bio-Rad) and used according to the manufacturer's instructions.

Reagents

Tank buffer	15.1g tris[Hydroxymethyl]aminomethane (Trizma® base) and 72g glycine dissolved in 500ml water and adjusted to pH 8.3. The buffer was stored at 4°C and diluted 10 fold immediately before use.
Sample buffer	0.42g tris[Hydroxymethyl]aminomethane hydrochloride (Trizma®-HCl) was dissolved in 3ml water and adjusted to pH 6.8. To this was added 1.2mg Bromophenol Blue and 3.6ml glycerol. This solution was then made to 10ml with water and stored in 0.5ml aliquots at -20°C. The buffer as prepared was a x6 concentrate and for use was added to sample 1 to 5.
Solution A	acrylamide/bis-acrylamide solution 30%.

Solution B	7.28g Trizma® base and 92µl N, N, N', N'-Tetramethylethylaminediamine (TEMED) were dissolved in a small volume of water and pH adjusted to pH 8.9 with 1N HCl. This solution was then diluted to 40ml with water.
Solution C	1.21g Trizma® base and 92µl TEMED were dissolved in a small quantity of water and pH adjusted to pH 6.9 with 1N HCl. This solution was then diluted to 20ml with water.
Solution D	10g sucrose was dissolved in water and made to 100ml to give a 10% solution.
Solution E	150mg capsule of ammonium persulphate was dissolved in 12.5ml water immediately before use.

To prepare the separating gel predetermined volumes of de-ionised water, solution A, solution B and solution D were mixed and degassed under vacuum, 1.0ml of freshly prepared solution E was added and mixed using a glass pipette avoiding aeration. The resulting solution was immediately poured into the glass sandwich gel system according to the manufacturer's instructions. A layer of amyl alcohol was pipetted along the top of the separating gel to ensure the gel set with an even surface. The system was then left at room temperature to allow polymerisation of the acrylamide. Following polymerisation the amyl alcohol was washed away with distilled de-ionised water and excess water removed from the top of the gel with absorbent tissue. The separating gel composition varied according to the final gel strength required. The volume of solution E remained constant for all gel strengths. Table 13 shows the volumes of each component solution required for various separating gel strengths.

Stacking gel composition remained constant for all separating gel strengths used and consisted of:

- 3.6ml distilled de-ionised water
- 0.8ml solution A
- 1.0ml solution C
- 2.0ml solution D

The mixture was degassed under vacuum prior to the addition of 0.6ml of freshly prepared solution E and mixed using a glass pipette avoiding aeration. The resulting

solution was then poured onto the separating gel and sample well combs inserted according to manufacturer's instructions. The gel was then left at room temperature to polymerise. When set, the sample combs were removed and any un-polymerised acrylamide solution present in the sample wells removed using a syringe fitted with a flat-ended needle taking care not to puncture the gel. The gels were then fitted to the Mini-Protean system and used immediately. Tank buffer was prepared as above and pre-cooled to 4°C before use.

Protein samples for analysis were prepared according to Section 2.8. Crude cell free extracts were prepared fresh before use and kept on ice until required. Standard proteins employed are as described in Section 2.11.3. Sample buffer was added (20µl to 100µl cell free extract), mixed and 10µl of the mixture loaded onto the gel per sample well using the syringe described above. For *C. butyricum* cell free extract, the sample buffer was added and the syringe loaded inside the anaerobic cabinet. The loaded syringe was withdrawn and the gel sample well loaded immediately to avoid exposure of the cell extract to oxygen. The syringe was washed with tank buffer between samples. The gel system was operated in a cold room at 4°C with the gel tank itself packed in crushed ice. Two gel tanks were operated at any one time at 12mA for approximately 6h until the tracking dye was 1cm from the bottom of the gel. After running the gels were removed and stained immediately by the appropriate technique.

Table 13. Separating gel strength and volume of components required.

Gel strength (%)	Solution A (ml)	Solution B (ml)	Solution D (ml)	Water (ml)
6	4.00	5.00	5.98	4.02
7	4.67	5.00	5.63	3.70
8	5.33	5.00	5.27	3.40
9	6.00	5.00	4.91	3.09
10	6.67	5.00	4.56	2.57
11	7.33	5.00	4.00	2.47
11.5	7.67	5.00	3.80	2.53
12	8.00	5.00	3.60	2.40

2.11.2. Gel staining.

(a) Azoreductase activity staining.

The method used for activity staining was based on that described by Zimmerman *et al.* (1982). Gels were soaked in a 0.1% (w/v) solution of Reactive Black for 10min. The excess dye was decanted and the gel washed in potassium phosphate buffer (100mM, pH7). The gel was incubated at room temperature in 1mM NADH. For *C. butyricum* cell free extract samples the gels were removed from the glass plates inside the anaerobic cabinet and all stages of staining performed therein. A colourless band in the blue background indicated the position of azoreductase activity. The R_f (electrophoretic mobility) of the decolourised band was determined relative to the tracking dye.

(b) Coomassie Blue stain.

Reagents

Fixative solution	180ml methanol and 50ml acetic acid made to 1l with water.
Stain	0.5g Coomassie Blue dissolved in 500ml fixative solution.

Gels were soaked in stain overnight with agitation then de-stained with several changes of fixative solution. Gels were stored at 4°C in fixative solution.

(c) Silver stain.

Reagents

Solution A	100ml methanol and 25ml acetic acid made to 250ml with distilled water.
Solution B	25ml methanol and 12.5ml acetic acid made to 250ml with distilled water.
Oxidiser	0.25g potassium dichromate and 35.5µl 68% nitric acid made to 250ml with distilled water.
Silver reagent	0.5097g silver nitrate made to 250ml with distilled water.
Developer	14.84g sodium carbonate made to 500ml with distilled water, 0.25ml formaldehyde added immediately before gel placed in developer.
Stop solution	12.5ml acetic acid made to 250ml with water.

Gels were soaked in solution A for 30min. Solution A was decanted and the gel soaked in solution B for 15min. This was then decanted and replaced by fresh solution B and incubated for a further 15min. The gel was removed from solution B and placed in oxidiser for 5min followed by two distilled water washes. The gel was incubated in the silver reagent for 20min then washed in distilled water for 1min. The developer solution was applied until the first bands were seen to develop. The developer was discarded and fresh developer applied until bands were stained to the required amount. The gel was then soaked in stop solution for 5min. All staining steps required agitation.

2.11.3. Determination of azoreductase molecular weight.

Azoreductase molecular weight was determined indirectly by electrophoresis on a set of gels of various polyacrylamide concentrations as detailed in the Sigma technical bulletin MKR-137. The R_f of the protein in each gel was calculated from the migration distance of the protein relative to the tracking dye. A plot was constructed of 100 Log (R_fx100) against percent gel concentration for samples and molecular weight markers. From these plots individual slopes were calculated (the retardation coefficients) and the negative slopes plotted against the molecular weight on a log-log scale. The molecular weight of the unknown was calculated from the resulting linear plot. Molecular weight markers and gel concentrations used were as given in Table 14. Markers were prepared according to the Sigma bulletin.

Table 14. Standard proteins used for azoreductase molecular weight determination.

Protein	Molecular weight	% gels run	Vol. Applied to gel (µl)
α-Lactalbumin, Bovine milk	14,200	7, 8, 9, 10	15
Carbonic anhydrase, Bovine Erythrocytes	29,000	6, 7, 8, 9	20
Albumin, Chicken Egg	45,000	7, 8, 9, 10	20
Albumin, Bovine Serum	66,000 (monomer) 132,000 (trimer)	7, 8, 9, 10	15
Unknown		10,11,11.5,12	10

2.12. Redox potential of chemical and biological decolourisation.

The redox electrode used was the Hanna Instruments H18720E.

2.12.1. Chemical.

All solutions were prepared with distilled deionised water (Millipore 18MΩ). Water used for preparation of the sodium dithionite solution was degassed with nitrogen for 5min immediately before use. The freshly prepared sodium dithionite solution (typically 0.16g per 60ml) was added to 100ml of dye solution (typically 0.05% (w/v)) in 0.5ml aliquots, with the redox electrode and a nitrogen gas line immersed in the dye solution. The redox potential was measured after each reducing agent addition and observations were made as to the colour of the dye solution. The quantity of reducing agent and concentration of the dye solution were varied as necessary to achieve decolourisation within approximately 20-30ml reducing agent solution added. Titration in buffered conditions was achieved via preparation of dye solutions as above in phosphate buffer (pH 7) and citrate-phosphate buffer (pH 3). After each measurement session the electrode was rinsed with distilled water.

2.12.2. Biological.

Assays with *E. faecalis* were prepared according to Section 2.8.1. Thick cell suspensions were typically a 50 fold increase in cell density from an 18h culture. Dye concentration was 0.05%. Assay volume was 10ml and incubation at 37°C in a thermostatically controlled water bath. The redox electrode was inserted into the assay and potential monitored over a defined period of time. Observations were made as to the colour of the assay. After each measurement session the electrode was cleaned with ethanol and rinsed with distilled water.

2.13. The Microtox® acute toxicity test.

2.13.1. Sample preparation.

All samples for the Microtox® acute toxicity test were prepared in 2.2% NaCl (diluent). This was achieved by diluting samples prior to testing in diluent to the required concentration or by osmotic adjustment with 22% NaCl (0.1ml to 1.0ml sample) where no dilution was required to obtain measurable readings. Diluent and

osmotic adjusting solutions were obtained from Azur Environmental (Reading, U.K.). All biologically reduced samples and HPLC fractions for Microtox® testing were prepared by the author and tests were performed by A. Gottlieb (Department of Life Sciences, The Nottingham Trent University). Other test solutions were prepared and tested by A. Gottlieb.

(a) Biologically reduced dyes.

Decolourisation assays were set according to Section 2.8. Cell suspensions were a 50 fold increase in density from an 18h culture. Dye concentrations were 0.05% w/v (500ppm). Post decolourisation the assays were centrifuged (1ml Eppendorf, microfuge, 11,600 x g) to remove the cells and the supernatants reserved for analysis. All samples were tested on the day of preparation. A 5 fold dilution of the decolourised material was prepared diluent prior to analysis giving a final top concentration in the test of 50ppm (100ppm from the dilution reduced to 50ppm at start of the test). Further dilutions of the original decolourised material were prepared as necessary to obtain a range covering the concentration causing a 50% light loss (measurable EC_{50} value) for the Microtox® test reagent (*Vibrio fischeri*).

(b) Chemically reduced dyes.

Solutions of Reactive Black (parent and hydrolysed) were prepared immediately before use in distilled water degassed with nitrogen. Dye concentrations were 0.05% w/v (500ppm). The sodium dithionite reducing agent was added as a powder to the dye solution and mixed gently avoiding aeration. The quantity of sodium dithionite added for each dye was the minimum weight of reducing agent required to produce decolourisation as determined according to Section 2.12.1. For a 0.05% w/v dye solution sodium dithionite was added at 3.9mg/10ml for the parent dye and 3.5mg/10ml for the hydrolysed dye. A 5 fold dilution of the decolourised material was prepared in diluent prior to analysis giving a final top concentration in the test of 50ppm. Further dilutions of the original decolourised material were prepared as necessary.

(c) HPLC fractions.

Decolourisation assays with *E. faecalis* and Reactive Black (parent and hydrolysed) were prepared according to Section 2.8. Cell suspensions were a 50 fold increase in

cell density from an 18h culture. Dye concentration was 0.05% w/v (500ppm). Post reduction and cell removal the assay supernatants were injected directly onto the HPLC column with conditions as specified in Section 2.3.2. The supernatants were injected repeatedly onto the HPLC column and the two product fractions were collected from the column exhaust as they eluted. Samples were osmotically adjusted with osmotic adjusting solution (22% NaCl) prior to testing. The number of injections and volume of material collected was recorded and the top concentration of the component being tested was calculated according to the following example:

Molecular weight of Reactive Black = 991.8, contribution to molecular weight from each component: H-acid = 389 and vinyl sulphone side chain = 301. So for each molecule of Reactive Black the vinyl sulphone chain constitutes 60% of the total weight of dye present (two side chains for each molecule of dye) whereas the H-acid component constitutes 40% of the total weight of dye.

If a sample is 0.05g dye / 100ml (500ppm) of reduced dye then in 1000µl of sample there will be 500µg of dye. For each 20µl HPLC injection there is 10µg dye. For each injection therefore there will be 6µg of vinyl sulphone side chain and 4µg of H-acid.

For hydrolysed Reactive Black vinyl sulphone peak:

Number of injections = 2 thus 12µg of component present

Total volume of collected fraction = 2.4ml 12µg / 2.4ml = 5µg / ml = 5ppm

With osmotic adjustment 22% saline to produce final 2.2% saline:

5 x (10/11) = 4.55ppm to give a top concentration in the test of 2.27ppm.

2.13.2. Test procedure.

The instrument used was the Microtox® model 500 Analyser from Azur Environmental (Reading, U.K.). The analyser consisted of 30 sample wells (6 rows of 5 wells) at 15°C, an exposure chamber controlled at 15°C and a storage well for the re-hydrated test reagent maintained at 5°C. All measurements of light output were recorded on a dedicated computer. All reagents were supplied by Azur Environmental.

Reagents

Microtox® test reagent	freeze dried preparation of *Vibrio fischeri*
Reconstitution solution	Organics free ultra pure water
Diluent	2% analytical grade sodium chloride solution
Osmotic adjusting solution	22% analytical grade sodium chloride solution

The basic test performed was according to the manufacturer's instructions and consisted of 8 sample dilutions (2 fold) with 2 controls.

The sample wells can be represented thus:

	1	2	3	4	5
A	O	O	O	O	O
B	O	O	O	O	O
C	O	O	O	O	O
D	O	O	O	O	O
E	O	O	O	O	O
Exposure chamber			O		

O Reagent well

The Microtox® test reagent was re-hydrated with 1ml reconstitution solution at 4°C and equilibrated in the storage well at 5°C for 15min before use. Sample wells were allocated for both dilutions and tests with 1ml diluent pipetted into the dilution well cuvettes (rows A and C, C5 being left empty) and 0.5ml pipetted into the test well cuvettes (rows B and D). Test reagent was pipetted (10µl) into all test wells (rows B and D) and left to equilibrate for 15min. A 2ml volume of the sample under test was pipetted into well C5. A series of 2 fold sample dilutions were prepared by transferring 1ml of this test solution to well C4, mixing with an automatic pipette and transferring 1ml of this dilution to well C3 and so on. Transfers were stopped at well A3 leaving A1 and A2 sample free controls. After the 15min equilibration period the instrument was set on one of the test cuvettes. The test was started by measurement of initial light levels in the test wells (rows B and D) prior to addition of 0.5ml aliquots of each dilution. Additions were made from each dilution well to the corresponding test well from the lowest concentration to the highest, e.g. 0.5ml transferred from A1 to B1 and from A2 to B2 etc. Light levels were read 5min and 15min after the start of the test. All data obtained was reduced by the Microtox®

model 500 software to produce EC_{50} values (concentration of sample under test producing 50% inhibition of luminescence). Data reduction formulae are given in the Microtox® model 500 instrument manuals.

2.13.3. Calculation of EC_{50} values for coloured compounds.

The Microtox® test reagent emits light at 490nm. The natural fall off in light with time (sample free control wells) and the reduction in light due to exposure to a toxicant (sample wells) is measured by the Microtox® model 500 instrument at this wavelength. During testing of a coloured compound however problems would be encountered in that the coloured compound is not present during the reading of initial light levels (I_0) but is present in the reading of the light levels after 5 and 15min (I_5 and I_{15}) following exposure of the reagent to a test compound. If the coloured compound under test absorbs at 490nm then some of the light emitted by the reagent will be absorbed by the coloured compound and will not be detected by the Microtox® model 500 instrument and hence a falsely high light loss due to toxicant response will be recorded. To overcome this the light levels I_0, I_5 and I_{15} were recorded manually, a correction applied for the presence of the coloured compound and then the corrected values entered manually into the Microtox® model 500 software to produce EC_{50} values for the compound under test. The correction applied was as follows (as recommended by Azur Environmental):

The absorbance (ABS_x) at 490nm was measured against a diluent blank using a spectrophotometer for each test dilution.

The contribution to absorbance (A_x) was calculated using the absorbance readings taken for each sample concentration using the equation: $A_x = 2.303 \times ABS_x$

The A_x was then used to calculated the transmittance (T_x) for each sample concentration using the equation: $T_x = (1-e^{-Ax}) / A_x$

The absorbance corrected I_0 was then calculated using the equation: $ACI_0 = I_0 \times T_x$

The corrected I_0 values (ACI_0) and the measured I_5 and I_{15} light levels were then entered manually into the Microtox® model 500 software.

3. RESULTS.

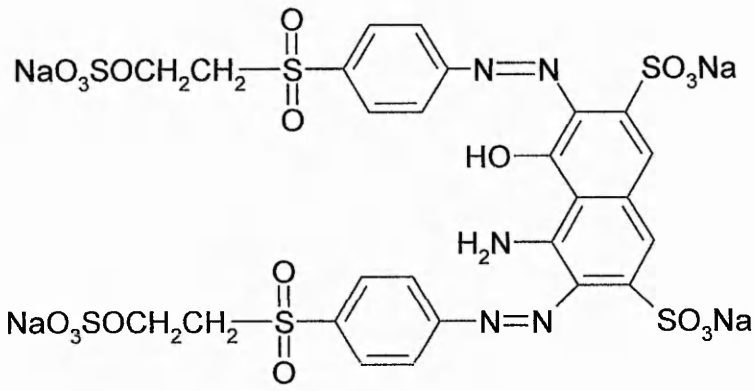
3.1. Identification of representative textile dyes.

Two East Midlands textile companies were consulted to reduce the large number of dyes commercially available (>2000) to a selection which would represent the different dye classes and colours presently in use. The first company, Welbeck fabric dyers (Somercotes, Derbyshire), was a commission dyer using a very large number of dyes of varied chemical constitution. Of the 200 dyes in use, 14 comprised 90% of the total dye usage. The second company, Courtaulds Jersey Underwear (Sherwood, Nottingham), concentrated on the dyeing of cotton with reactive dyes. Four reactive dyes were identified as those most commonly used. The dyes and their respective trade names are given in Table 15. Five of the 18 dyes listed have structures declared according to the *Colour Index* (1971) and these are given in Figure 3. These 18 dyes, although representative of those currently in use, may be present in a textile effluent intermittently and at a range of concentrations due to the batch-wise nature of the dyeing process. In view of this, to develop a working decolourisation system and analytical methods for monitoring colour removal it was deemed practical to narrow this range further and to nominate a model dye. As the Reactive dyes are considered to be the most problematic to treat in conventional treatment plants it was decided to concentrate on dyes of this class. Reactive Black 5 was chosen as the primary model dye for the following reasons:

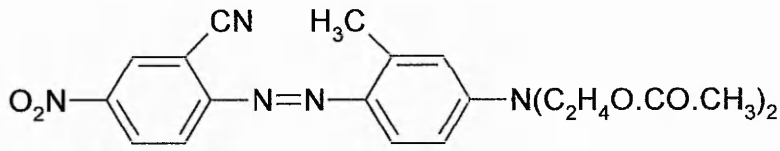
- Pierce (1994) states that this dye is used almost exclusively for the dyeing of black shades.
- There is sustained fashion for black clothing throughout the year.
- The chemical structure of Reactive Black is known which allows prediction of decolourisation end products.
- The Reactive Black intermediate 8-amino-1-naphthol-3,6-disulphonic acid (H-acid) is commercially available.

The 3 Procion dyes from Courtaulds were also used in some studies as further examples of reactive dyes currently in use.

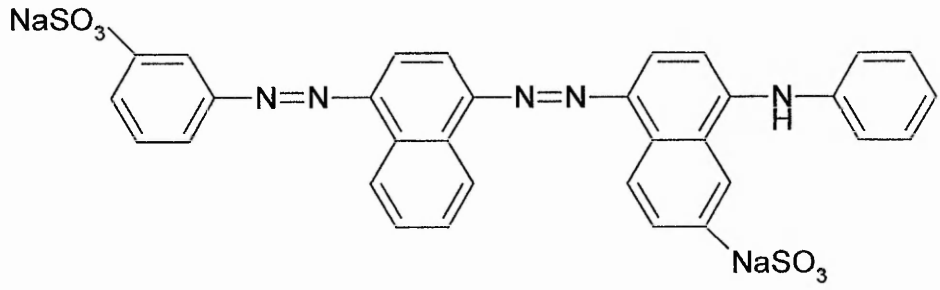
Aqueous solutions of all 18 representative dyes were prepared in sterile distilled water and UV/VIS scanned according to Section 2.3.1. The resulting profiles provided fingerprints for the unreduced dyes for comparison with decolourisation end products. The λ_{max} of each dye determined from the profiles (Appendix 2) is given in Table 15. Measurements of dye concentration and decolourisation rate were subsequently performed at the λ_{max} of the dye under test. The UV/VIS profiles for Reactive Black and the 3 Procion dyes are given in Section 3.11.1. Aqueous solutions of the 18 dyes (and the hydrolysed form of Reactive Black) were also characterised by Thin Layer Chromatography (TLC) using the solvent system described in Section 2.3.3. Separation was achieved for all 18 dyes and hydrolysed Reactive Black. Four dyes contained components that showed no movement from the starting line (Solophenyl Black, Solophenyl Navy, Indosol Blue and Indosol Rubinole). All dyes tested separated into more than 1 component indicating that more than one chemical species may be present and that none were 100% pure. Many dyes contained non-coloured components invisible in natural light but which fluoresced under UV and were thought to be unreacted species carried over from manufacture. The resultant band patterns and hR_f values are given in Table 16. For Reactive Black (parent and hydrolysed form) numerous bands were observed for each form of the dye. However one band of each dye appeared to predominate with regards to size and depth of colour at hR_f 73 for the parent and hR_f 67 for the hydrolysed dye. For each form of Reactive Black the TLC bands obtained were marked, scraped from the surface of the plate and extracted into methanol. The extracted samples were then UV/VIS scanned against a blank portion of the TLC plate developed with the same solvent and extracted as for samples. The resultant scans are given in Figures 4 and 5. The scans show that the bands observed at hR_f 73 for the parent and hR_f 67 for the hydrolysed dye were the only bands to have UV/VIS profiles characteristic of those obtained for aqueous solutions of the dye (Figure 26, Section 3.10). Some of the other bands present also absorbed at 600nm (λ_{max} of Reactive Black) and were thought to be traces of partially coupled intermediates carried over from the dye's manufacture.



REACTIVE BLACK 5

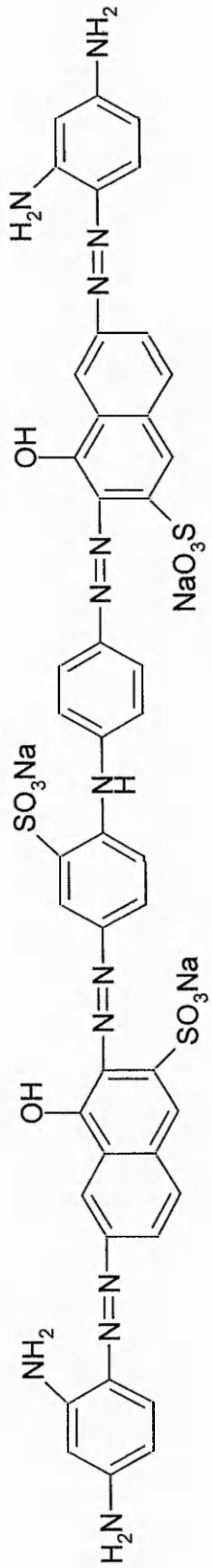


SERILINE RUBINE

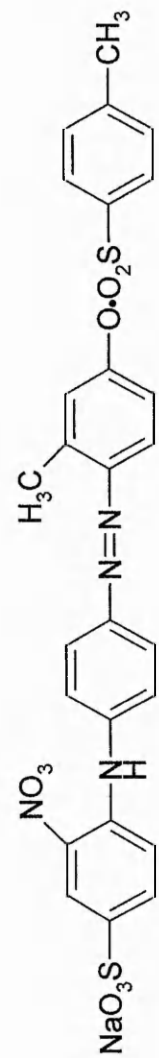


ERIONYL NAVY BLUE R

Figure 4. **Structures of representative textile dyes declared in the *Colour Index* (1971).**



SOLOPHENYL BLACK FG



ERIONYL YELLOW RXL

Figure 4. Continued.

Table 15. Representative textile dyes.

Trade Name	CI Name	Dye Type	λmax (nm)
Remazol Black B	Reactive Black 5	Reactive diazo vinyl sulphone	600
Procion Crimson H-EXL		Reactive azo bismonochlortriazine	550
Procion Navy H-EXL		Reactive azo bismonochlortriazine	600
Procion Yellow H-EXL	Reactive Yellow 138:1	Reactive azo	420
Dispersol Black C-VS		Disperse azo blend	570
Dispersol Blue C-RN	Disperse Blue 369	Disperse azo	580
Dispersol Navy C-2G		Disperse azo blend	580
Erionyl Navy Blue R	Acid Blue 113	Acid azo	570
Erionyl Yellow RXL	Acid Orange 67	Acid azo	372 and 440
Foron Black		Azo	600
Indosol Blue SF-2G		Metal complex azo	612
Indosol Rubinole	Direct Red 262	Metal complex azo	530
Indosol Yellow SF-2RL	Direct Yellow	Direct azo	390
Serilene Rubine	Disperse Violet 33	Disperse azo	520
Serilene Yellow 4GN-LS	Disperse Yellow 211	Disperse mono azo	456
Serilene Yellow Brown 2RL	Disperse Orange 37	Disperse azo	520
Solophenyl Black FG	Direct Black 22	Direct azo	492
Solophenyl Navy BL	Direct Blue 94	Metal complex azo	588

All dyes were prepared as aqueous solutions with pH unadjusted.

Table 16. TLC separation of the representative textile dyes and resultant hR_f values.

Dye	Band No.	HR_f	Comment
Reactive Black	1	67	Pale purple
	2	70	Pale purple
	3	73*	Dark purple
	4	74	Pink
	5	76	Pale purple
	6	79	Pale pink
	7	81	Pale pink
Hydrolysed Reactive Black	1	65	Blue
	2	67*	Dark purple
	3	72	Pale blue
	4	75	Greyish pink
	5	86	Pale blue
Procion Crimson	1	35	Pink
	2	60	Pink
	3	63	Pale pink
	4	66*	Dark pink
	5	69	Pale pink
	6	71	Pink
Procion Navy	1	35	Pale purple
	2	66	Pale purple
	3	68	Purple
	4	70*	Blue
	5	71	Pale blue
Procion Yellow	1	37	Fluorescent yellow
	2	65	Pale yellow
	3	69*	Yellow
	4	78	Pale yellow
Dispersol Black	1	36	Fluorescent yellow
	2	69	Fluorescent yellow
	3	86	Pink
	4	89	Brown
	5	91	Blue
	6	94	Yellow
Dispersol Blue	1	34	Fluorescent yellow
	2	72	Fluorescent blue
	3	94*	Purple
Dispersol Navy	1	38	Fluorescent blue
	2	70	Fluorescent blue
	3	90	Purple
	4	94	Blue

Table 16. Continued.

Dye	Band No.	HR_f	Comment
Erionyl Navy Blue	1	71	Grey
	2	76	Fluorescent blue
	3	81*	Purple
	4	85	Fluorescent yellow
	5	91	Fluorescent yellow
	6	94	Fluorescent yellow
	7	96	Fluorescent blue
Erionyl Yellow	1	69	Pale yellow
	2	79	Pale yellow
	3	88*	Yellow
Foron Black	1	37	Pale grey
	2	71	Pale grey
	3	90	Pinkish brown
	4	93	Blue
Indosol Blue	1	0	Pale blue spot
	2	34	Blue
	3	68	Purple
Indosol Rubinole	1	0	Pink spot
	2	33	Pink
	3	67	Pink
Indosol Yellow	1	36	Fluorescent yellow
	2	67*	Yellow
	3	78	Pale yellow
	4	81	Pale yellow
Serilene Rubine	1	37	Fluorescent yellow
	2	90	Fluorescent yellow
	3	91*	Pink
Serilene Yellow	1	37	Fluorescent yellow
	2	69	Fluorescent yellow
	3	93*	Yellow
Serilene Yellow Brown	1	36	Fluorescent yellow
	2	70	Fluorescent blue
	3	91*	Yellow
Solophenyl Black	1	0	Black spot
	2	71	Grey
	3	80	Grey
	4	83	Grey
Solophenyl Navy	1	0	Purple spot
	2	36*	Purple
	3	69	Violet
	4	75	Pink

KEY * **Major Band.**

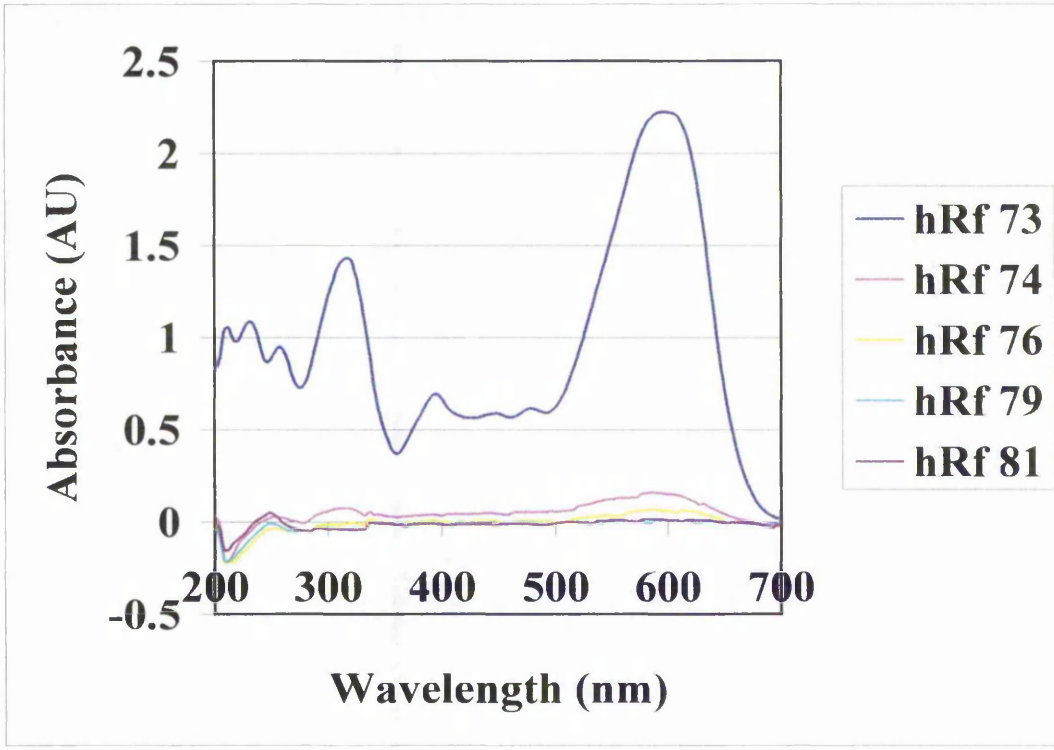


Figure 5. **UV/VIS profiles of TLC bands for Reactive Black.**

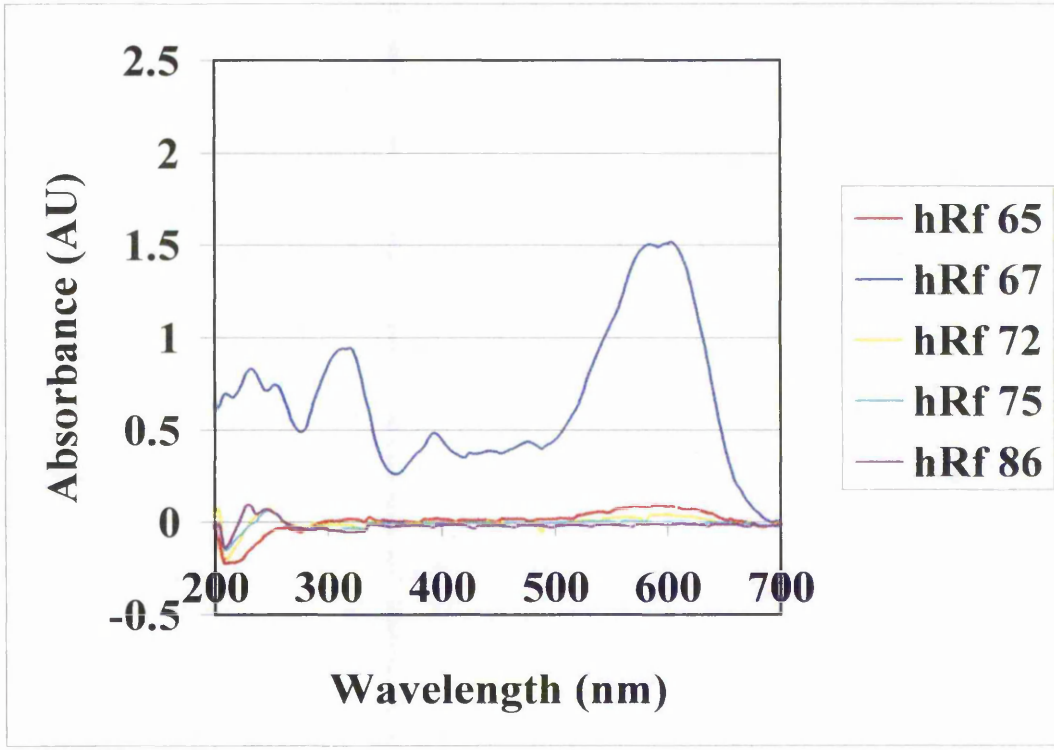


Figure 6. **UV/VIS profiles of TLC bands for hydrolysed Reactive Black.**

3.2. Chemical decolourisation of Reactive Black and three Procion dyes.

Initial studies concentrated on the chemical decolourisation of Reactive Black. It was assumed that dye decolourisation proceeds via azo bond cleavage alone and hence the quantity of reducing agent required for azo reduction could be predicted from knowledge of the dye structure (see Figure 4). It was anticipated that for Reactive Black 1 mole of dye would require 8 moles of reducing agent as the 2 azo bonds present per molecule required the donation of 4 H atoms per bond for complete reduction to two amine groups. Expression of molar amounts of reducing agent required for decolourisation of a dye was only of use for Reactive Black since the Procion dye molecular structures are undeclared. Calculation of the minimum weight of reducing agent required for decolourisation of a known weight of dye was investigated to overcome this.

The minimum quantity of reducing agent required to produce dye decolourisation was thought to depend on the purity of the dye, the pH of the medium, the number of azo groups present in the dye and the presence of other reducible groups on the dye molecule. The purity of Reactive Black was estimated to be approximately 55% based on product information supplied by Aldrich. The purity of Procion Crimson was estimated to be >75% (w/w) according to the hazard data sheet supplied by Zeneca colours. The purity of Procion Yellow and Procion Navy was unknown. To simplify calculations the weights of dyes used in the titrations against reducing agent were taken as if the dyes were 100% pure. For Reactive Black and the 3 Procion dyes both the parent and hydrolysed forms of the dyes were studied.

The effect of pH on reduction was investigated for Reactive Black using unbuffered aqueous solutions of dye and solutions of dye prepared in a series of buffers (pH 3, pH 7 and pH 10) as detailed in Section 2.12.1. In the unbuffered aqueous solutions initial pH was pH 5 for the parent dye and pH 7 for the hydrolysed form of the dye.

A series of titrations were performed as detailed in Section 2.12.1. initially for Reactive Black and its hydrolysed form to determine the weight of reducing agent required for total decolourisation under the various conditions applied. All titrations were performed in triplicate. Observations were made as to the colour of the dye

solution and redox potential after each sodium dithionite addition. The weights of reducing agent per g of dye to produce decolourisation for Reactive Black and hydrolysed Reactive Black are given in Table 17. Reactive Black buffered at pH 10 did not decolourise completely with sodium dithionite added at an equivalent of 33.4g reducing agent per g dye, a colour shift from dark blue to violet was observed indicating partial bond breakage. For comparison, aqueous solutions of Reactive Black and hydrolysed Reactive Black were adjusted to pH 10 (unbuffered) and titrated as for the other conditions.

Table 17. Weight of sodium dithionite (g) required per g of dye and redox potential at total decolourisation (mV).

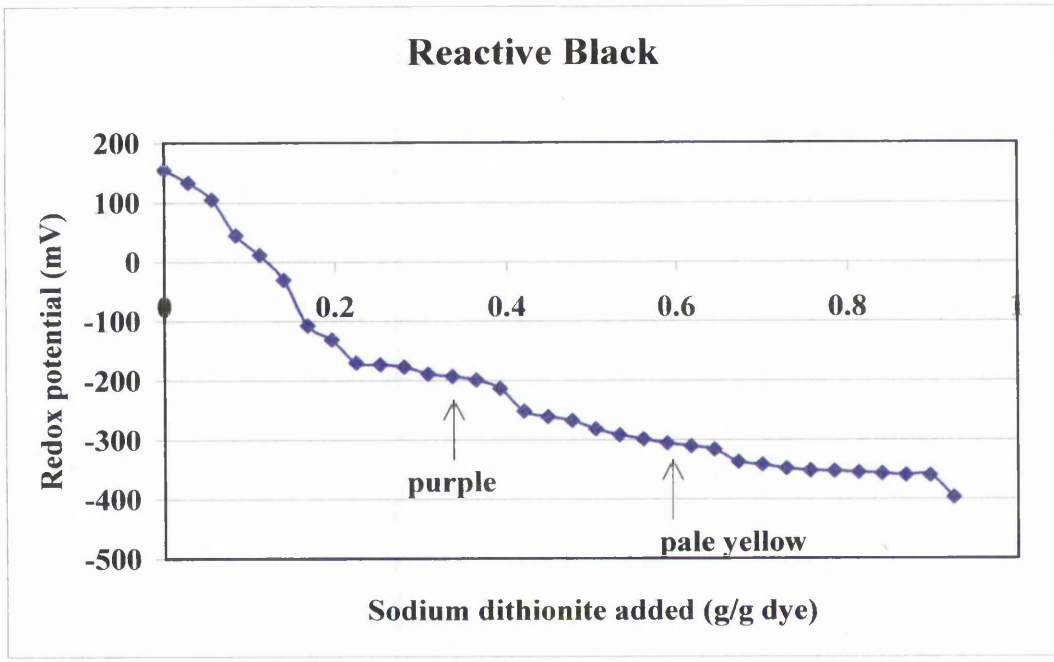
Condition	Reactive Black				Hydrolysed Reactive Black			
	g		mV		g		mV	
	mean	SD	mean	SD	mean	SD	mean	SD
Water	**0.8**	0.03	**-266**	22	**0.7**	0.03	**-396**	11
pH3	**0.6**	0.05	**-306**	0.6	**0.6**	0.03	**-202**	12
pH7	**1.2**	0.01	**-617**	4	**2.8**	0.06	**-609**	4
pH10 unbuffered	**24.3**	0.30	**-507**	10	**20.2**	0.60	**-467**	17

Values quoted represent the mean of 3 determinations.

From Table 17 it can be seen that the pH of the medium had an effect on the quantity of reducing agent required per g of dye. At pH 3 no difference was observed between the parent and hydrolysed dye with regards to the weight of reducing agent required (both forms of the dye requiring 0.6g sodium dithionite per g of dye). A difference in redox potential at decolourisation was observed however being –306mV for the parent form and –202mV for the hydrolysed form. At pH 7 the weight of reducing agent required for decolourisation increased, for the hydrolysed form of the dye requiring a greater weight of sodium dithionite (at 2.8g per g dye) than the parent form (1.2g per g dye). At this pH the redox potential at decolourisation was more negative (in the region of –600mV for both forms of the dye). In a buffered system at pH 10 only partial decolourisation was observed as stated previously and it can be seen from Table 17 at this pH (unbuffered) the weight of reducing agent required per g of dye is greatly increased at 24.5g (parent) and 20.6g (hydrolysed). For the aqueous solutions of dye with no pH adjustment the quantities of reducing agent required for decolourisation resemble those observed at pH 3. Starting pH for these solutions were pH 5 and pH 7 for the parent and

hydrolysed dye respectively. For the dye solutions in water and the unbuffered solutions adjusted to pH 10 the pH of the reduced dye solution at the end of the titrations was pH 4. For Reactive Black the decolourisation was deemed to be pH dependent with greater quantities of reducing agent required with increasing pH. If addition of sufficient reducing agent was required to produce acidic conditions before decolourisation could occur then this may explain the greatly increased quantity of sodium dithionite required for a dye solution at pH 10 and the pH reduction of the solution to pH 4 at the end of the titration. The buffered systems at pH 7 and pH 10 appeared to be inhibitory to decolourisation and at pH 7, although decolourisation did occur, the redox potential was much more negative than that for an unbuffered system. During addition of reducing agent to the solutions of Reactive Black gradual colour changes were observed. This indicated that the 2 azo bonds present in the dye molecule were not broken simultaneously. Figure 7 shows the colour changes and redox potential observed for the parent and hydrolysed forms of Reactive Black.

The structures of the Procion dyes were unknown therefore the number of azo bonds in the Procion dye molecules and the presence of other reducible groups on the dye structures could not be determined. The quantity of reducing agent required for decolourisation of the 3 Procion dyes with that required for Reactive Black (known to contain 2 azo bonds) was compared to gain information as to the number of azo bonds present in the Procion dyes. The sodium dithionite titrations were repeated for the 3 Procion dyes for unbuffered aqueous solutions of dye and buffered solutions at pH 3 and pH 7. The ratio of reducing agent to that required for Reactive Black is given in Table 18. As for Reactive Black, Procion Crimson and Procion Navy underwent gradual colour changes throughout the titrations thought to be due to the presence of multiple azo bonds being broken sequentially. For Procion Yellow the only colour change observed was from yellow to colourless indicating that either 1 azo bond was present or that the dye was of such a structure that multiple bonds would be broken simultaneously. Example curves for the 3 Procion dyes are given in Figures 9 to 11.



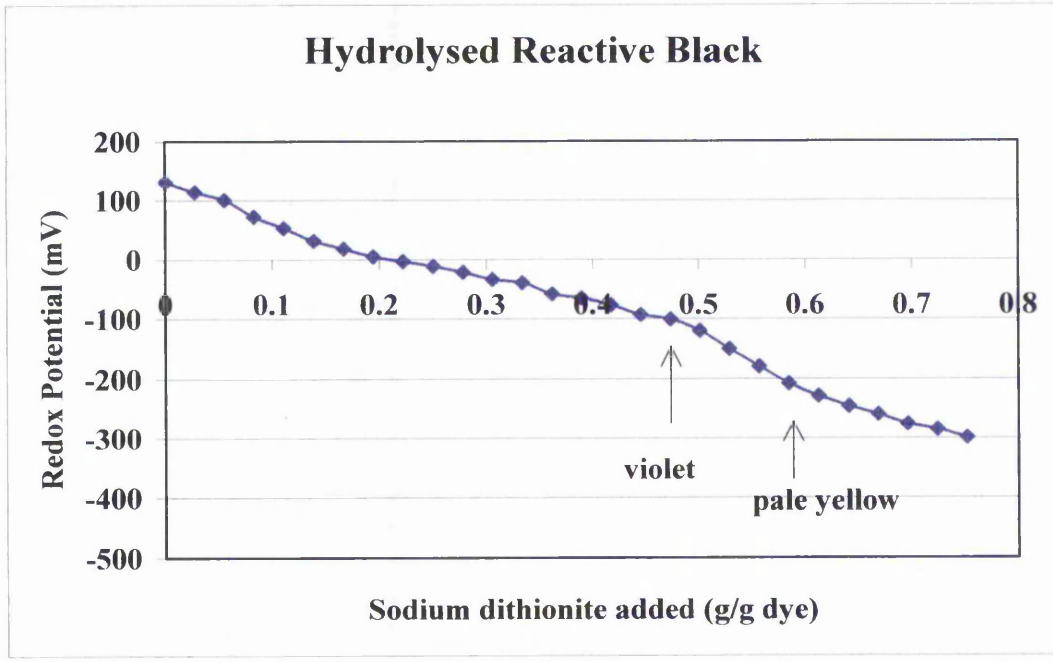


Figure 7. **Colour changes and corresponding redox potential observed for the parent and hydrolysed forms of Reactive Black decolourised by sodium dithionite at pH 3.**

Table 18. Ratio of sodium dithionite required for decolourisation to that required for Reactive Black and redox potential at total decolourisation (mV).

Dye	Ratio of sodium dithionite and redox potential					
	Water	(mV)	pH 3	(mV)	pH 7	(mV)
Procion Navy	2.7	-409	2.5	-361	21.0 [PD (1)]	
Hydrolysed Procion Navy	3.2	-424	2.2	-356	53.3 [PD (2)]	
Procion Crimson	4.6	-420	7.0	-382	22.9 [PD (3)]	
Hydrolysed Procion Crimson	6.6	-437	7.8	-398	41.9 [PD (4)]	
Procion Yellow	0.8	-369	1.7	-344	27.1	-631
Hydrolysed Procion Yellow	1.2	-471	1.5	-350	42.9	-634

KEY

PD Partial decolourisation observed. Sodium dithionite added at (1) 24.5g/g dye for Procion Navy, (2) 62.3g/g dye for hydrolysed Procion Navy, (3) 26.7g/g dye for Procion Crimson and (2) 49.0g/g dye for hydrolysed Procion Crimson.

Values quoted represent the mean of 3 determinations.

Correlation between the amount of reducing agent required for decolourisation and redox potential at decolourisation was observed for the Procion dyes at pH 3 only and is illustrated in Figure 8. No such correlation was observed with the amounts of reducing agent required in unbuffered aqueous solution.

From Table 18 it can be seen that for Procion Navy more than double the quantity of reducing agent compared to that required for Reactive Black was needed to produce decolourisation in the unbuffered aqueous solution, the ratios being 2.7 for the parent dye and 3.2 for the hydrolysed dye. Reactive Black was known to contain 2 azo bonds and be of approximately 55% purity. Several reasons may account for the greater quantity of reducing agent required for Procion Navy: the dye could be of a higher purity, contain twice the number of azo bonds as Reactive Black or contain other reducible groups on its structure. It was thought unlikely that the dye would contain four azo bonds. The most probable explanation for the increased reducing agent required for decolourisation was thought to be due to either the presence of other reducible groups on the dye molecule or differences in purity of the two dyes. The pH of the dye solution at the start of the titration was pH 9 for the parent dye and pH 7 for the hydrolysed dye. For Reactive Black it was noted that in unbuffered

conditions the quantity of reducing agent required for decolourisation was higher at higher pH. It may therefore be expected that the amount of reducing agent required for the parent dye would be higher than that for the hydrolysed dye due to the difference in pH. This was not found to be the case. Some pH dependence was observed however as at pH 3 the amount of sodium dithionite required was less than the unbuffered solution and at pH 7 only partial decolourisation was observed. With reducing agent added at 24.5g/g dye for the parent dye (ratio to Reactive Black: 21.0) and 62.3g/g dye for the hydrolysed form (ratio to Reactive Black: 53.3) colour shifts from dark blue to purple were observed for both forms of the dye indicating only partial bond breakage.

Similar results were obtained for Procion Yellow in that the hydrolysed dye required a greater quantity of reducing agent than the parent dye in unbuffered aqueous solution. The pH of the parent and hydrolysed dyes at the start of the titrations were pH 9 and pH 7 respectively. At pH 3 the ratio of sodium dithionite required to that for Reactive Black was slightly higher at 1.7 for the parent and 1.5 for the hydrolysed form. The ratio of reducing agent required at pH 7 was considerably higher at 27.1 for the parent and 42.9 dye for the hydrolysed dye. The ratio of reducing agent required for Procion Yellow to that for Reactive Black in unbuffered aqueous conditions was close to 1. Procion Yellow underwent only one colour change during its reduction (i.e. yellow to colourless) suggesting the presence of only one azo bond. If one bond were present it may be expected that the amount of sodium dithionite required would be less than that for Reactive Black. The presence of other reducible groups on the dye molecule and differences in the purity of the two dyes may account for the actual amount of reducing agent required.

For Procion Crimson some similarities were observed for the overall pattern of reducing agent amounts required for decolourisation as was observed for Procion Navy and Procion Yellow. As for the other two Procion dyes the hydrolysed Procion Crimson required a greater amount of reducing agent (ratio 6.6) compared to the parent Procion Crimson (ratio 4.6) in unbuffered aqueous solutions. Starting pH was pH 9.5 for the parent dye and pH 7 for the hydrolysed dye. Procion Crimson was estimated to be >75% pure according to hazard data sheets provided by Zeneca colours. The greater quantity of reducing agent required for decolourisation

compared to that for Reactive Black (more than 4-fold higher) was in part thought to be due to the higher purity of the dye. As for the other Procion dyes it was thought likely that other reducible groups may be present on the dye structure which may account in part for the higher amount of sodium dithionite required for decolourisation. As for Procion Yellow the ratio of reducing agent required at pH 3 was greater at 7.0 for the parent and 7.8 for the hydrolysed form. At pH 7 complete decolourisation did not occur with sodium dithionite added at 26.7g/g dye for the parent (ratio to Reactive Black 22.9) and 49.0g/g dye for the hydrolysed dye (ratio to Reactive Black 41.9). For both forms of the dye a colour shift from dark pink to orange/yellow was observed indicating partial bond breakage.

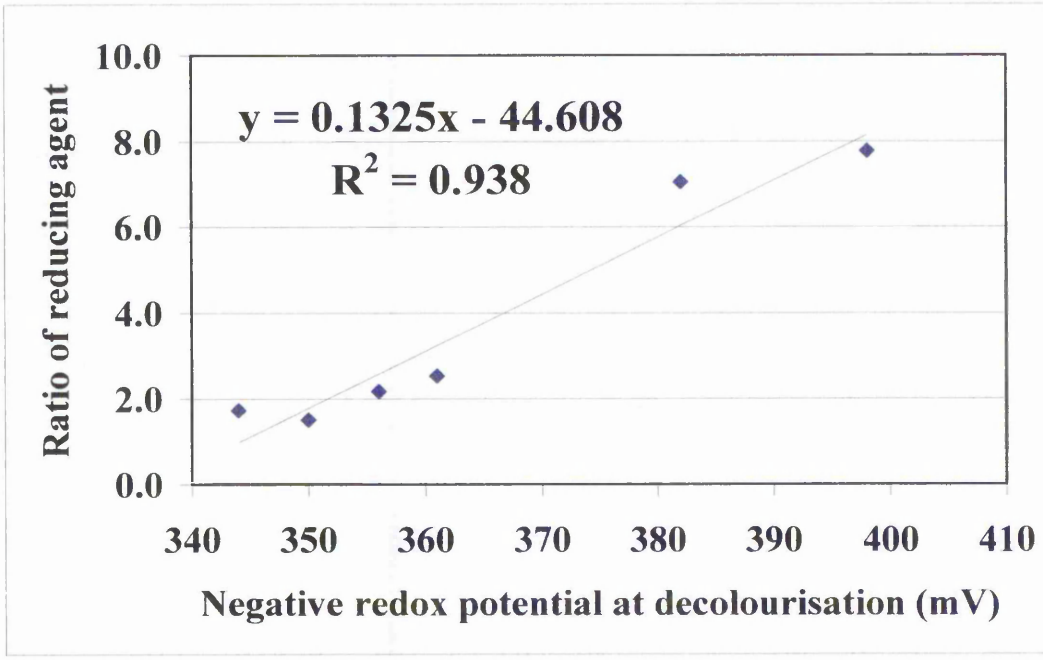
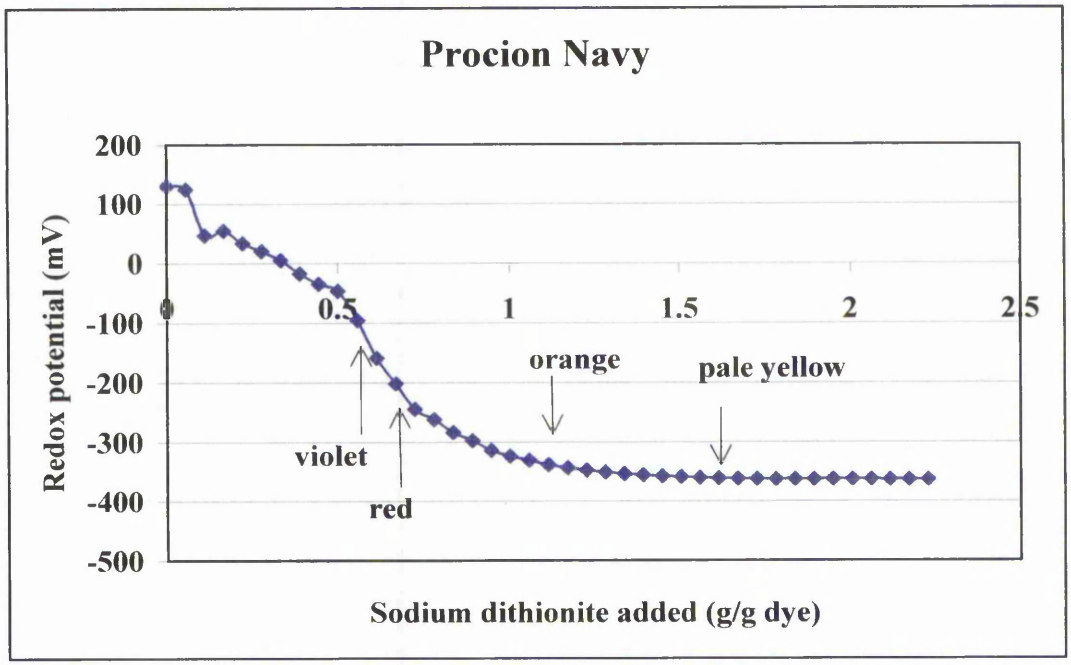


Figure 8. **Correlation between the ratio of reducing agent to that required for Reactive Black and the redox potential at decolourisation for the three Procion dyes in their parent and hydrolysed forms.**



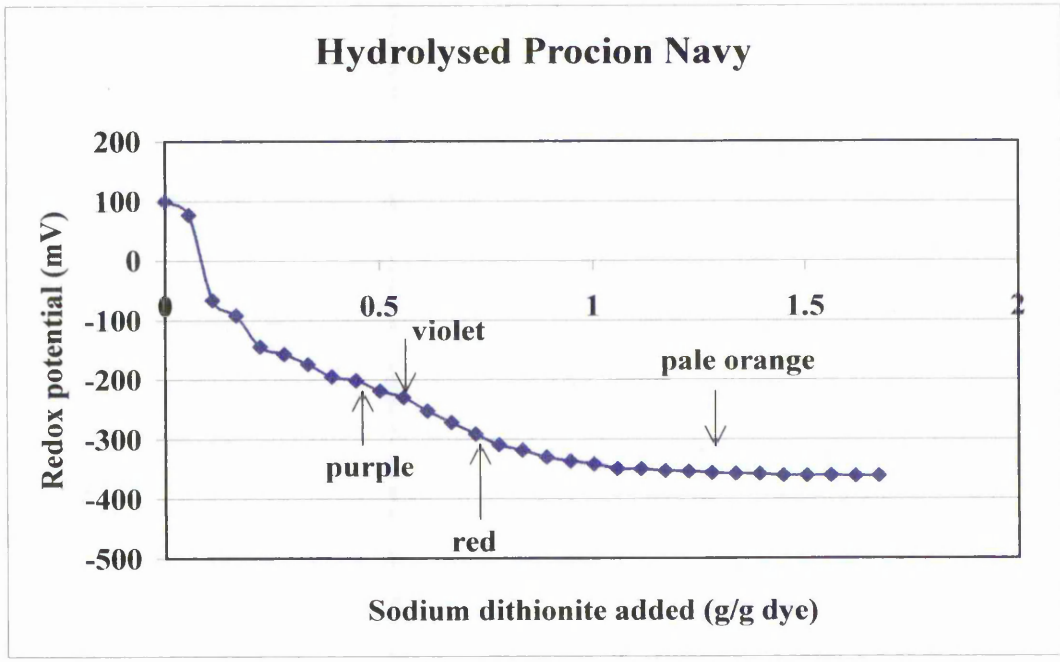
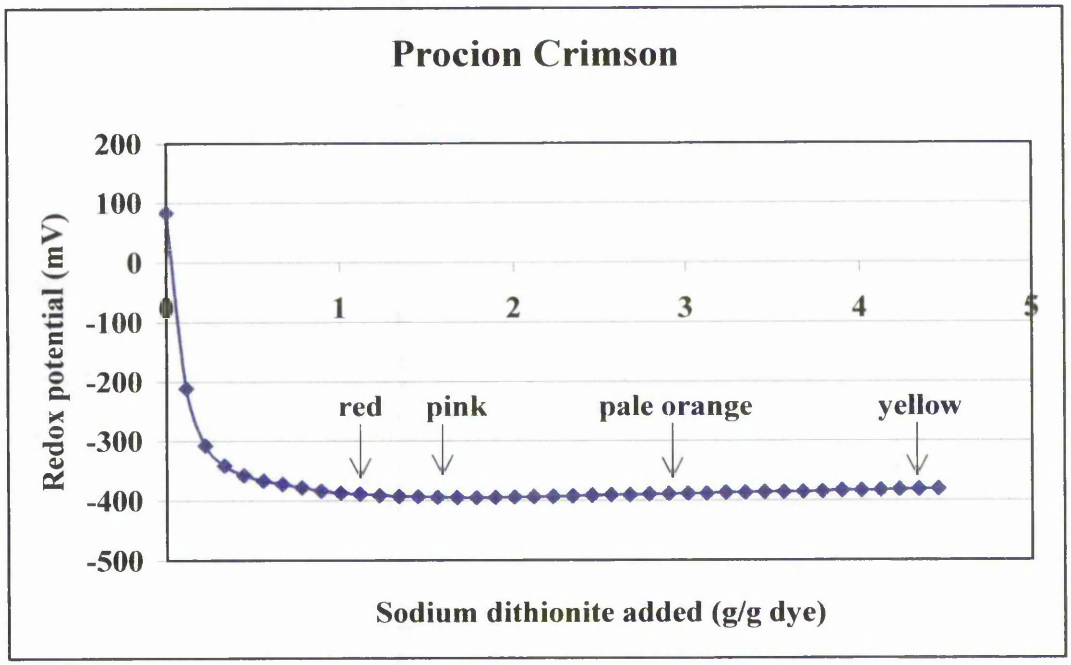


Figure 9. **Colour changes and corresponding redox potential observed for the parent and hydrolysed forms of Procion Navy decolourised by sodium dithionite at pH 3.**



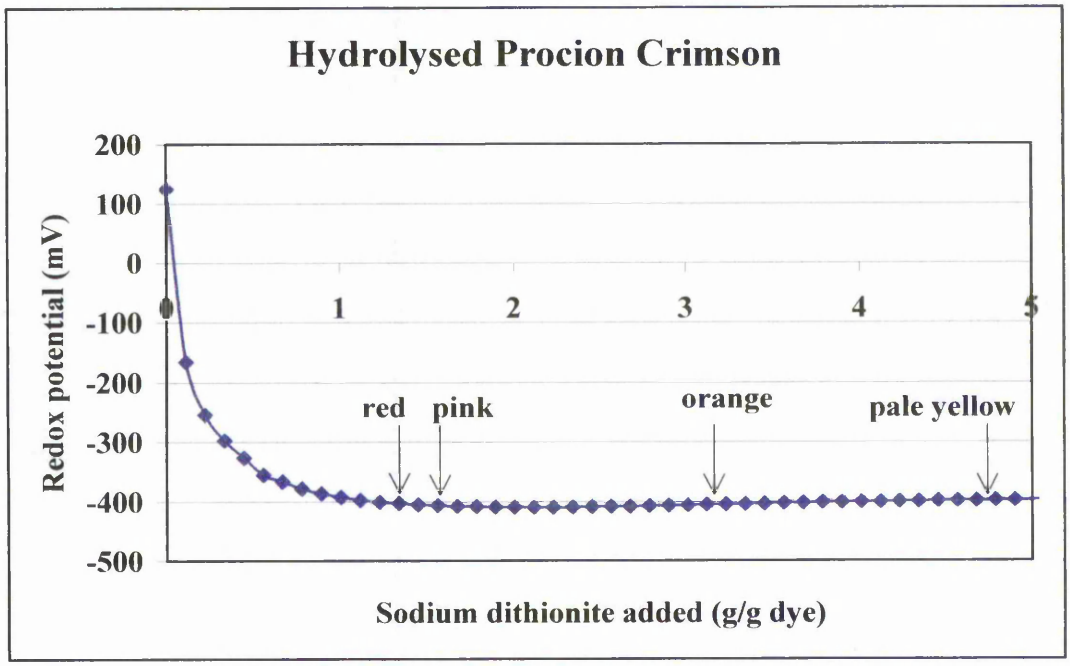
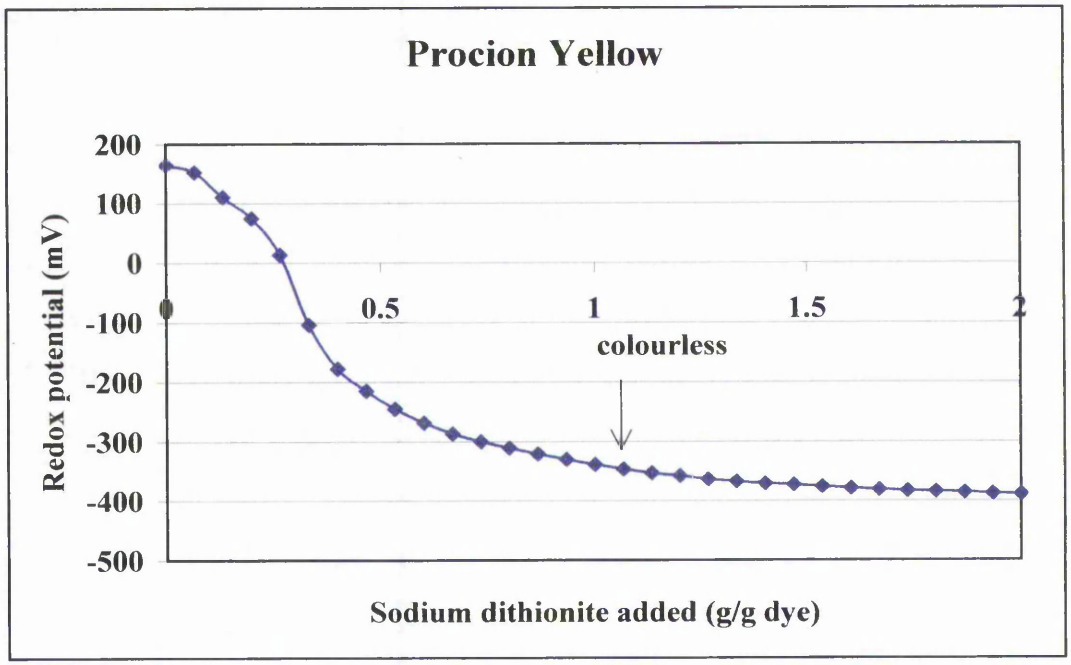


Figure 10. **Colour changes and corresponding redox potential observed for the parent and hydrolysed forms of Procion Crimson decolourised by sodium dithionite at pH 3.**



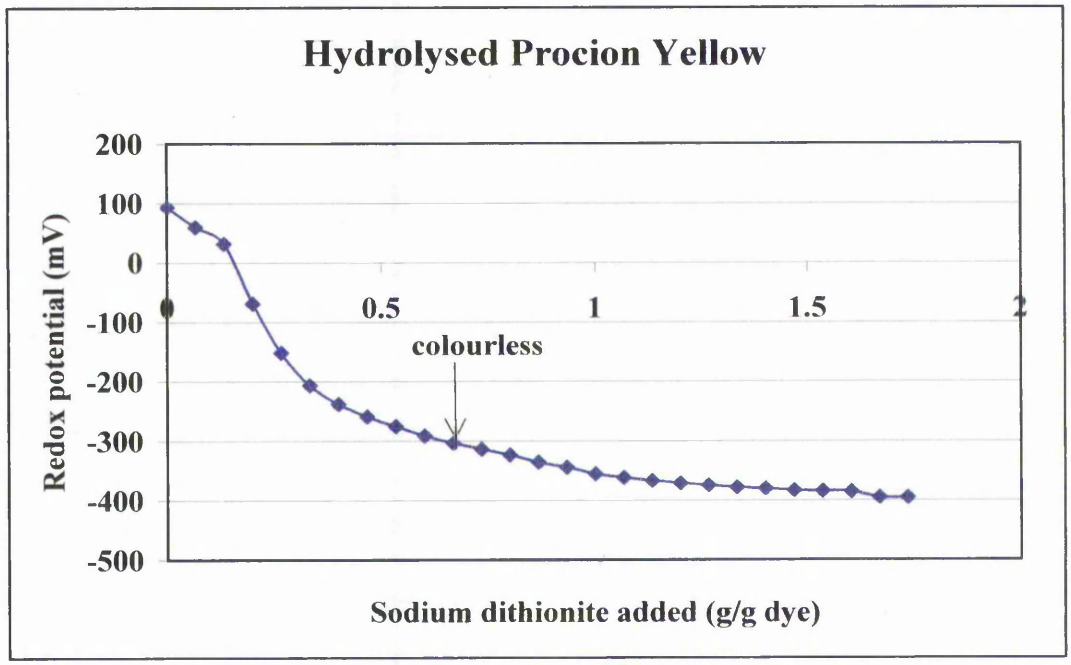


Figure 11. **Colour changes and corresponding redox potential observed for the parent and hydrolysed forms of Procion Yellow decolourised by sodium dithionite at pH 3.**

3.3. Isolation and Identification of Dye Decolourising Bacteria.

Decolourising bacteria were isolated according to Section 2.5. from Welbeck fabric dyers effluent. Initially dye plates contained the five dyes with declared structures (Figure 4). It was found that only Reactive Black and Acid Blue produced a strong enough colour when mixed with the agar medium to enable decolourisation zones around the bacterial growth to be seen easily. The aerobic, anaerobic and microaerophilically incubated plates all showed growth with decolourisation zones being seen mainly on the Reactive Black dye plates. Colonies producing decolourisation on the Acid Blue plates showed various levels of purple/blue discolouration. Some colonies were present that appeared dark blue in colour. It was thought that the predominant mechanism of dye removal from the agar was adsorption rather than decolourisation. The use of Acid Blue plates was discontinued. Colonies producing decolourisation on the Reactive Black plates however showed no accumulation of dye with the clearest zones being seen on the anaerobically incubated plates. Typical counts for dye decolourisers were 3×10^6 cfu/ml effluent for the aerobic plates and 2×10^2 cfu/ml effluent for the anaerobic plates. Figure 12 shows decolourising colonies on Reactive Black and Acid Blue plates incubated anaerobically. Decolourising colonies were streaked to purity on Reactive Black plates as detailed in Section 2.5. Once pure the isolates were identified according to Section 2.6.

3.3.1. Aerobes.

Eleven strains were purified from the aerobically incubated Reactive Black dye plates. Isolates were Gram stained, examined for motility and reaction to the oxidase test. All isolates were Gram negative rods and were positive to the oxidase test. Four strains exhibited motility. Two of the isolates displayed a red pigmentation and hence reaction to the oxidase reaction was difficult to determine. Inconclusive identity results were obtained from the API tests for these two isolates. The remainder of the isolates were identified as follows:

Shewanella putrefaciens (four strains)
Aeromonas masoucida
Pseudomonas capacia
Pseudomonas vesicularis
Pseudomonas chloraphis
Sphingomonas paucimobilis

During the purification and identification process a number of isolates became difficult to culture and/or lost the ability to decolourise Reactive Black dye plates. Work with these strains was therefore discontinued. It was thought possible that the media used for isolation and identification (TSA) was too nutrient rich for these strains and/or an essential requirement for growth may have been present in the original textile effluent which was absent in the laboratory media. Media used for the remaining isolates was switched to NA. The two strains showing pigmentation were also abandoned as difficulty in observing decolourisation with these isolates was anticipated due to their inherent colour. Of the remaining four *Shewanella putrefaciens* strains two (designated V1 and V2) were chosen which displayed the strongest growth for further study.

3.3.2. Anaerobes.

Twelve strains were purified from the anaerobically incubated Reactive Black dye plates. Isolates were Gram stained and spore stained. Reproducible Gram staining was difficult to achieve and varied with culture age. Immersion oil used for viewing microscope slides on the 100X magnification was found to partially de-stain the stained cells giving them a Gram negative appearance. Staining of 12h cultures and viewing through a coverslip revealed that all strains were Gram positive rods. All isolates contained spores which were circular, terminal and distended the cell. None exhibited motility. The 12 strains divided into two categories with respect to colony morphology. Six strains showed convex, circular colonies with entire edges. All were positive to the catalase test and produced identical biochemical profiles in the API 20A test strips. These were identified as *Clostridium butyricum /beijerinckii.* The API 20A biochemical identification system was unable to distinguish between *C. butyricum* and *C. beijerinckii* and for simplicity these strains will be referred to as *C. butyricum*. One strain was chosen for further study (designated N1). The other six strains displayed irregular colonies with undulate edges and were negative to the catalase test. Five of the six were identified as *Clostridium butyricum /beijerinckii* (these will again be referred to as *C. butyricum*). Within the five, two different biochemical profiles were observed and one of each were chosen for further studies (designated F3 and F5), together with the remaining sixth isolate which was identified as *Clostridium inocuum* (designated F6).

Reactive Black **Acid Blue**

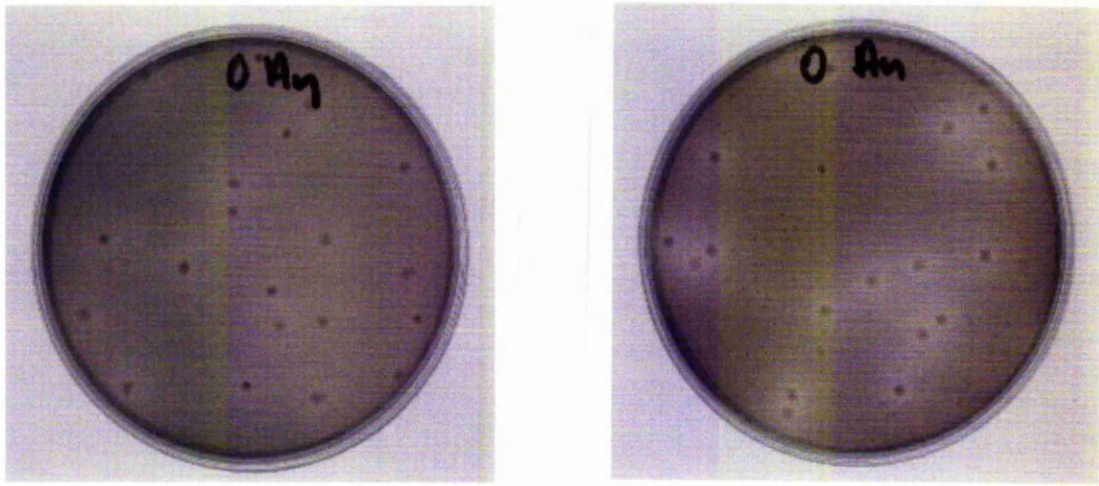


Figure 12. Decolourising colonies from Welbeck Fabric Dyers effluent on Reactive Black and Acid Blue NA plates.

3.4. Decolourisation screen.

3.4.1. Range of dyes decolourised by *E. faecalis* and selected isolates.

The range of dyes decolourised by the selected six isolates was investigated according to Section 2.7.1. Dye plates were prepared using all 18 representative dyes as in Section 2.4.4. The laboratory strain *E. faecalis* , known to produce azo fission, was included in the screening study for comparison purposes. Results are given in Table 19. Figure 13 shows selected plates from the screening study.

Observations

The only dye decolourised by all strains was Reactive Black. One dye, Dispersol Blue, was not visually decolourised by any of the strains. This dye however was very pale in colour making visual observation of decolourisation zones difficult. Where this problem was encountered the dyes in question are marked with an asterix in Table 19. In general the environmental isolates decolourised a wider range of dyes than *E. faecalis* with the decolourisation zones more defined and easier to see on the anaerobic culture plates. Two dyes, Procion Crimson and Solophenyl Black,

were decolourised by all six isolates but not *E. faecalis*. Accumulation of dyes by several strains was observed. For the anaerobes, (N1, F3, F5 and F6), accumulation was seen for five dyes: Acid Blue, Indosol Blue, Indosol Rubinole, Indosol Yellow and Solophenyl Navy. With the exception of Acid Blue all of these dyes belong to the direct dyeing class suggesting that adsorption may be a function of dye type. Similar accumulation of these dyes was observed with *E. faecalis* although some adsorption was also seen for Erionyl yellow with this strain. Isolates F3 and F5 both identified as *C. butyricum* although having different biochemical profiles showed almost identical results for decolourisation of the 18 dyes, the only difference being that F5 showed no accumulation of Indosol Yellow. The *S. putrefaciens* V1 and V2 displayed slight differences in decolourising ability but did however produce the same results for 14 of the 18 dyes. Adsorption of three dyes was observed with the two aerobic isolates (V1 and V2): Dispersol Black, Erionyl Yellow and Foron Black. No accumulation of the reactive dyes (Reactive Black and the three Procion dyes) was observed with any strain.

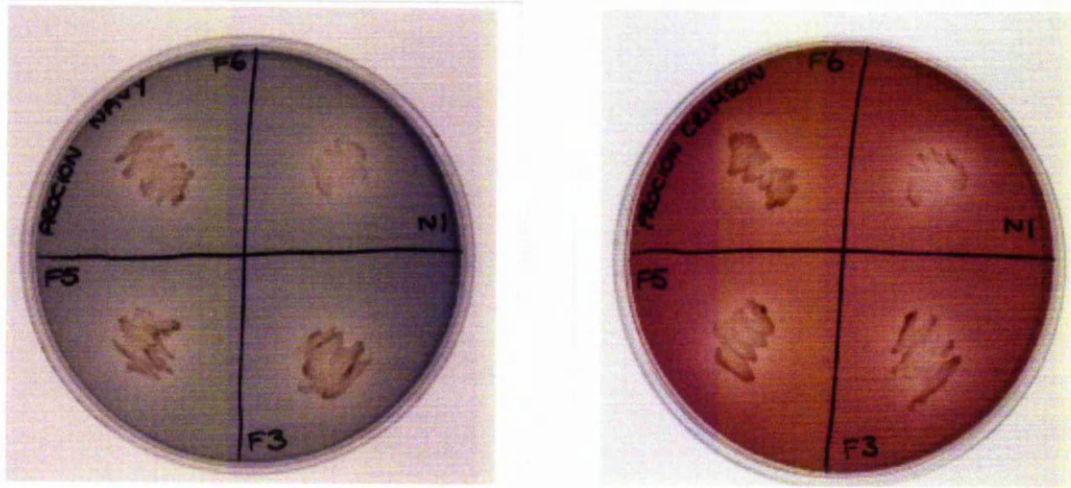


Figure 13. Dye plates from the screening study with selected isolates.

Table 19. Decolourisation of textile dyes by selected isolates and *E. faecalis*.

Dye	*E. faecalis*	V1	V2	N1	F3	F5	F6
Reactive Black	+	+	+	+	+	+	+
Procion Crimson	-	+	+	+	+	+	+
Procion Navy	+/-	-	-	+	+	+	+
Procion Yellow	+/-	-	+	+	+	+	+
Dispersol Black*	-	+(a)	+(a)	+	+	+	+
Dispersol Blue*	-	-	-	-	-	-	-
Dispersol Navy*	+/-	-	+/-	+/-	+	+	+
Erionyl Navy Blue	+(a)	+	+	+(a)	+(a)	+(a)	+(a)
Erionyl Yellow	-(a)	+(a)	+(a)	-	+	+	+
Foron Black*	-	-(a)	-(a)	+	-	-	+/-
Indosol Blue	+/-(a)	+	+	+	+(a)	+(a)	+
Indosol Rubinole*	-(a)	-	+	+(a)	+(a)	+(a)	+(a)
Indosol Yellow	-(a)	+/-	+/-	+(a)	+(a)	+	+(a)
Serilene Rubine	-	+	+	+/-	+	+	+/-
Serilene Yellow*	+/-	-	-	+	+	+	+
Serilene Yellow Brown	-	-	-	-	+	+	+
Solophenyl Black*	-	+	+	+	+	+	+
Solophenyl Navy	+/-(a)	+	+/-	+(a)	+(a)	+(a)	+(a)

KEY

V1 and **V2**	*S. putrefaciens*	+	decolourised zone
N1, **F3** and **F5**	*C. butyricum*	+/-	weak decolourisation zone
F6	*C. inocuum*	-	no decolourisation
*	plates very pale in colour	**(a)**	accumulation of dye

3.4.2. Range of dyes decolourised by selected fungal cultures.

The range of dyes decolourised by selected fungal cultures from the culture collection of the Department of Life Sciences, The Nottingham Trent University, was investigated mainly for comparison of decolourising ability with the six decolourising isolates described in Section 3.3. In an initial screen the following brown and white rot fungal cultures were tested for the ability to decolourise Reactive Black and Acid Blue dye plates: *Poria placenta*, *Coniophora puteana* (formerly *Coniophora cerebella*), *Polystictus versicolor*, *Coriolus versicolor*, *Serpula lacrymanus*, *Poria monticola*, *Lentinus lepideus* and *Gloeophylluro trabeum*. Following 5 days incubation at 25°C clear decolourisation for both dyes was observed for *Polystictus versicolor* and *Coriolus versicolor* only. Weak decolourisation was observed for *Poria placenta* and *Coniophora puteana* for both dyes with some discolouration of the growth on the Acid Blue plates indicating dye accumulation. These four fungal strains were further tested for the ability to decolourise all 18 representative dyes. Dye plates were prepared using all 18 dyes as in Section 2.4.4. Observations were made after 3 and 5 days incubation at 25°C. Results after 5 days are given in Table 20. Figure 14 shows selected plates from the screening study.

Observations

Two dyes were decolourised by all 4 fungal strains namely Reactive Black and Procion Navy, although decolourisation of Reactive Black was weak for *P. placenta.* Acid Blue (Erionyl Navy Blue), Indosol Blue and Indosol Rubinole were decolourised by 3 of the strains with some evidence of bond breakage by the fourth strain in the form of different coloured products observed surrounding the fungal growth on the dye plate. Serilene Yellow was the only dye not to be decolourised by any strain although if weak decolourisation had occurred colour changes for this dye were difficult to see due to the colour of the plate. The efficiency of decolourisation with regards to range of dyes decolourised was highest for *C. versicolor* and *C. puteana* with these strains showing decolourisation of 17 of the 18 dyes tested with clear decolourisation for 12 and 11 and weak decolourisation for 5 and 6 of the remaining dyes respectively.

The lowest decolourisation ability was observed for *P. placenta* with only 4 of the 18 dyes showing clear decolourisation and weak decolourisation being observed for one other only. In view of the time taken for decolourisation of the dyes by the fungal strains (3 days incubation required before decolourised zones were apparent) work on the fungal cultures was discontinued.

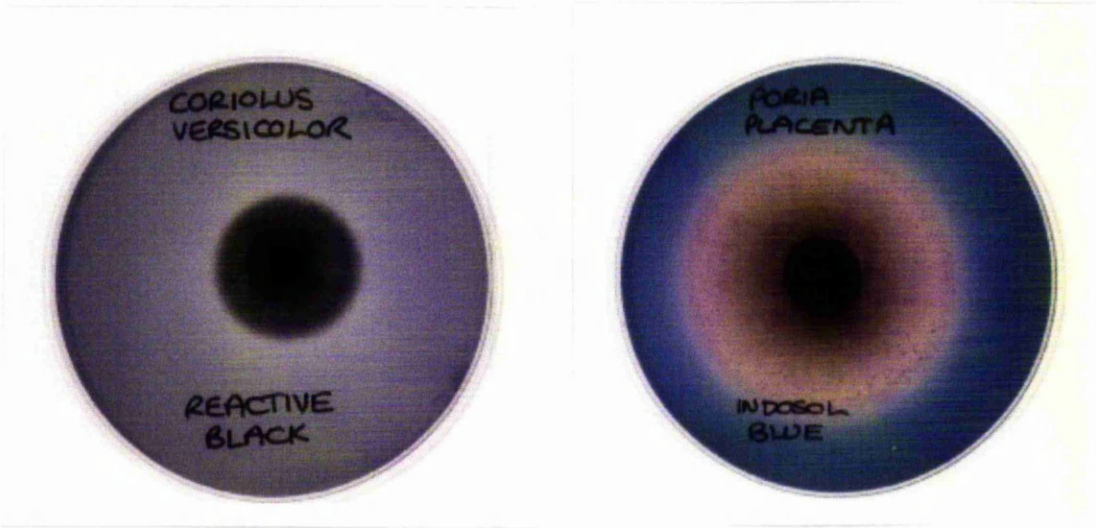


Figure 14. Dye plates from the screening study with selected fungal cultures.

Table 20. Decolourisation of textile dyes by selected fungal cultures.

Dye	*C. versicolor*	*P. placenta*	*P. versicolor*	*C. puteana*
Reactive Black	+	+/-	+	+
Procion Crimson	+	-	+	+
Procion Navy	+	+	+	+
Procion Yellow	+	-	+/-	+
Dispersol Black*	+	-	+/-	+/-
Dispersol Blue*	+	-	-	+/-
Dispersol Navy*	+/-	-	+/-	+/-
Erionyl Navy Blue	+	+(c)	+	+
Erionyl Yellow	+	-	+/-	+/-
Foron Black*	+/-	-	+/-	+
Indosol Blue	+	+(c)	+	+
Indosol Rubinole*	+	+	+(c)	+
Indosol Yellow	+	-	+	+
Serilene Rubine	+/-	-	+/-	+/-
Serilene Yellow*	-	-	-	-
Serilene Yellow Brown	+/-	-	+/-	+/-
Solophenyl Black*	+/-	-	+/-	+
Solophenyl Navy	+	-	+	+

KEY

+	decolourised zone	+/-	weak decolourisation zone
-	no decolourisation		
*	plates very pale in colour	**(c)**	coloured product

3.5. Preliminary decolourisation enzyme assays.

Development of a decolourisation assay concentrated on Reactive Black and *E. faecalis*. It had two main aims:

(a) A reproducible decolourisation assay was required to allow comparison of the specific decolourisation activity of different strains.

(b) A method for production of decolourised material in a clean matrix for further analysis.

Due to this dual requirement it was decided to minimise experimental variables and define assay conditions applicable to all strains. The assay was to consist of washed cells of the strain under test re-suspended in a phosphate buffer (100mM, pH 7) with glucose as electron donor. Using this system variation due to different growth media requirements for different strains could be avoided. Cells could be concentrated or diluted by any amount as required to speed up or slow down reactions to make them measurable. Assuming that the glucose concentration was not limiting the main variable would be the dye under test. Decolourised material produced in the assay would be in a relatively clean matrix, free from complex chemical and organic interference that may have been present had the assay been performed in growth media for example. Thus assay conditions could be kept constant and reproducible for all strains tested and decolourised material could be utilised for further analysis.

3.5.1. Dye concentration.

Dye concentration in the decolourisation assay was limited predominantly by the measurement technique employed. UV/VIS spectrophotometry was the technique of choice but was limited due to the linear measuring range of the instrument. Two options for dye concentration were available:

(a) Dye concentration such that starting absorbances of the assay were in the region of 1AU thus allowing spectrophotometer readings at the λ_{max} of the dye under test to be read directly by measurement of the whole assay absorbance.

(b) Dye concentration such that starting absorbances in the assay would be too high to be read directly in the spectrophotometer but from which aliquots could be withdrawn at timed intervals, centrifuged and the supernatant absorbances measured with dilution as necessary to bring readings on scale.

For measurement of decolourisation rate it was decided to use option (a). This would allow automatic data collection by the Labtech Aquire package connected to the spectrophotometer hence the number of readings taken being less limited and easily varied as necessary in relation to the reaction rate. It would also avoid disruption and possible aeration of the assay and changes in volume due to sampling. Option (b) was considered to be a more practical way of preparing larger quantities of decolourised material for further analysis.

Dye concentration was defined in terms of percentages on a weight per unit volume basis as the molecular weights for the three Procion dyes were unknown. For Reactive Black a concentration of 1% w/v equated to 10mM. Calibration curves for dye concentration versus absorbance at λ_{max} were prepared to allow direct conversion of spectrophotometer readings to dye concentration remaining in the assay and hence the rate of dye decolourisation by the strain under test. Calibration curves for the dyes tested are given in Appendix 2. For measurement of decolourisation rate a typical starting concentration giving an absorbance of 1AU for Reactive Black was 0.0025% w/v (0.125mg in 5ml assay). For preparation of decolourised material for further analysis higher dye concentrations were used. All measurements of dye concentration were based on an assumption that the dyes were 100% pure, however in practice this was deemed unlikely as purity varies between dyes and may even vary between batches of the same dye. The Reactive Black obtained from Aldrich was estimated to be approximately 55% whereas the purity of Procion Crimson was estimated to be >75% and the purities of Procion Navy and Procion Yellow were unknown. For measurement of decolourisation rate the dye concentration in the assays was varied according to starting absorbance at λ_{max} for the dye under test. This produced a visually equivalent strength of all dyes based on the composition of the commercially available preparations although actual concentrations may have been different.

3.5.2. Glucose concentration.

(a) Production of decolourised material.

The effect of glucose concentration on decolourisation was investigated using *E. faecalis* and Reactive Black. Initial assays utilised glucose concentrations of 1%, 0.5%, 0.2%, 0.1% and 0.05% to determine any visual differences in decolourisation. Assays were prepared according to Section 2.8.1. Dye concentration was 0.1% (w/v). Observations were as follows:

Colour of assay tubes after 24h incubation.

0.5-1.0%	Green/yellow opaque solution with brown layer at surface.
0.2%	Dark grey/purple with brown layer at top. Blue ring seen around top of solution.
0.05-0.1%	Light brown with blue ring around top of solution.

Samples were withdrawn from each tube by automatic pipette and centrifuged (1ml Eppendorf tube, microfuge 5min 11,600 x g). Following centrifugation, supernatants were visually assessed for decolourisation. Assays with the two lowest glucose concentrations appeared to be decolourised but when samples were centrifuged to remove the cells the supernatants were blue. It was thought that this might be due to a redox effect. Under the low redox conditions produced within the assay the dye molecule may be forced into the partially reduced hydrazo form which reverts back when the solution is disturbed. This would explain the blue ring seen around the surface of the assay tube where the solution is exposed to oxygen. The 0.2% assay supernatant was purple implying that the dye may be partially reduced either by only some molecules having complete azo reduction or by asymmetric azo bond fission within a molecule. Cleavage of one of the azo bonds in a dye molecule would not produce complete decolourisation but would change the colour of the dye. The 0.5% and 1.0% glucose assay samples remained green/yellow after centrifugation. The brown layer observed at the surface of the assays was thought to be due to oxidation of the aromatic amines produced from decolourisation. At glucose concentrations lower than 0.5% therefore it was observed that decolourisation of Reactive Black by *E. faecalis* was not complete. As the same visual decolourisation was observed for 0.5% and 1.0% glucose it was decided to use 1.0% to ensure that the concentration in the assays would not become limiting.

The assays were terminated upon visual decolourisation (with a check absorbance measurement at λ_{max} of the dye under test to ensure azo bond cleavage had occurred), the cells removed and supernatants stored in glass bottles with minimal headspace to avoid oxidation of the amines.

It was intended that all assays be performed under the same conditions for all dyes tested. In order to check that this glucose level would be appropriate a series of assays were set with *E. faecalis* and the 3 Procion dyes. Procion Navy and Procion Yellow both visually decolourised although no change in colour was observed for Procion Crimson. A series of assays were set for *E. faecalis* and Procion Crimson with the range of glucose concentrations detailed above.

Observations were as follows:

0.2-1.0%	No decolourisation observed. A gelatinous mass of cells at the bottom of the tube seen.
0.05-0.1%	Pale orange/brown solution.

Decolourisation was observed at 0.1% and 0.05% glucose whereas no decolourisation was observed with 1.0%, 0.5% or 0.2% glucose. These results indicate that the higher glucose concentrations were inhibitory to decolourisation of Procion Crimson by *E. faecalis*.

For preparation of decolourised material it was decided to use 1.0% glucose for Reactive Black, Procion Navy and Procion Yellow for the reasons given above. For Procion Crimson it was recognised that for production of decolourised material a glucose concentration of 0.1% was required.

(b) Effect on initial decolourisation rate.

The level of glucose for measurement of initial decolourisation rate was investigated using *E. faecalis,* Reactive Black and Procion Crimson. Assays for rate measurements were set according to Section 2.8.1. Glucose concentrations used were 1.0%, 0.1% and 0%. Assays were performed in triplicate and rates were determined from the initial linear slope of the curve. Initial decolourisation rates were 22.95 and 21.35 mg dye/h/g dry cell wt for the 1.0% and 0.1% glucose assays respectively. No significant difference in initial rate was observed between the 1.0% and 0.1% glucose concentrations for Reactive Black at the 95% confidence level

(see Appendix 3). Negligible decolourisation rates (initial rate only) were observed for Procion Crimson at all glucose concentrations tested over the assay period. As no significant difference was observed between 1.0% and 0.1% glucose for Reactive Black it was decided to standardise all assays for initial decolourisation rate measurements on 1.0% glucose.

3.5.3. Choice of isolate for detailed decolourisation studies.

Twelve anaerobic strains and 11 aerobic strains were isolated and identified from Welbeck fabric dyers effluent capable of decolourising a range of dyes, all of which decolourised the model dye Reactive Black. This number was reduced to six as discussed in Section 3.3. according to biochemical profile and viability (growth and decolourisation). It was deemed impractical to study all six strains in depth and so it was decided to choose a single isolate for further study to compare with *E. faecalis*. Difficulties were encountered with the two *Shewanella* strains (V1 and V2) when producing large volumes of culture required for the thick cell suspension decolourisation assays. Compact pellets were not formed during centrifugation making decanting of the supernatant difficult without losing cells to waste. The problem was not alleviated by prolonged spin times or by washing the cells in buffer. It was thought that the cell surfaces may be charged and hence would resist compaction. Storage of the cultures on agar slopes, beads and in glycerol broths for longer than two months resulted in strains being difficult to revive. When decolourisation assays were prepared similar redox effects to those observed with *E. faecalis* and the lower glucose concentrations (Section 3.5.2.) were observed. The assays appeared to be visually decolourised but when samples were removed and centrifuged the sample supernatants were blue. For these reasons study of the two *Shewanella* strains (V1 and V2) was discontinued. The anaerobes (N1, F3, F5 and F6) did not suffer any of these problems and produced more defined clearer decolourisation on dye plates than the *Shewanella* strains in the screening study. All four isolates were similar with respect to viability with storage and range of dyes decolourised. No real differences were observed between the anaerobic isolates except that strain F5 (*C. butyricum*) exhibited denser growth in broth culture. For this reason it was decided to concentrate on strain F5.

3.5.4. Variation in rate between batches of cells.

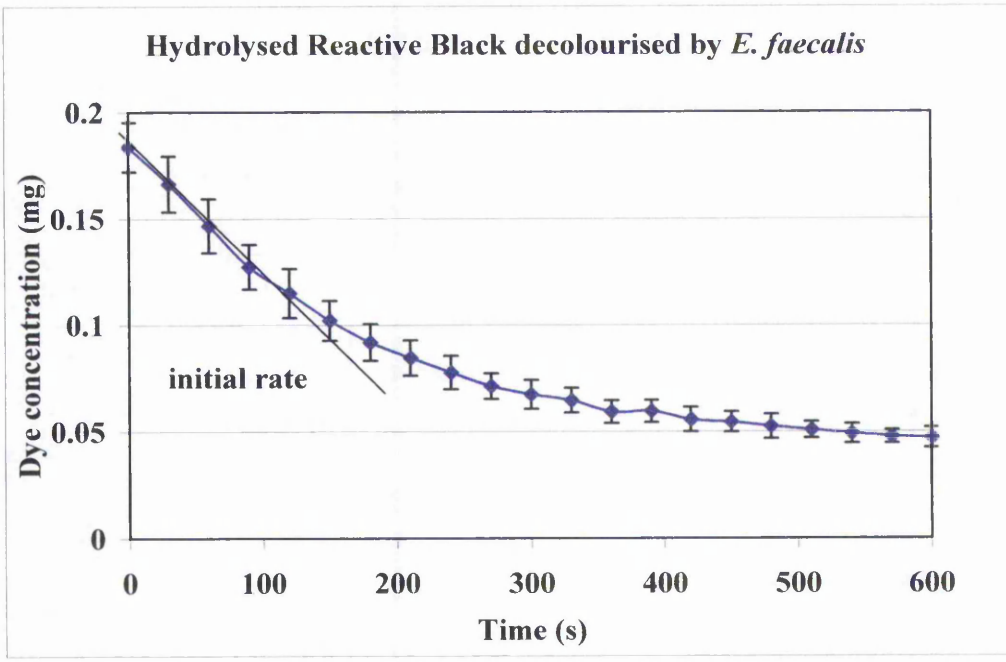
The thick cell suspensions used for the decolourisation enzyme assays were used within 90min of preparation. As one batch of cells could not be used for measurement of all rates and assays would be performed over a period of time it was essential to determine that results obtained using different batches of cells gave comparable results. To achieve this assays were performed on three separate days for *E. faecalis* and *C. butyricum* (F5) decolourising the parent and hydrolysed forms of Reactive Black. Culture age was kept constant and all assays were performed in triplicate prepared according to Section 2.8. Example curves are given for hydrolysed Reactive Black and the two strains in Figure 15. Rates were calculated from the initial linear portion of the graph. The results obtained for the triplicate assays on the three different days were compared by analysis of variance using the Microsoft Excel 97 spreadsheet package. Calculations are given in Appendix 3. All calculated F values were < critical F at the 95% confidence level. It was assumed therefore that for both strains there was no significant difference in decolourisation rate between cultures and results from different batches of cells would be comparable.

3.5.5. Linearity of decolourisation rate with cell density.

Confirmation that decolourisation rate was linear with cell density was required for two main reasons:

(a) If decolourisation occurred via biologically induced azo bond breakage, a higher concentration of cells in an assay would be expected to increase the decolourisation rate and vice versa. If this was not observed the decolourisation reaction may be proceeding via a different mechanism.

(b) In order to obtain a measurable reaction rate it may be necessary to concentrate or dilute cell suspensions to speed up or slow down a reaction as required. All rates measured need to be corrected for variation in cell density to allow comparison of results on an equal basis.



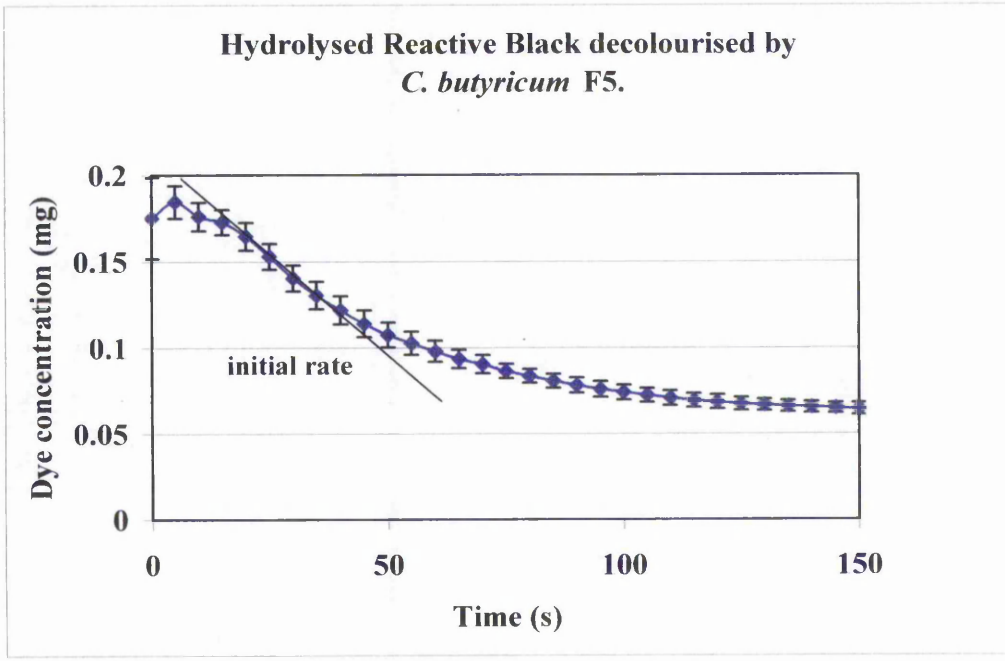


Figure 15. **Decolourisation of hydrolysed Reactive Black by *E. faecalis* and *C. butyricum* F5.**

A series of decolourisation assays were prepared with *E. faecalis* and *C. butyricum* (F5) decolourising the parent and hydrolysed forms of Reactive Black according to Section 2.8. Cell density was varied by diluting the initial thick cell suspension prepared and was determined as dry cell weight according to Section 2.8.4.

From the example given in Figure 15 for *E. faecalis* and hydrolysed Reactive Black it can be seen that the decolourisation reaction has two phases, the first being an initial linear reduction in dye concentration followed by a second phase exponential type decay. The exponential decay curve indicates that the decolourisation reaction is proceeding to an end point. It is not known whether the reaction is becoming limited due to glucose exhaustion or an inhibition of activity by accumulation of decolourisation end products. The form of the curves suggest the decolourisation reaction follows first order kinetics. If this were the case a plot of ln[dye concentration] versus time should produce a straight line of the form:

$\ln[A] = -kt - \ln[A_0]$

where A = dye concentration at time t

A_0 = initial dye concentration

t = time

and k = rate constant

Re-plotting the rate data as ln[A] versus time and calculation of the slope yielded values for the rate constant k. A plot of rate constant against dry cell weight for the parent and hydrolysed form of the dye produced straight lines (Figure 16) with correlation coefficients R^2=0.99 for Reactive Black and R^2=0.97 for hydrolysed Reactive Black. It was assumed that decolourisation rate was linear with cell density for *E. faecalis*.

From the example decolourisation curve for *C. butyricum* (F5) and hydrolysed Reactive Black (Figure 15) it can be seen that the decolourisation reaction with *C. butyricum* has three phases. The linear reduction in dye concentration followed by the exponential type decay are both present as with *E. faecalis*. In addition to these an initial lag phase was observed. The time of the lag phase before decolourisation commences varied with cell density. In the assays to determine linearity with cell density at the highest cell density (34mg dry cell weight) the lag was approximately 1-1.5min increasing to 2min for the 17mg dry cell weight assay and 10min for the

lowest density used (8.5mg dry cell weight). In the example given in Figure 15 the cell density for this assay was 79mg dry cell weight and it can be seen that at this cell concentration the initial lag phase was much reduced being approximately 15s. It is thought that this lag phase was due to a definitive period of time required for the production of reducing equivalents by *C. butyricum* from the glucose in the assay. This would explain the increase in lag time with reduction in cell density.

Calculation of rate constants from plots of ln[A] versus time was performed as for *E. faecalis* and plots against dry cell weight again produced straight lines as for *E. faecalis*. Decolourisation rate was assumed to be linear with cell density for *C. butyricum* (F5). Correlation coefficients obtained were R^2=0.93 for Reactive Black and R^2=0.96 for hydrolysed Reactive Black.

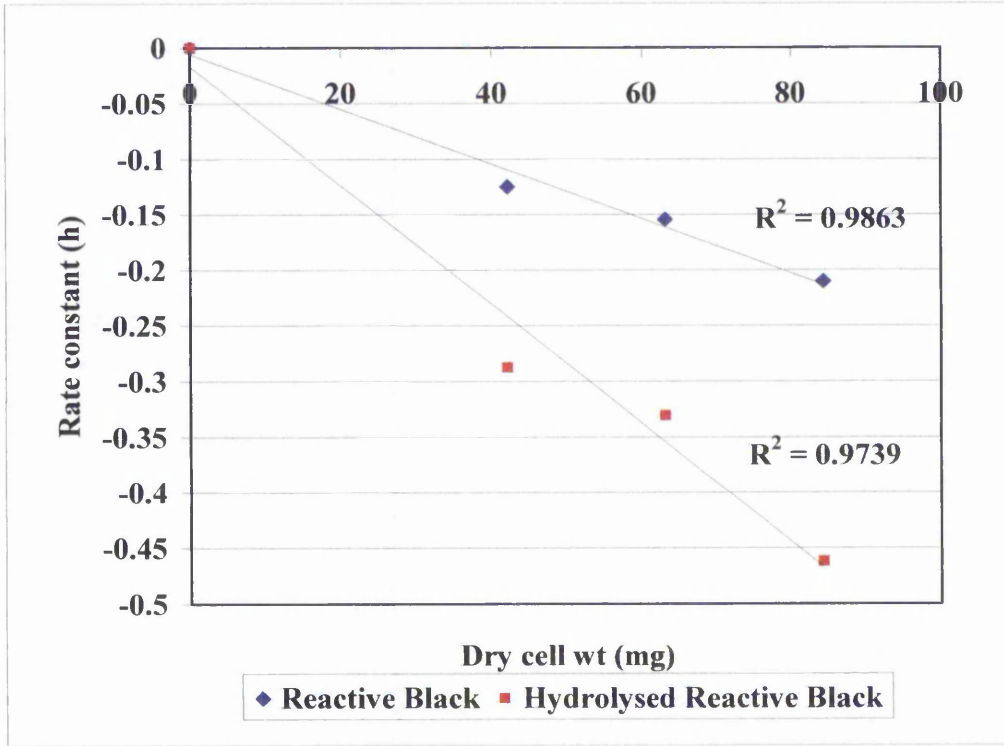


Figure 16. Variation in rate constant with dry cell weight for *E. faecalis*.

3.6. Decolourisation rates with whole cells.

Decolourisation assays were prepared according to Section 2.8. For comparative purposes assays were prepared with and without glucose. Decolourisation rates were measured for *E. faecalis* and *C. butyricum* (F5) with Reactive Black and the three Procion dyes (both parent and hydrolysed forms). Dye concentrations were such that starting absorbances were approximately 1AU at the λ_{max} of the dye under test. Rates were calculated from the initial linear slope of the dye concentration-time curves and expressed as mg dye/h/g dry cell weight. Results are given in Tables 21 and 22. For Reactive Black and hydrolysed Reactive Black with glucose the number of experimental batches equalled 9, for all other dyes the number of batches was equal to 3.

Observations

(a) *E. faecalis.*

The decolourisation rates for Reactive Black (both parent and hydrolysed) were higher than for all 3 Procion dyes with and without glucose. The initial decolourisation rate observed for hydrolysed Reactive Black was faster than the initial rate measured for the parent dye, in the presence of glucose being more than twice the magnitude (51.7 and 21.2 mg dye/h/g dry cell wt respectively). If decolourisation was an intra-cellular reaction then the difference could be due to variation in the degree of sulphonation of the two forms of the dye. The parent dye has four sulphonate groups per molecule (Figure 4). Upon hydrolysis two of these groups will be lost from the two vinyl sulphone side chains. As the sulphonate groups confer a high degree of water solubility to the dye structure their presence may inhibit passage of a dye molecule through the cell membrane of *E. faecalis*. The hydrolysed dye, being less sulphonated, may pass through the cell membrane faster and hence be decolourised at a faster rate. In the absence of glucose the difference in rate for the parent and hydrolysed forms of Reactive Black was also observed but was less pronounced (8.9 and 12.5 mg dye/h/g dry cell wt respectively). In the absence of glucose production of reducing equivalents for dye decolourisation relies on residual substrates within the cell which may not be as readily available as an external energy source which may explain why the rates without glucose are

lower. A distinction in rate between the parent and hydrolysed forms of the Procion dyes was not as clear. The rate for the parent Procion Navy was only slightly higher at 4.1 mg dye/h/g dry cell wt than for the hydrolysed form at 3.5 mg dye/h/g dry cell wt with no significant differences observed with and without glucose. For Procion Yellow slight differences were observed with and without glucose the initial rates being 6.1 and 3.8 mg dye/h/g dry cell wt respectively for the parent and 5.7 and 4.2 mg dye/h/g dry cell wt respectively for the hydrolysed form. The parent dye initial rate was higher than the hydrolysed dye initial rate only in the presence of glucose. For the two forms of Procion Crimson measured initial decolourisation rates were the lowest for all dyes tested and no significant difference was observed with and without glucose (0.8 and 0.9 mg dye/h/g dry cell wt respectively). It is not known at this stage whether the overall lower rates for the three Procions are due to a slower passage across the cell membrane due to the presence of sulphonate groups or whether the dyes have structures such that steric effects may hinder access to the azo bonds. If decolourisation occurs via azo bond breakage the number of azo bonds in each dye molecule may affect the rate to an extent. As the number of azo bonds per molecule for the Procion dyes is not known the effect of this variable on the rates measured cannot be assessed.

(b) *C. butyricum* (F5)

For *C. butyricum* (F5) the decolourisation rate for Reactive Black and hydrolysed Reactive Black were also higher than for the Procion dyes as observed with *E. faecalis*. The rate for the hydrolysed form of Reactive Black was again much higher than the parent dye in the presence of glucose (102.7 and 59.0 mg dye/h/g dry cell wt respectively) possibly due to the mechanism discussed above. Differences in rates with and without glucose were observed for the three Procions but again no clear distinction was present between the parent and hydrolysed forms. In general the decolourisation rates with *C. butyricum* (F5) were higher than with *E. faecalis* although these cannot be compared directly due to the assays being performed at different temperatures (30°C for *C. butyricum* and 37°C for *E. faecalis*). The overall trend appeared to be the same as for *E. faecalis* with the decolourisation rates descending in the order Reactive Black>Procion Yellow>Procion Navy>Procion

Crimson. Difference in rates between parent and hydrolysed forms was only evident with Reactive Black.

Table 21. Initial decolourisation rates for *E. faecalis*.

Dye	Azoreductase (mg dye/h/g dry cell wt)			
	+ glucose		- glucose	
	Mean	**SD**	**Mean**	**SD**
Reactive Black	**21.2**	2.4	**8.9**	2.6
Hydrolysed Reactive Black	**51.7**	5.8	**12.5**	4.2
Procion Navy	**4.1**	0.1	**4.1**	0.5
Hydrolysed Procion Navy	**3.5**	0.2	**3.4**	0.1
Procion Yellow	**6.1**	0.3	**3.8**	0.2
Hydrolysed Procion Yellow	**5.7**	0.5	**4.2**	0.1
Procion Crimson	**0.8**	0.2	**0.9**	0.1
Hydrolysed Procion Crimson	**0.8**	0.1	**0.9**	0.1

For Reactive Black and hydrolysed Reactive Black with glucose the number of measurements equalled 9, all other values quoted represent the mean of 3 determinations.

Table 22. Initial decolourisation rates for *C. butyricum* (F5).

Dye	Azoreductase (mg dye/h/g dry cell wt)			
	+ glucose		- glucose	
	Mean	**SD**	**Mean**	**SD**
Reactive Black	**59.0**	7.5	**31.7**	3.0
Hydrolysed Reactive Black	**102.7**	15.1	**49.9**	6.6
Procion Navy	**7.5**	0.5	**3.3**	0.1
Hydrolysed Procion Navy	**5.4**	1.3	**3.5**	0.4
Procion Yellow	**7.9**	1.1	**6.7**	0.1
Hydrolysed Procion Yellow	**8.5**	1.2	**5.9**	0.2
Procion Crimson	**2.9**	0.3	**1.1**	0.1
Hydrolysed Procion Crimson	**2.3**	0.4	**1.4**	0.1

For Reactive Black and hydrolysed Reactive Black with glucose the number of measurements equalled 9, all other values quoted represent the mean of 3 determinations.

3.7. Decolourisation rates with cell free extracts.

Decolourisation assays with cell free extracts were prepared according to Section 2.10. Decolourisation rates were measured for *E. faecalis* and *C. butyricum* (F5) with Reactive Black and the three Procion dyes (parent and hydrolysed forms). Dye concentration was such that starting absorbances at λ_{max} for the dye under test was approximately 1AU. Rates were calculated from the initial linear slope of the dye concentration-time curves and expressed as mg dye/h/g protein. For every batch of cell free extract prepared control assays were performed with Sunset Yellow to determine variation in decolourisation activity. This was deemed necessary as it was thought possible that localised heating leading to denaturation of enzymes may occur during preparation that would not be apparent from a measurement of protein content. The decolourisation rates for the Sunset Yellow check assays were compared using an analysis of variance (Appendix 3). No significant difference between cell free extracts was observed with regards to decolourisation rate for Sunset Yellow for *E. faecalis* or *C. butyricum* (F5). It was assumed therefore that decolourisation rates for Reactive Black and the Procion dyes measured with different batches of cell extract could be compared directly for each strain and are given in Table 23. An example decolourisation curve for a cell free extract of *E. faecalis* decolourising hydrolysed Reactive Black is given in Figure 17.

Observations

(a) *E. faecalis*

The rate of decolourisation for the Sunset Yellow check assay was much higher than the measured rates for any of the textile dyes tested (521.1 mg dye/h/g protein). Sunset Yellow, being a food dye with a simpler structure than the textile dyes was known to have a single azo bond which may explain why the observed decolourisation rate for this dye was much higher than the textile dyes tested. For Reactive Black, the measured rates were again higher than for the Procion dyes as was observed with the whole cell experiments (72.7 and 54.1 mg dye/h/g protein for parent and hydrolysed dye respectively). In cell free extract however it was the parent dye that had the higher decolourisation rate and not the hydrolysed form. This reinforces the theory that the greater number of sulphonate groups on the parent

dye structure inhibits transport across the cell membrane and reduces the measured rate. In a cell free assay this rate-limiting factor is removed thus allowing a better comparison of the measured rates for the two forms of the dye. For the Procion dye rates a similar pattern is observed as for the whole cell assays in that measured rates descended in the order Procion Yellow>Procion Navy>Procion Crimson. A difference between parent and hydrolysed forms is apparent for Procion Navy and Procion Crimson, the measured rates for the hydrolysed forms of the Crimson and Navy dyes being approximately double those for the parent forms (22.7 and 9.7 mg dye/h/g protein for Procion Navy and 8.2 and 4.5 mg dye/h/g protein for Procion Crimson). No distinct difference in rate was observed for the parent and hydrolysed forms of Procion Yellow.

It is recognised that the whole cell rates quoted in Section 3.6. have been calculated on a dry cell weight basis whereas the cell free extract results have been calculated on the protein content of the extracts and as such cannot be compared directly. It is estimated however that proteins comprise 50% of total dry cell mass (Green, 1991). Based on this assumption if the rate values obtained for the whole cell measurements given in Table 21 are multiplied by a factor of 2 an estimation of the whole cell rate based on protein can be obtained. Estimation of results in this way shows that rates for the Procion dyes are generally higher in cell free assays than in whole cell assays.

For Reactive Black a difference is apparent for the two forms of the dye. For the parent form of the dye the rate is increased in cell free measurement (from 42.4 (estimated) to 72.7 mg dye/h/g protein). For the hydrolysed form of the dye the rate is much reduced (from 108.2 (estimated) to 54.1 mg dye/h/g protein). It is not known at this stage whether this may be due to the hydrolysed dye having a toxic effect on the cell free extract and hence reducing the decolourisation rate.

(b) *C. butyricum* (F5)

The rate of decolourisation for the Sunset Yellow check assay was considerably lower with *C. butyricum* (F5) (12.3 mg dye/h/g protein) than with *E. faecalis* (521.1mg dye/h/g protein). It is thought unlikely that the measurement could have missed the reaction if the rate was actually faster as the initial dye concentration measured at the start of the assay was as expected in terms of the amount of dye added. As the Sunset Yellow is a simpler dye structure with one azo bond it may be

expected that the decolourisation for this dye would proceed at faster rate. This however is not the case and may indicate that *E. faecalis* had a greater specificity towards this dye than *C. butyricum* (F5). The main difference in observed rates was that the measured rates for Reactive Black are of the same order as for the Procion dyes. The highest rate measured was for Procion Yellow (26.8 mg dye/h/g protein for the parent and 41.8 mg dye/h/g protein for the hydrolysed dye). With *C. butyricum* (F5) the rates for the hydrolysed dyes were higher than for the parent forms for all dyes tested.

Table 23. Initial decolourisation rates for *E. faecalis* and *C. butyricum* (F5) cell free extracts.

Dye	Azoreductase (mg dye/h/g protein)			
	E. faecalis		*C. butyricum*	
	Mean	**SD**	**Mean**	**SD**
Reactive Black	**72.7**	4.7	**13.9**	1.1
Hydrolysed Reactive Black	**54.1**	6.8	**16.5**	1.8
Procion Navy	**9.7**	0.2	**10.1**	0
Hydrolysed Procion Navy	**22.7**	0.1	**11.2**	2.9
Procion Yellow	**17.6**	3.0	**26.8**	0.1
Hydrolysed Procion Yellow	**15.4**	2.1	**41.8**	9.1
Procion Crimson	**4.5**	0.5	**4.3**	0.4
Hydrolysed Procion Crimson	**8.2**	1.1	**11.0**	3.4
Sunset Yellow	**521.1**	141.3	**12.3**	3.5

For *E. faecalis* and Sunset Yellow number of measurements equalled 18, all other values quoted are the mean of 3 determinations. For *C. butyricum* (F5) and Sunset Yellow number of measurements equalled 16, all other values quoted are the mean of 2 determinations.

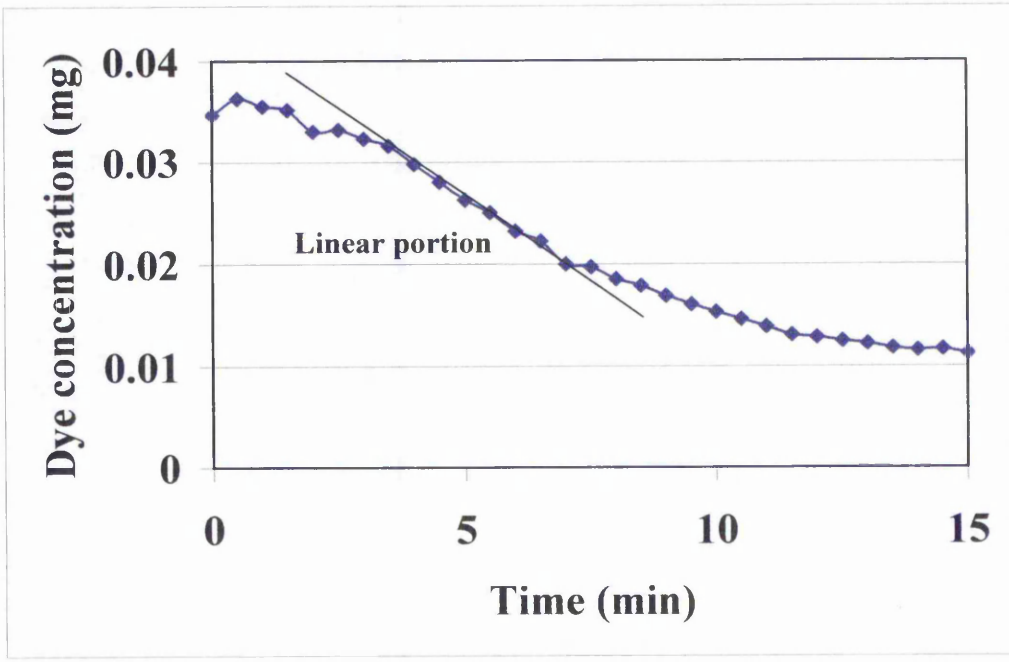


Figure 17. Decolourisation of hydrolysed Reactive Black by a cell free extract of *E. faecalis*.

3.8. Redox Potential and decolourisation.

3.8.1. Redox potential at biological decolourisation.

The redox potential at decolourisation for Reactive Black and the 3 Procion dyes decolourised by a chemical reducing agent has been investigated in Section 3.2. For comparison the redox potential at decolourisation in a biological system was investigated for Reactive Black (parent and hydrolysed) decolourised by *E. faecalis*. Decolourisation assays for measurement of redox potential were prepared according to Section 2.12.2. As was observed for the titrations of dye solutions with chemical reducing agent (Section 3.2) the two forms of Reactive Black underwent gradual colour changes during incubation with *E. faecalis* indicating sequential azo bond breakage. The measured redox potential and corresponding colour changes for the two forms of the dye are given in Figure 18. It can be seen from Figure 18 that a sharp drop in redox potential is apparent at the start of the assay for both forms of Reactive Black and the control assay (consisting of cells and glucose only). The sharp drop in redox potential is however of the same magnitude (approximately

250mV) for both forms of Reactive Black and the control assay. Assays for both forms of Reactive Black changed colour from dark blue to purple after approximately 2min indicating changes occurring to the dye structure before the redox potential had fallen. Concomitant with the sharp drop in redox potential complete decolourisation was not observed indicating that it was not redox potential alone causing decolourisation of the dyes. Further colour changes were observed from purple through to grey before the dyes appeared to be completely decolourised taking on a characteristic pale yellow colour after approximately 100min from the start of the assay. The redox potential at decolourisation was similar for the two forms of Reactive Black at –295mV for the parent and –292mV for the hydrolysed form. Comparison with the redox potential at chemical decolourisation (Table 17, Section 3.2) shows that only the redox potential at decolourisation for Reactive Black in an unbuffered aqueous solution was similar at –306mV.

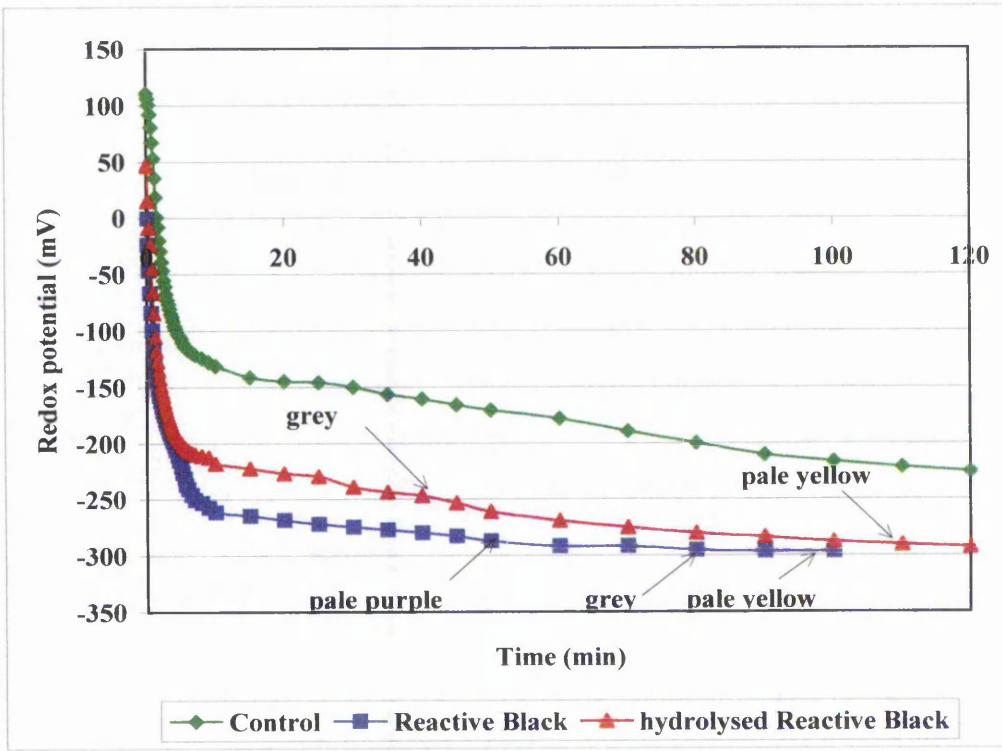


Figure 18. **Redox potential during decolourisation of Reactive Black and hydrolysed Reactive Black by *E. faecalis*. Values represent the mean of 3 determinations.**

For comparison, the food dye Sunset Yellow (having a simpler structure than the textile dyes) was also decolourised in assays with *E. faecalis* and the results are given in Figure 19. As was expected the Sunset Yellow decolourised faster than Reactive Black with decolourisation observed at a potential of –80mV. Decolourisation was complete before the redox potential had dropped considerably indicating it was not the fall in potential alone that is responsible for decolourisation.

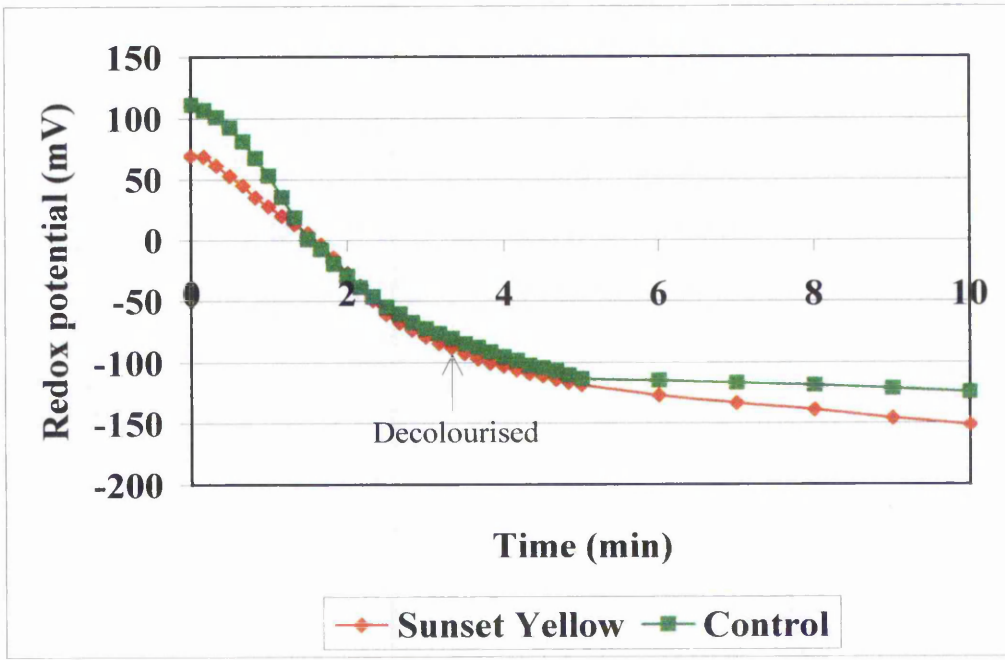


Figure 19. Redox potential during decolourisation of Sunset Yellow by *E. faecalis*. Values represent the mean of 3 determinations.

3.8.2. Correlation between initial decolourisation rate and redox potential at decolourisation.

Data for initial decolourisation rates with whole cells of *E. faecalis* (Table 21) and *C. butyricum* F5 (Table 22) were compared with redox potential at chemical decolourisation (Table 18). A correlation was observed between initial rate for both strains in the presence of glucose and redox potential at decolourisation for the dyes titrated at pH 3. Figure 20 shows a plot of initial rate versus redox potential for these conditions. No correlation was observed for assays without glucose or cell free extract assays with redox potential at decolourisation for chemical reduction.

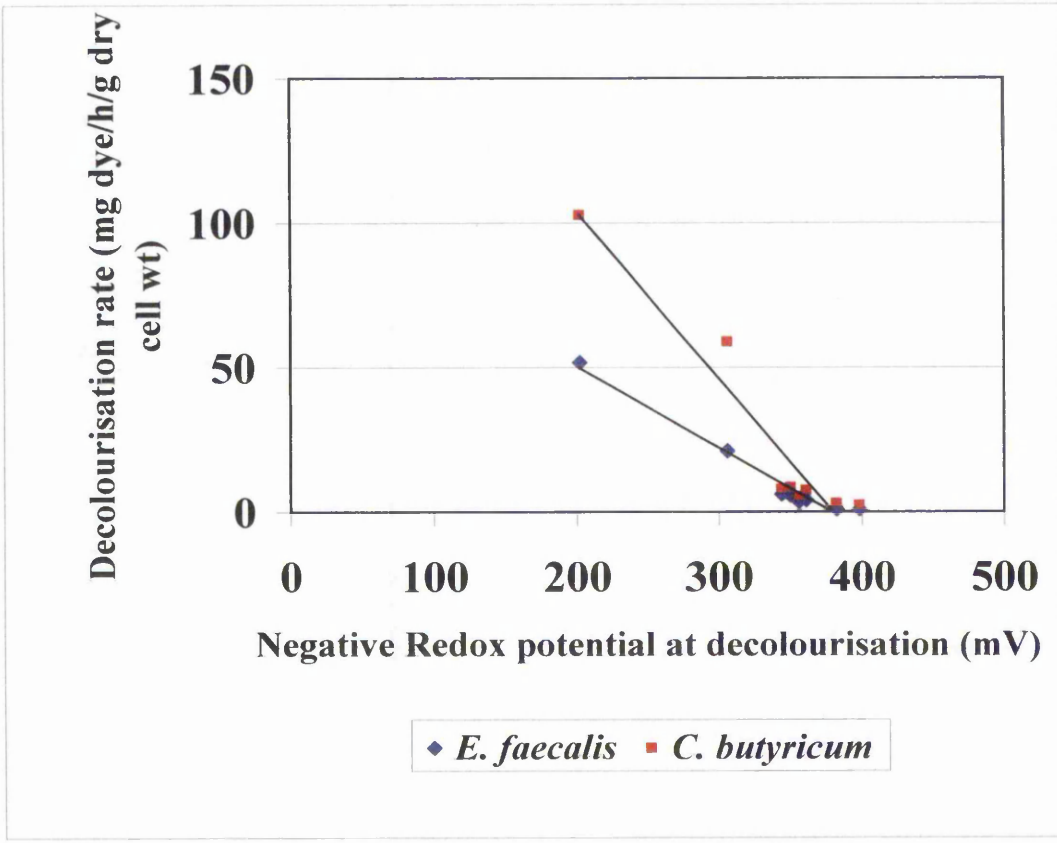


Figure 20. **Correlation between initial decolourisation rate in whole cell assays with glucose for *E. faecalis* and *C. butyricum* (F5) and redox potential at decolourisation for Reactive Black and 3 Procion dyes decolourised by sodium dithionite at pH3. For *E. faecalis* R^2=0.97 and for *C. butyricum* R^2=0.92.**

3.9. Azoreductase Molecular Weight.

Prior to loading cell extract samples on to gels decolourisation activity was checked using the Sunset Yellow check assay as in Section 3.7. Gels with molecular weight standards were Coomassie Blue stained only. Gels with cell free extracts were initially activity stained followed by Coomassie Blue and silver staining where appropriate.

Initial attempts to obtain activity staining on a gel were performed with *E. faecalis* and Sunset Yellow due to the high rate of decolourisation observed for this dye in cell free extract. The cell free extract of *E. faecalis* was freshly prepared immediately before use. Following addition of the concentrated sample buffer to the extract a series of two fold dilutions were prepared in single strength sample buffer in order to produce a range of protein loads across the gel. It was not known how intense the decolourisation would be or whether overloading the gel would be necessary to obtain a reaction. The original extract and subsequent dilutions were loaded onto a 10% acrylamide gel. After separation the gel was soaked in Sunset Yellow but no decolourisation was observed upon addition of the NADH solution. As the cell extracts were checked for activity prior to loading onto the gel it was thought unlikely that the extract had been denatured during preparation. It was not known whether activity was lost during the 6h electrophoretic separation although the system was run at 4°C packed in ice to avoid denaturation. Decolourisation in the check assay was achieved with the crude cell extract used. If however decolourisation relies on various cellular components present in the crude preparation these may not be available after separation hence decolourisation may not be observed. A more likely explanation for the lack of decolourisation was considered to be the difficulty encountered when visually assessing a yellow gel for a decolourised region. As was noted from the screening experiments (Section 3.4) decolourisation zones for certain dyes were difficult to see due to their pale colour. The clearest decolourisation observed on the dye plates was for Reactive Black. It was therefore decided to repeat the separation of an *E. faecalis* extract and to activity stain using Reactive Black. The parent form of Reactive Black was chosen in preference to the hydrolysed form as a higher rate of decolourisation was observed in

cell free assay for the parent and it was suspected that the hydrolysed dye may have a toxic effect on the cell free extract.

A fresh cell free extract of *E. faecalis* was prepared, checked for activity via the Sunset Yellow check assay, serially diluted as above and loaded onto a 10% gel. Following electrophoretic separation the gel was soaked in 0.1% (w/v) Reactive Black. Addition of NADH and incubation at room temperature produced two decolourised zones against the blue background of the gel within 5min. No decolourisation was observed without the NADH addition. An example of a gel displaying decolourisation activity staining is given in Figure 21. Two decolourisation sites were observed having R_f values of 0.72 and 1.0, the second band co-eluting with the tracking dye. The gel was Coomassie Blue stained according to Section 2.11.2. and is shown in Figure 22. From Figure 22 it can be seen that with Coomassie Blue staining no protein bands were seen where decolourisation occurred. Silver staining of the gels did show the presence of a protein band at the decolourisation site with R_f 0.72 (Figure 23). No protein bands were seen at the second site of decolourisation (R_f 1.0) suggesting a chemical mechanism in operation for the dye loss in this region or the presence of a protein of molecular weight below the detection limit of the silver staining method.

Decolourisation at the second site could be due to a chemical present in the cell free extract moving with the tracking dye, the tracking dye itself or a combination of the two. To determine whether this site of reduction was due to the tracking dye a second sample buffer was prepared as in Section 2.11.1. omitting the Bromophenol Blue tracking dye. A fresh cell free extract of *E. faecalis* was prepared and divided into two portions. To the first was added the sample buffer without the Bromophenol Blue. The second portion was boiled for 5min to denature the proteins present and the original sample buffer containing Bromophenol Blue added. The separation and activity staining for the two extract portions and Reactive Black was repeated (Figure 24). Activity staining of the resultant gel produced two sites of decolourisation as before for the first extract portion containing sample buffer with no tracking dye. No decolourisation was observed for the second boiled extract portion containing the original sample buffer. The second site of decolourisation was not therefore due to the tracking dye. As no decolourisation was seen for the

boiled sample it is thought that the chemical entity present in the extract was not thermally stable.

The molecular weight of the first protein band observed (R_f 0.72 on the 10% acrylamide gel) was determined according to Section 2.11.3. For each molecular weight standard a plot of 100[log(R_fx100)] versus gel concentration was constructed where R_f was the migration distance relative to the tracking dye. The slope of these curves were calculated (the retardation coefficients) and plotted against the molecular weight of each standard. The resultant plot is given in Figure 26. R_f values and calculated retardation coefficients of the molecular weight standards are given in Appendix 4. The *E. faecalis* extract was separated and activity stained on a series of gels as given in Table 14. The R_f value of the decolourising band was determined as for the standards and the retardation coefficient calculated from a plot of 100[log(R_fx100)] versus gel concentration.

The molecular weight of the band was then calculated as follows:

Equation of the line from Figure 26.

$y = 0.0783x + 2.7913$ y = retardation coefficient

x = molecular weight

Retardation coefficient for *E. faecalis* decolourising band 1 = 11.75

Rearranging the equation

$x = (11.75-2.7913)/0.0783$ x = 114.4

Multiplying by 1000 gives a molecular weight of 114,415 Daltons or 114.4 kDa.

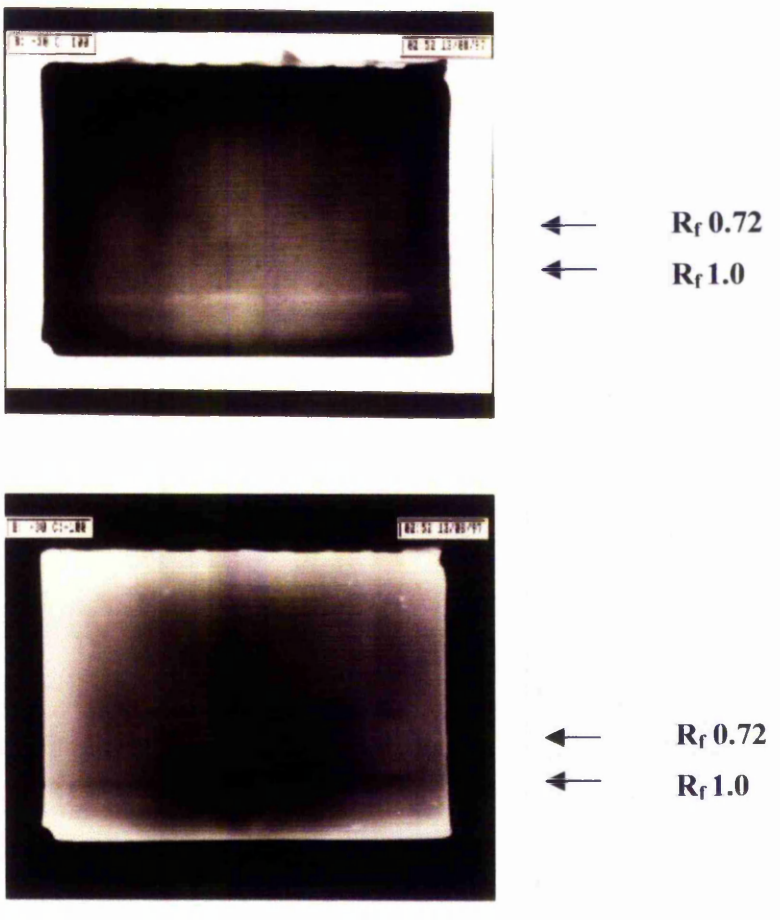


Figure 21. ***E. faecalis*** **cell free extract on a 10% gel displaying decolourisation zones when activity stained with Reactive Black and the reverse image showing dark bands at the decolourisation sites.**

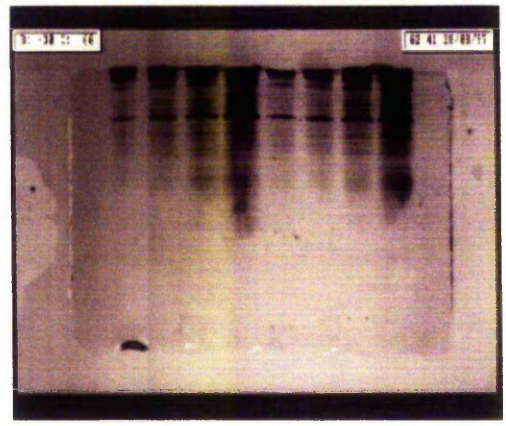


Figure 22. **Coomassie Blue stained gel of *E. faecalis* cell free extract showing absence of protein bands at the sites of decolourisation observed in Figure 21.**

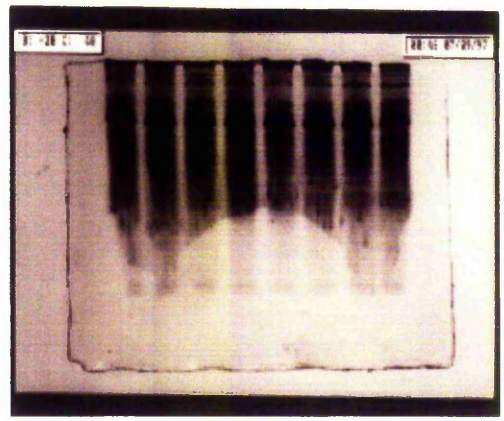
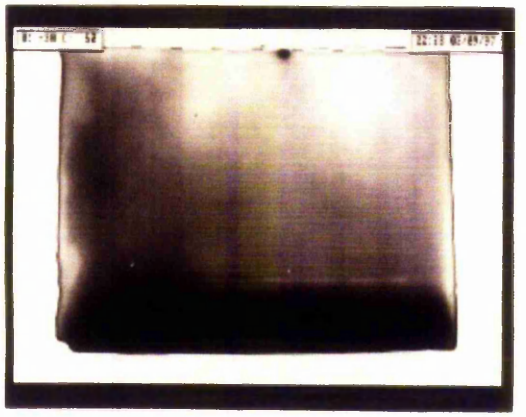
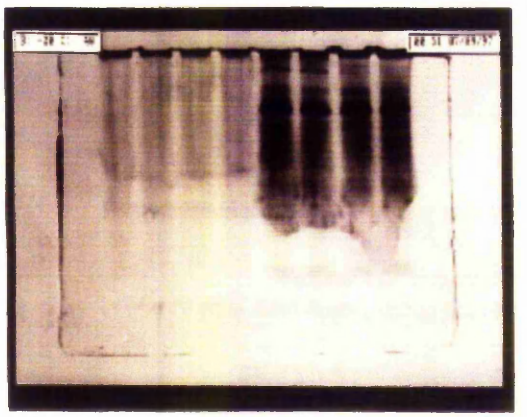


Figure 23. **Silver stained gel of *E. faecalis* cell free extract showing protein band at decolourisation site 1 (R_f=0.72) observed in Figure 21.**



1 8

Figure 24. **Activity staining of *E. faecalis* cell free extract. Lanes 1 to 4 show no decolourisation with a boiled sample of cell free extract. Lanes 4 to 8 show decolourisation sites observed for the cell free extract with no tracking dye.**



← R_f 0.72

Figure 25. **Silver stained gel for samples in Figure 24 showing denaturation of proteins in the boiled cell extract sample and the presence of protein bands at decolourisation site 1 for the sample run with no tracking dye.**

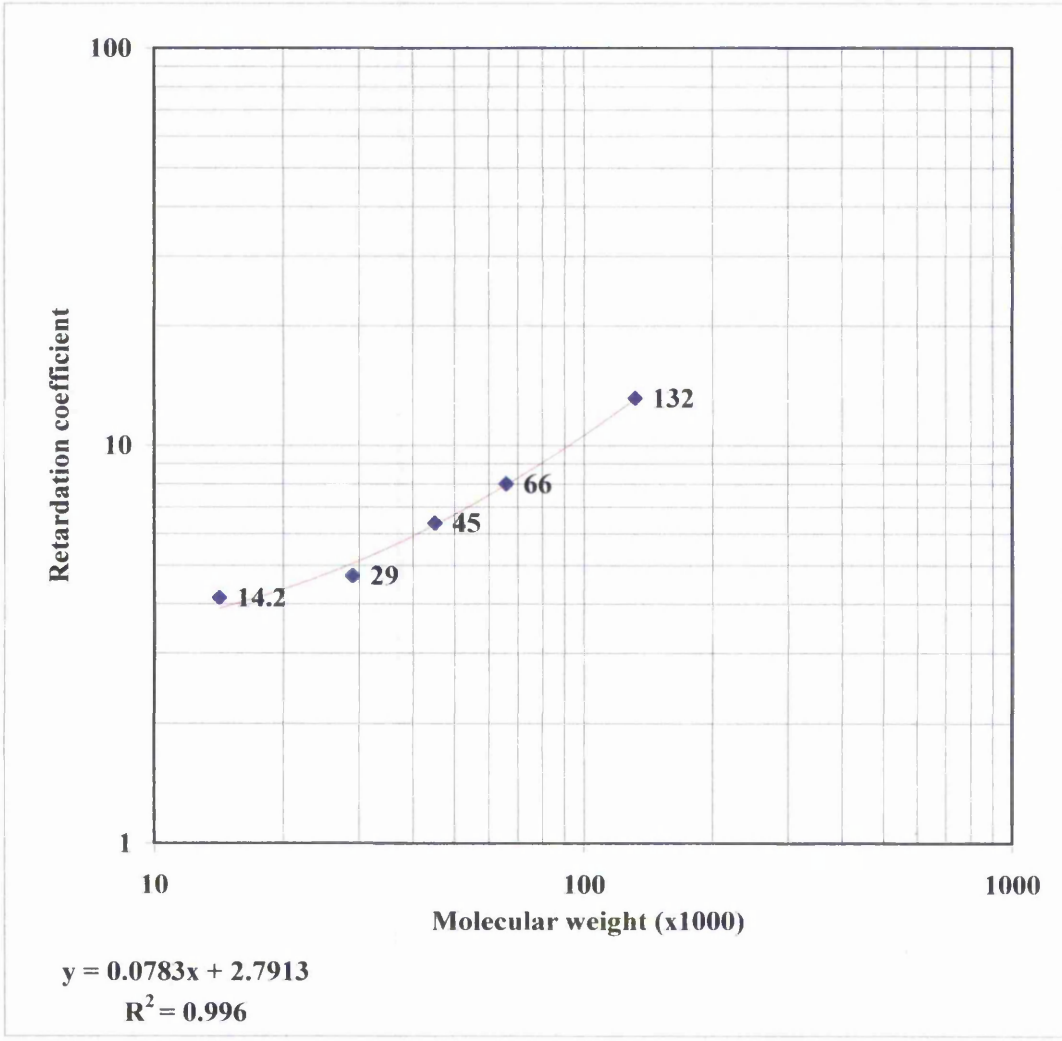


Figure 26. **Plot of retardation coefficient versus molecular weight for non-denatured protein standards. Data labels represent the molecular weight (x1000) for standards employed: α-lactalbumin (14.2), carbonic anhydrase (29), chicken egg albumin (45), bovine serum albumin monomer (66) and bovine serum albumin dimer (132).**

The separation and activity staining on gels was repeated for *C. butyricum* (F5) cell free extracts but no decolourisation was observed with or without the NADH addition. The absence of a decolourised band may be due to several reasons. It was thought unlikely that the cell free extract was denatured during preparation as decolourisation was observed in the Sunset Yellow check assay performed prior to loading the gel. The precautions taken to avoid oxygen exposure of the cell free extract may not have been effective and exposure of the cell free extract to oxygen may have resulted in irreversible inactivation. It is also not known whether the extract lost activity over the 6h separation time. The decolourisation reaction may also require a co-factor present in the crude extract that may not be available after separation. The quantity of protein that can be loaded onto a gel is limited and it is possible that the amount used was too low to detect decolourisation on the gel.

3.10. The mechanism of decolourisation of Reactive Black.

An investigation into the degradation pathway of Reactive Black was undertaken to gain an insight into the mechanism of decolourisation, presence of intermediate breakdown products and the extent of their persistence and an assessment of the number of non-coloured aromatic species produced from dye reduction. Changes in the absorbance spectra and the number of compounds present in an *E. faecalis* assay with Reactive Black were monitored over time.

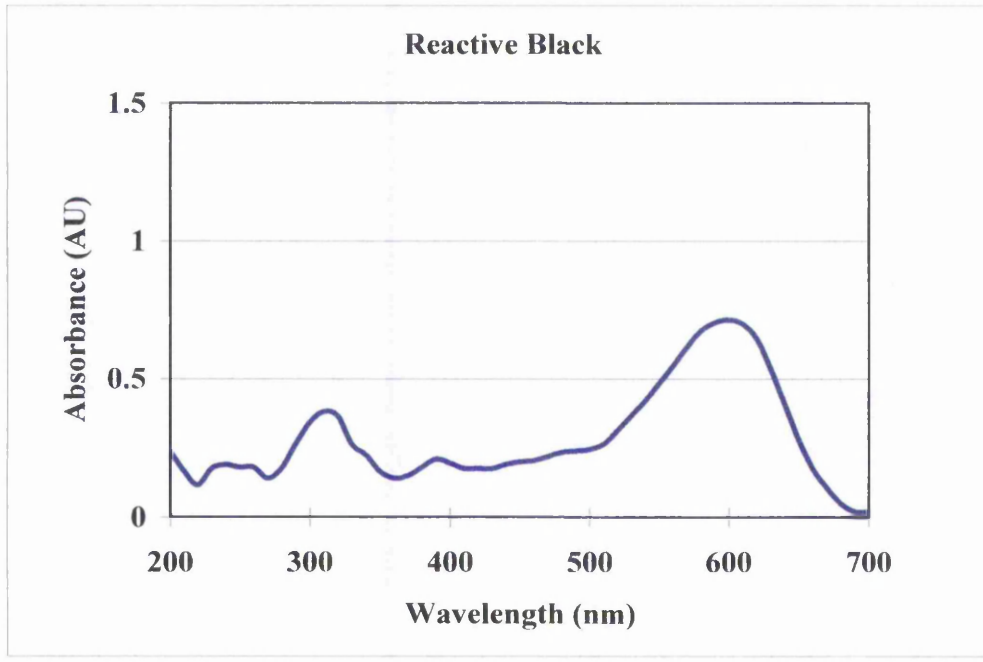
Assays were set according to Section 2.8.1. for *E. faecalis* and Reactive Black (parent and hydrolysed). Samples were withdrawn by automatic pipette at timed intervals (0, 2.5, 5, 10, 20, 40, 60, 90 and 120min). The 0min sample was effectively the time taken to start the assay by addition of thick cell suspension and immediate removal of a sample by pipette. Samples were centrifuged (1ml Eppendorf tube, microfuge 11,600 x g 5min) to remove the cells and supernatants analysed by UV/VIS spectrophotometry (20-fold dilution in phosphate buffer prior to scanning) and HPLC (injected directly). Instrument conditions were as in Section 2.3.

For comparison UV/VIS scans for unreduced Reactive Black and hydrolysed Reactive Black are given in Figure 27. Figures 28 and 29 show selected UV/VIS scans from assays with the two forms of Reactive Black. For the parent dye (Figure 28) it can be seen that after 2.5 minutes three product peaks have started to form in the UV region at 220, 260 and 340nm. After 5 minutes the height of the peak at 340nm is reduced, further reducing in subsequent samples suggesting the formation and loss of an intermediate product. Some reduction in absorbance was seen in the 200nm-250nm region from 2.5 minutes to 10 minutes however following sample scans showed a gradual increase in absorbance at 220nm and 260nm indicating the formation of the final decolourisation products. The scans for hydrolysed Reactive Black (Figure 29) show an intermediate product formed at 340nm as seen for Reactive Black.

HPLC chromatograms of selected timed samples are given in Figures 31 and 32. Chromatograms for unreduced parent and hydrolysed dye at the equivalent concentration used in the assay are given in Figure 30 for comparison. The retention times of the peaks observed in the HPLC chromatograms will be referred to as R_t. For the unreduced parent dye it was expected that more than one peak would be present due to the dyes purity being estimated at 55%. The main peak can be seen at R_t=29min with minor peaks at R_t=23min and R_t=32min. From diode array data the peak at R_t=29min

(main peak) and the peak at R_t=32min had identical UV spectra and were thought to be essentially the same compound with minor differences in chemical structure accounting for the different retention times observed. Collection of eluent fractions from the HPLC separation revealed that both components were blue. The minor peak at R_t=23min was thought to be a component carried over from the dyes manufacture as its UV spectrum did not match the other two and the eluent fraction containing the component was pale pink. For the unreduced hydrolysed dye the main peak is seen at R_t=35.5min. The numerous minor components of unreduced Reactive Black (parent and hydrolysed) observed from the TLC separation (Table 16, Section 3.1) may not be detected by HPLC due to their low absorbance at 260nm (Figures 5 and 6).

From Figure 31 following the time course for the parent dye it can be seen that for the zero time sample the main peak for the unreduced dye was seen at R_t=29min and two product peaks had started to form at R_t=23.5min and R_t=7.5min. By 5 minutes the unreduced dye peak (R_t=29min) was absent while the two product peaks R_t=23.5min and R_t=7.5min had increased in size. After 10 minutes a third product peak at eluting at R_t=21min had started to form which increased over time concomitant with a reduction in the peak observed at R_t=23.5min. By 120 minutes from the start of the assay the R_t=23.5min peak had disappeared completely leaving two main products from the decolourisation at R_t=7min and R_t=21min. This confirmed the presence of a decolourisation intermediate observed from the UV scans (Figure 28) eluting at R_t=23.5min which, as the sample supernatant was blue/purple at this stage, may be due to the azo bonds in the dye molecule being broken asymmetrically. If one of the azo bonds remains intact at this stage the molecule will still be coloured. The product at R_t=7min, once formed, increased in size and was persistent suggesting the direct formation of a final product. The final product at R_t=21min appeared to form via the intermediate at R_t=23.5min.



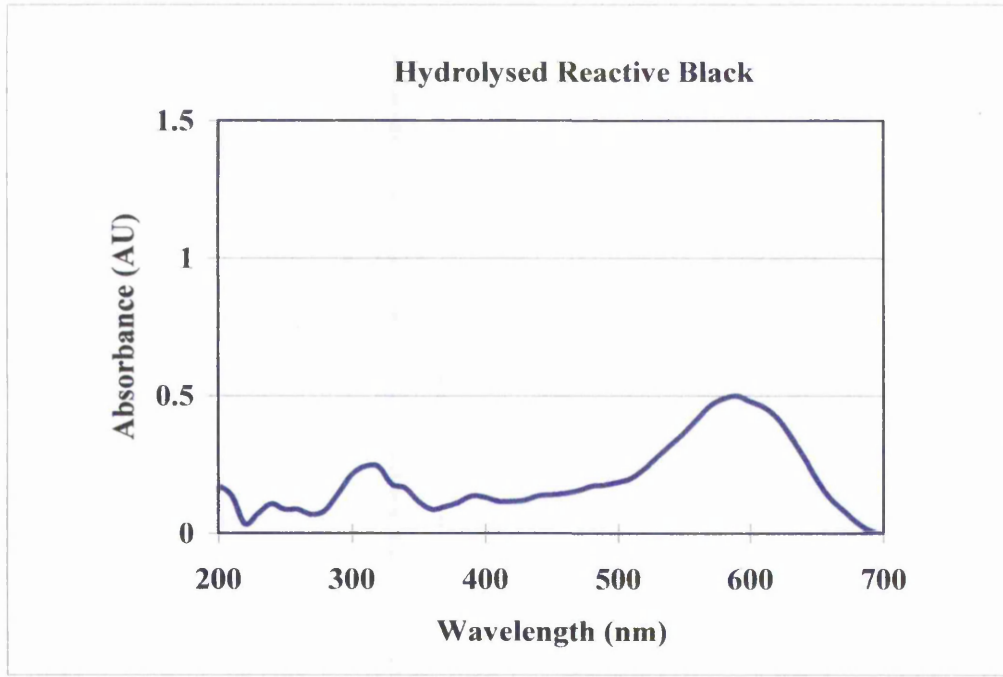


Figure 27. **UV/VIS scans of unreduced Reactive Black and hydrolysed Reactive Black. Dye concentration 0.002% (w/v).**

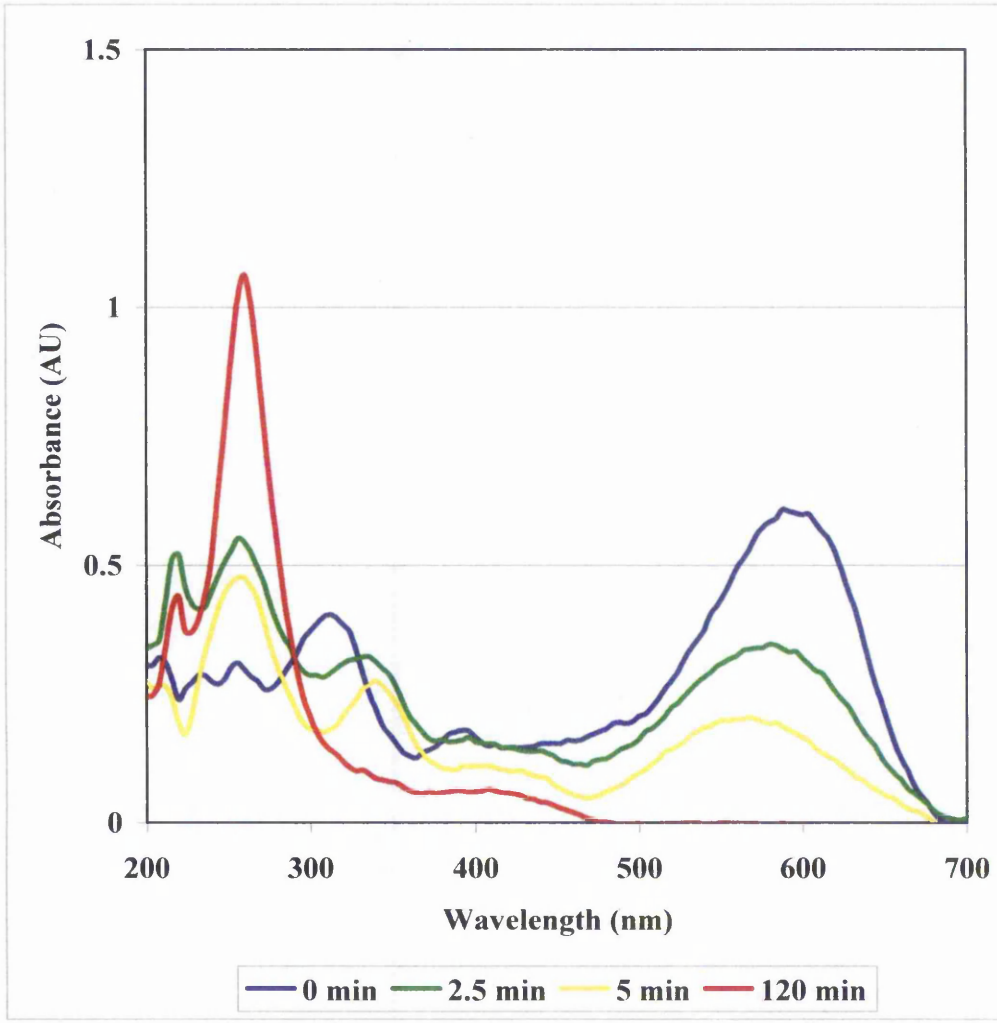


Figure 28. **UV/VIS scans at selected times from a decolourisation assay with *E. faecalis* and Reactive Black parent dye. Dye concentration 0.05% (w/v). Dye samples diluted 1 in 20 prior to scanning. 300mg dry cell mass in a 10ml assay.**

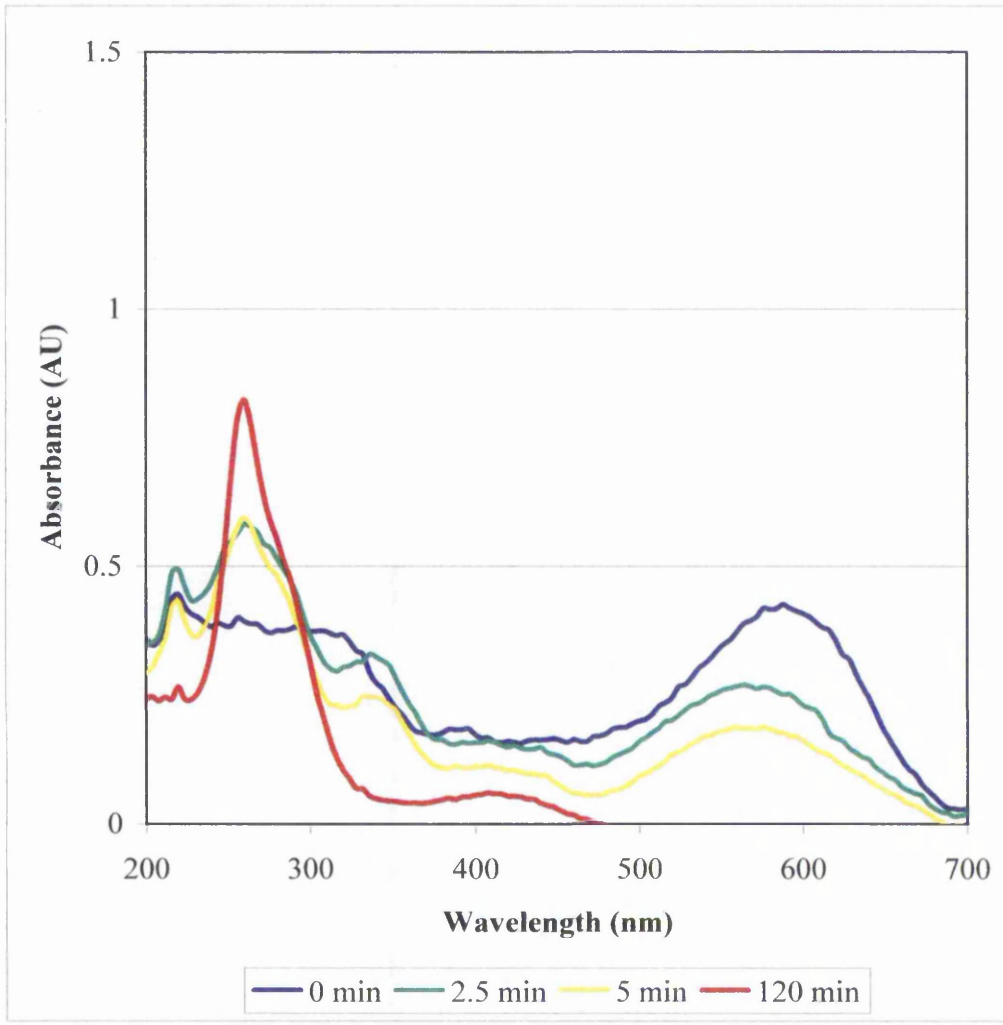
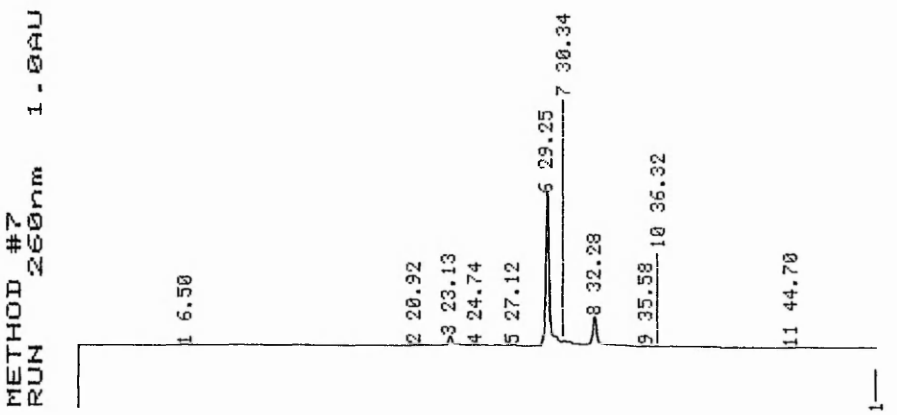


Figure 29. **UV/VIS scans at selected times from a decolourisation assay with *E. faecalis* and hydrolysed Reactive Black. Dye concentration 0.05% (w/v). Dye samples diluted 1 in 20 prior to scanning. 300mg dry cell mass in a 10ml assay.**

(a) parent Reactive Black



(b) hydrolysed Reactive Black

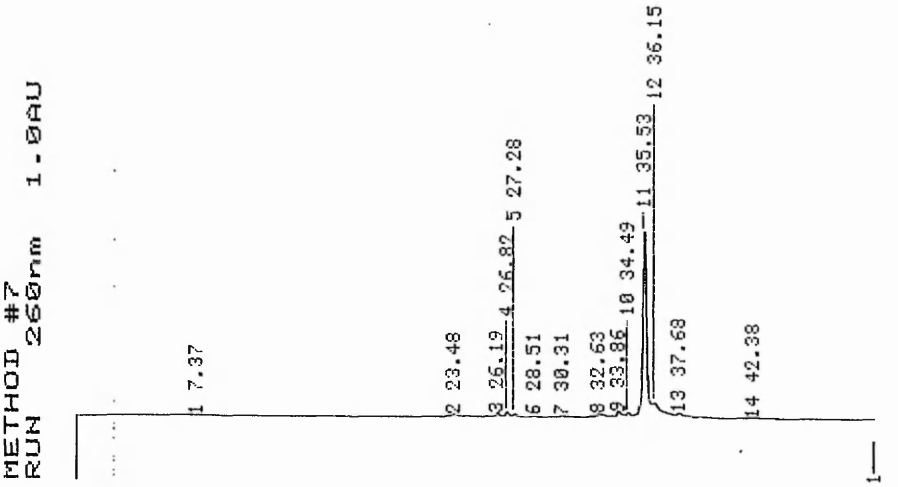


Figure 30. **Chromatograms of unreduced Reactive Black (a) parent and (b) hydrolysed. Dye concentration 0.05% w/v. Detection wavelength 260nm. Injection volume 20μl.**

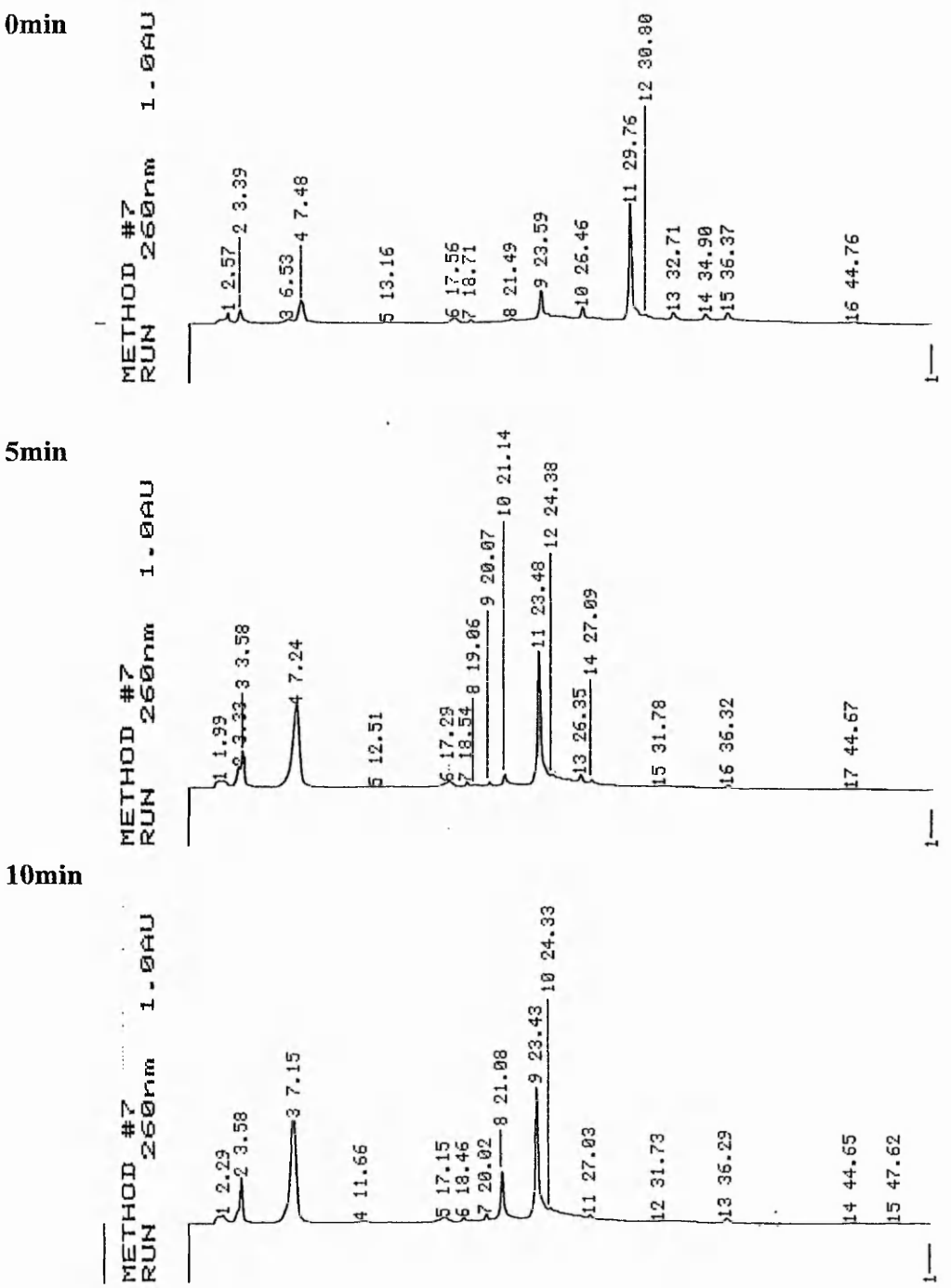


Figure 31. **Chromatograms and peak spectra of samples from a decolourisation assay with *E. faecalis* and Reactive Black parent dye. Dye concentration 0.05% w/v. Detection wavelength 260nm. Injection volume 20μl.**

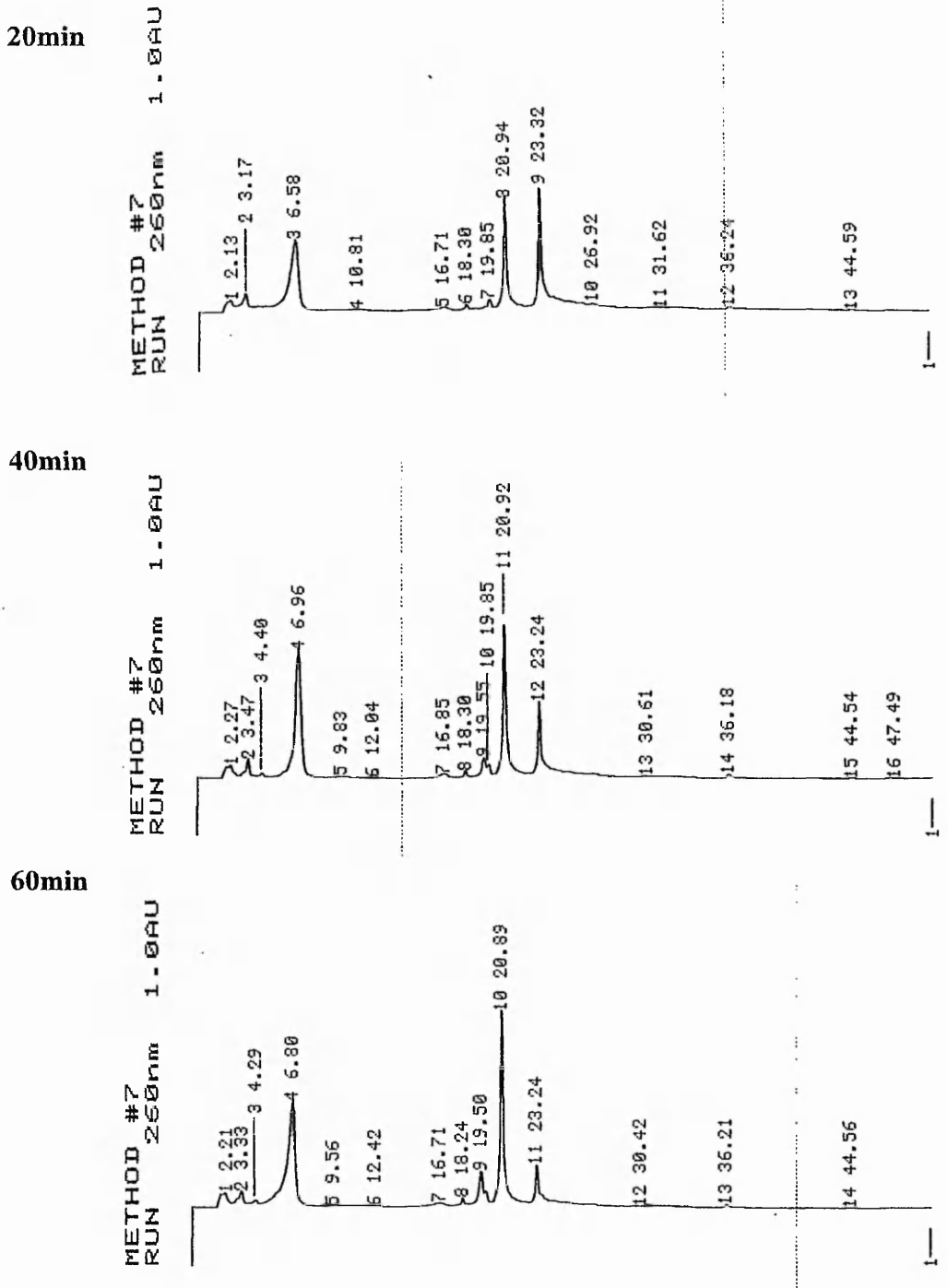
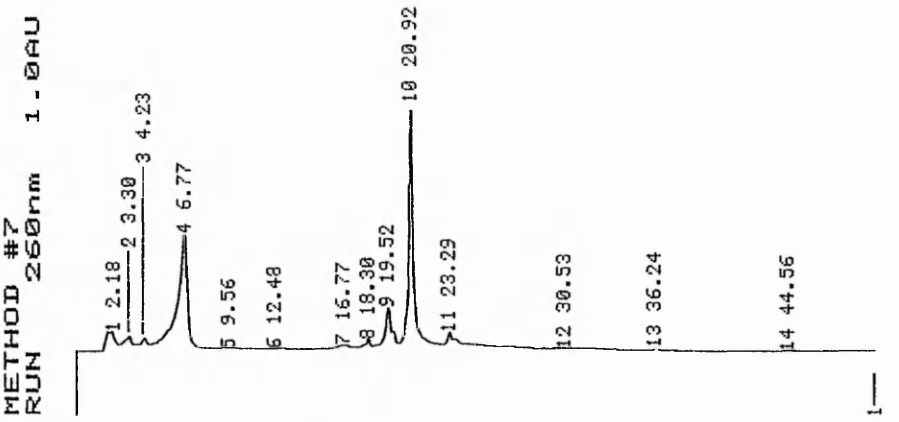
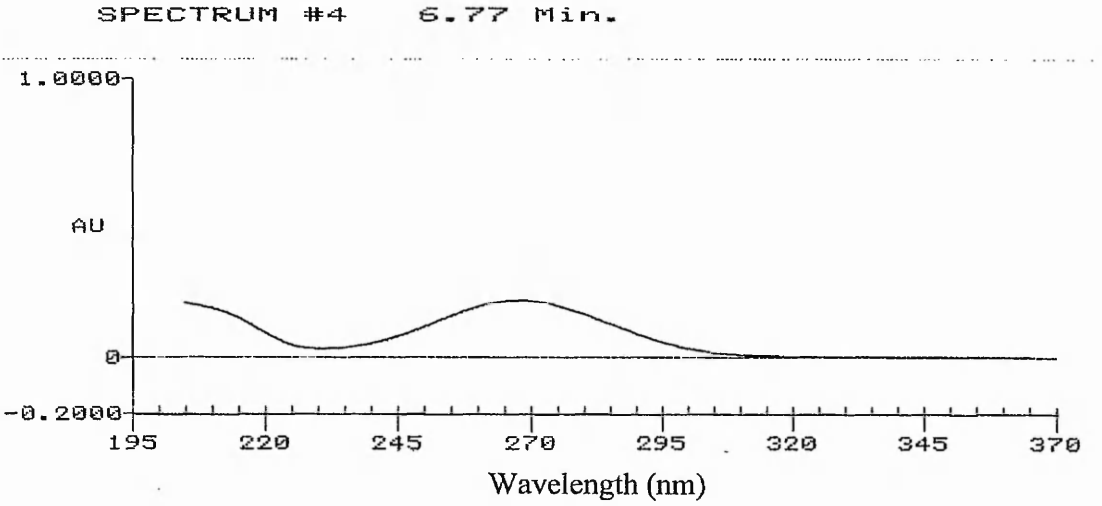


Figure 31. **Continued. Chromatograms and peak spectra of samples from a decolourisation assay with *E. faecalis* and Reactive Black parent dye. Dye concentration 0.05% w/v. Detection wavelength 260nm. Injection volume 20µl.**

120min





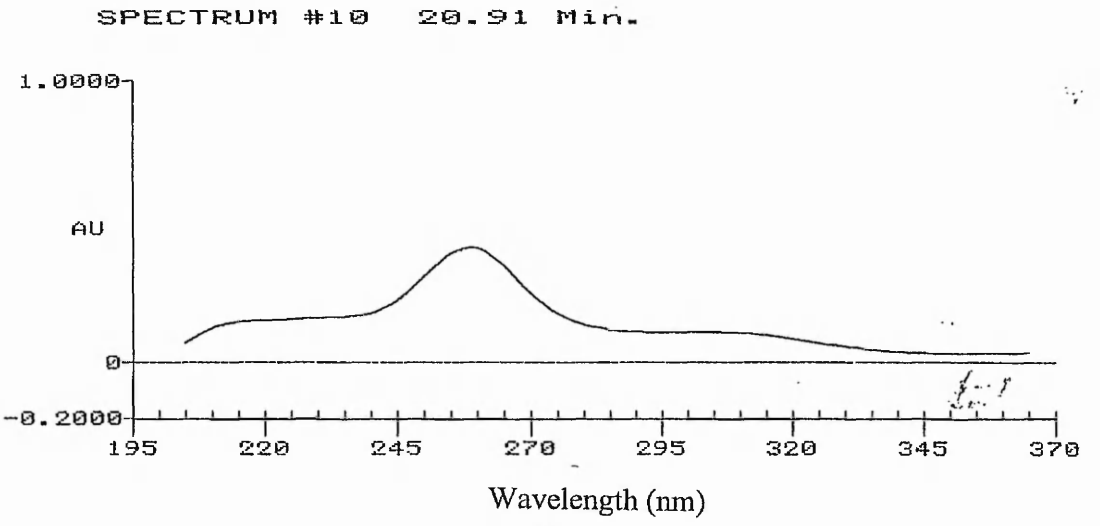


Figure 31. **Continued. Chromatograms and peak spectra of samples from a decolourisation assay with *E. faecalis* and Reactive Black parent dye. Dye concentration 0.05% w/v. Detection wavelength 260nm. Injection volume 20µl.**

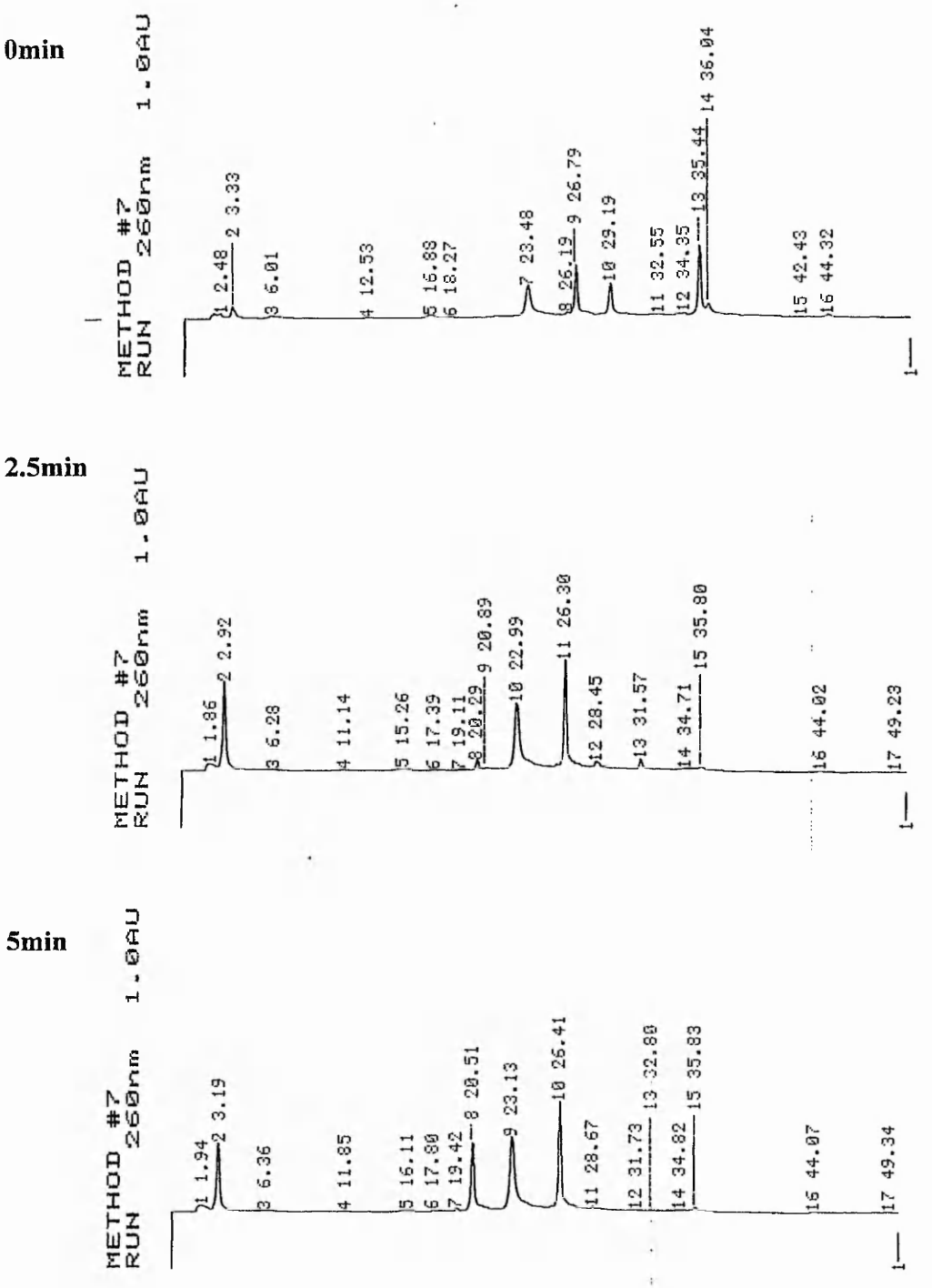


Figure 32. **Chromatograms and peak spectra of samples from a decolourisation assay with *E. faecalis* and hydrolysed Reactive Black. Dye concentration 0.05% w/v. Detection wavelength 260nm. Injection volume 20μl.**

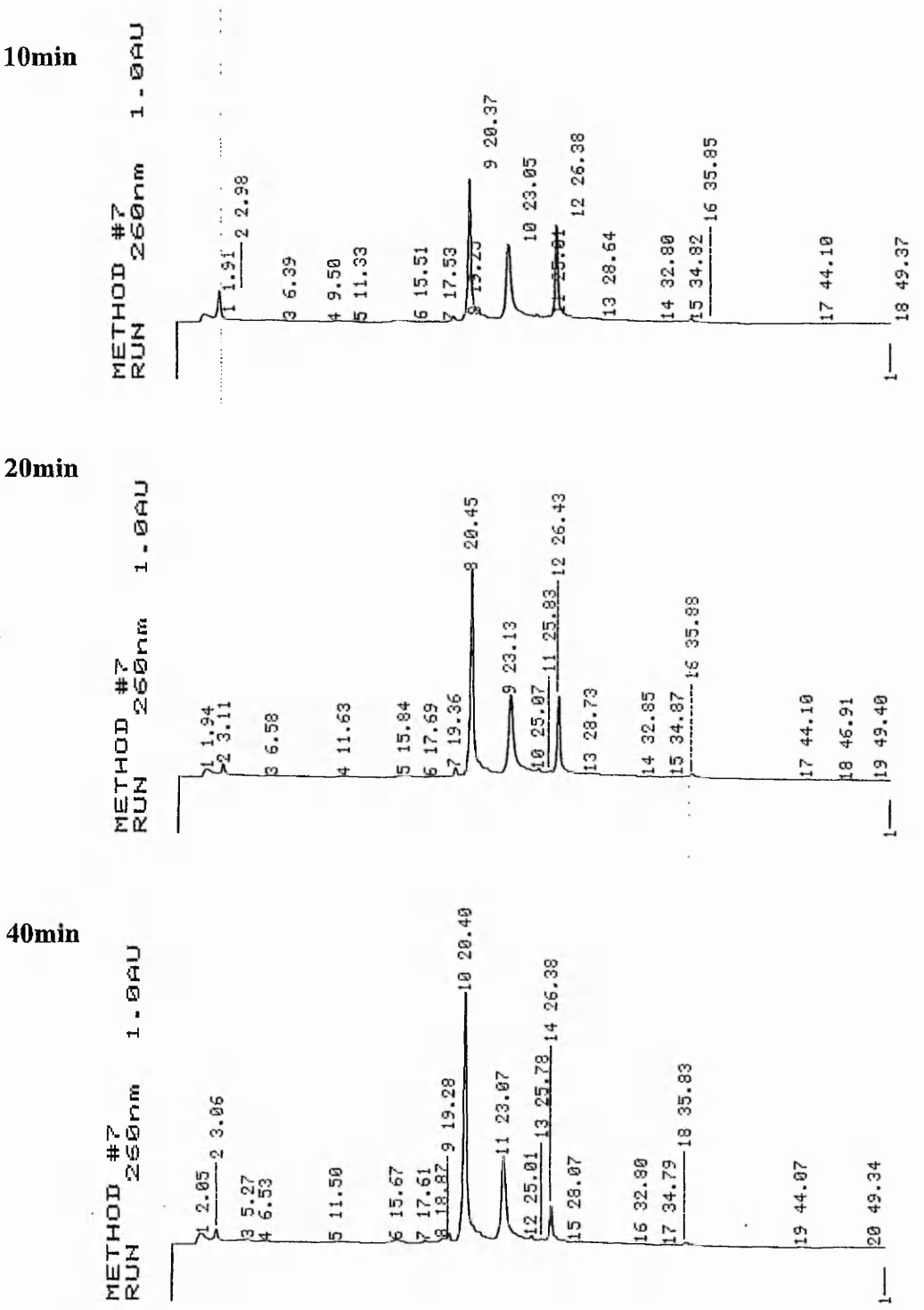


Figure 32. **Continued. Chromatograms and peak spectra of samples from a decolourisation assay with *E. faecalis* and hydrolysed Reactive Black. Dye concentration 0.05% w/v. Detection wavelength 260nm. Injection volume 20µl.**

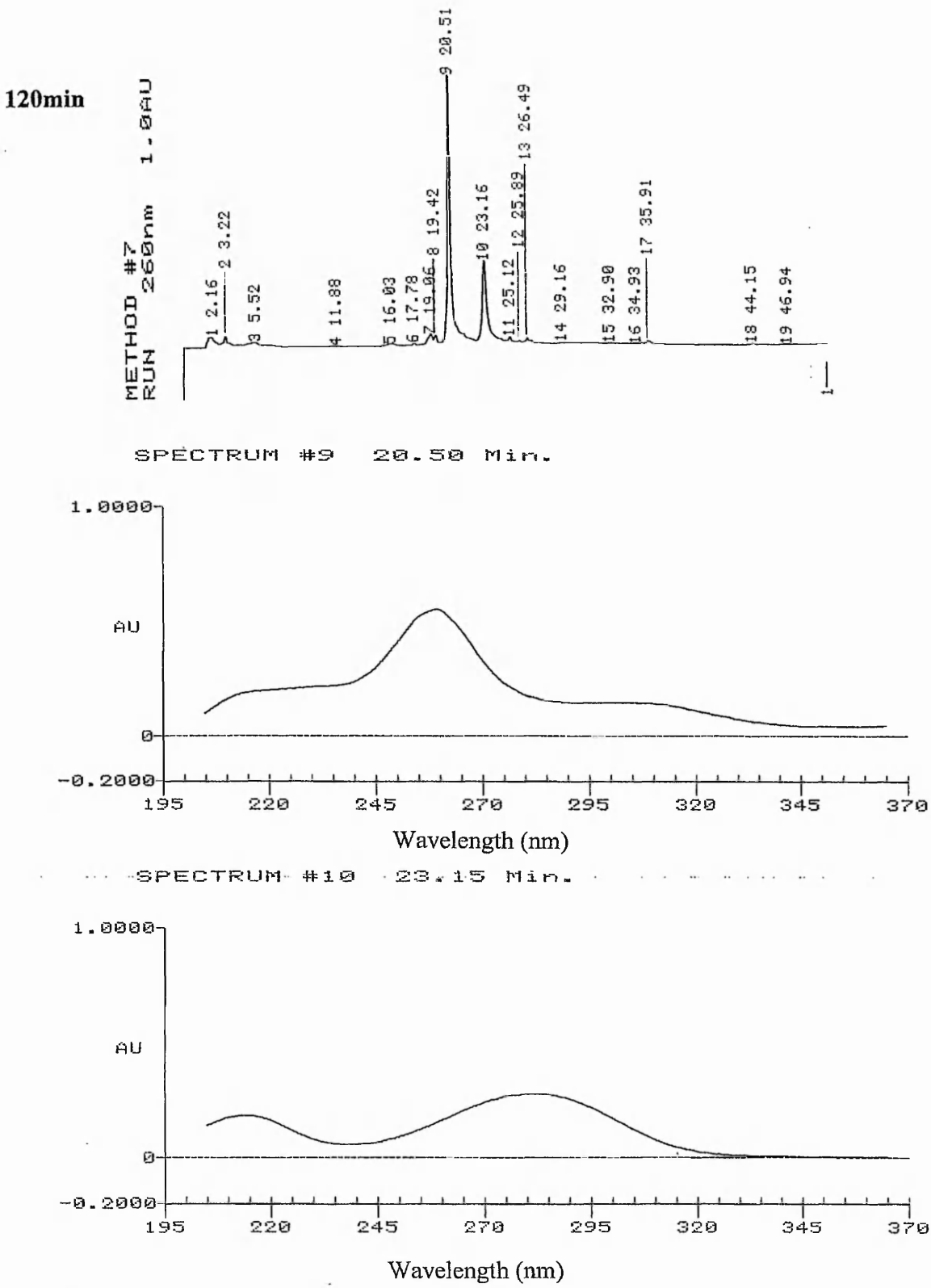


Figure 32. **Continued. Chromatograms and peak spectra of samples from a decolourisation assay with *E. faecalis* and hydrolysed Reactive Black. Dye concentration 0.05% w/v. Detection wavelength 260nm. Injection volume 20µl.**

For the hydrolysed dye (Figure 32) the sample at zero time shows the peak for the unreduced dye at R_t=35.5min and product peaks at R_t=23.5min, R_t=26.8min and R_t=29min forming. By 2.5 minutes the unreduced dye peak eluting at R_t=35.5min was absent as was the peak at R_t=29min. The R_t=23.5min and R_t=26.8min peaks had increased in size and two new peaks were seen at R_t=3min and R_t=20.5min. From 2.5min to 20min the peak at R_t=3min was seen to reduce in size with an increase in height for the 20.5min peak. After 20 minutes the peak eluting at R_t=3min had disappeared. The peak eluting at R_t=20.5min continued to increase in size simultaneously with a decrease at R_t=26min until by 120 minutes from the start of the assay only two main product peaks were present. It appeared that for the hydrolysed dye decolourisation intermediates are also formed as was observed for Reactive Black. For the hydrolysed dye three intermediates were observed at R_t=3min, R_t=26min and R_t=29min. It is not known whether there is a delay in the appearance of final products due to a necessity for all the dye being converted to an intermediate before complete decolourisation occurs.

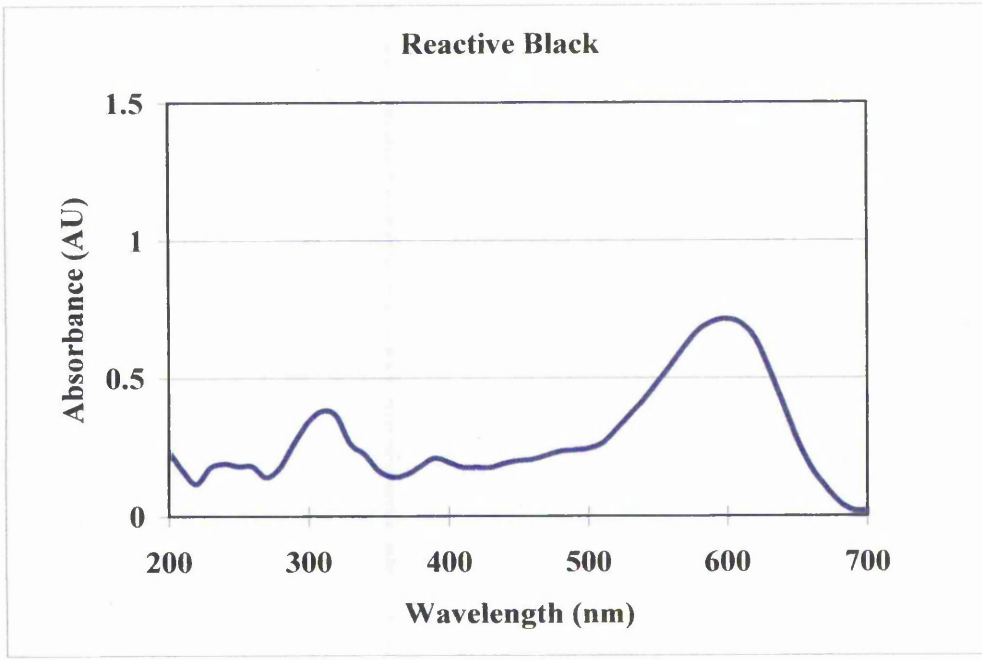
If the decolourisation does occur via asymmetric breakage of the azo bonds it is not known whether this is due to one of the bonds being more accessible and therefore preferentially attacked. If this were the case the second azo linkage may not be broken until all of the first azo bonds have been cleaved. If decolourisation occurs via this mechanism two products would be expected for Reactive Black (di-amino H-acid and 2 vinyl sulphone side chains) providing structural breakdown did not proceed any further. Breakdown of the parent and hydrolysed forms of Reactive Black should yield a common product for each form of the dye. During hydrolysis it is assumed that only the vinyl sulphone side chain undergoes transformation whereas the substituted H-acid component remains unchanged. Comparison of chromatograms for the two forms of reduced dye (Figures 31 and 32, 120min samples) shows that the product peak at R_t=20min is common to both and has identical UV spectra. It was assumed therefore that the R_t=20min product peak is due to the substituted H-acid component. By elimination it was assumed that the R_t=7min product peak for the parent dye is due to the vinyl sulphone side chain and the R_t=23min peak for the hydrolysed dye is due to the hydrolysed vinyl sulphone side chain. This was confirmed by the expectation that the vinyl sulphone side chain for the parent dye would elute from the HPLC column faster than the vinyl sulphone side chain for the hydrolysed dye due to it's increased polarity caused by the terminal sulphonate group.

3.11. Comparison of products from the decolourisation of Reactive Black and three Procion dyes by *E. faecalis* and *C. butyricum* (F5).

3.11.1. UV/VIS Spectrophotometry.

It is assumed that decolourisation of a dye proceeds via azo bond breakage giving rise to non-coloured aromatic breakdown products. If degradation does occur via this mechanism, different strains would be expected to produce identical end products for a particular dye. A convenient method for comparison of end products is by assessing similarity between the UV/VIS spectra of dye samples decolourised by different strains. A series of decolourisation assays were prepared according to Section 2.8. for *E. faecalis*, *C. butyricum* (F5), Reactive Black and the three Procion dyes (parent and hydrolysed forms). Dye concentration was 0.05% (w/v). Samples were taken by automatic pipette after 18h incubation and centrifuged to remove the cells. Supernatants were UV/VIS scanned against a control sample (assay set without the addition of dye) with dilution in phosphate buffer (pH7, 100mM) as required to bring the measurements on scale. The resultant scans are given in Figures 33 to 40.

For Reactive Black (Figures 33 and 34) it can be seen that although slight differences are observed in the profiles for the parent and hydrolysed dye decolourised by *E. faecalis* and *C. butyricum* (F5) the overall the profiles are the same. The scans for the decolourised samples of parent dye show a main product peak in the UV region at 260nm for both strains although for the *C. butyricum* (F5) decolourised sample the peak is broader with a slight shoulder at 290nm and minor differences in the low UV 200-230nm region. The scans for the decolourised samples of hydrolysed dye also show a main product peak at 260nm. Samples scans for the hydrolysed dye decolourised by both strains show a more pronounced shoulder on this peak at 290nm and a second product peak at 230nm. All scans show absorbance in the 400nm region that is concurrent with the yellow colour of the decolourised samples.



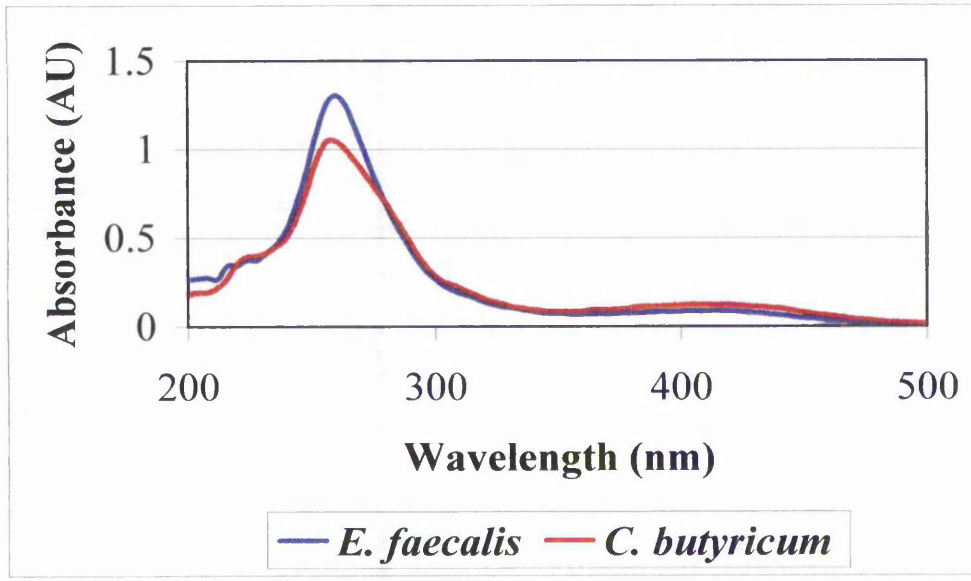
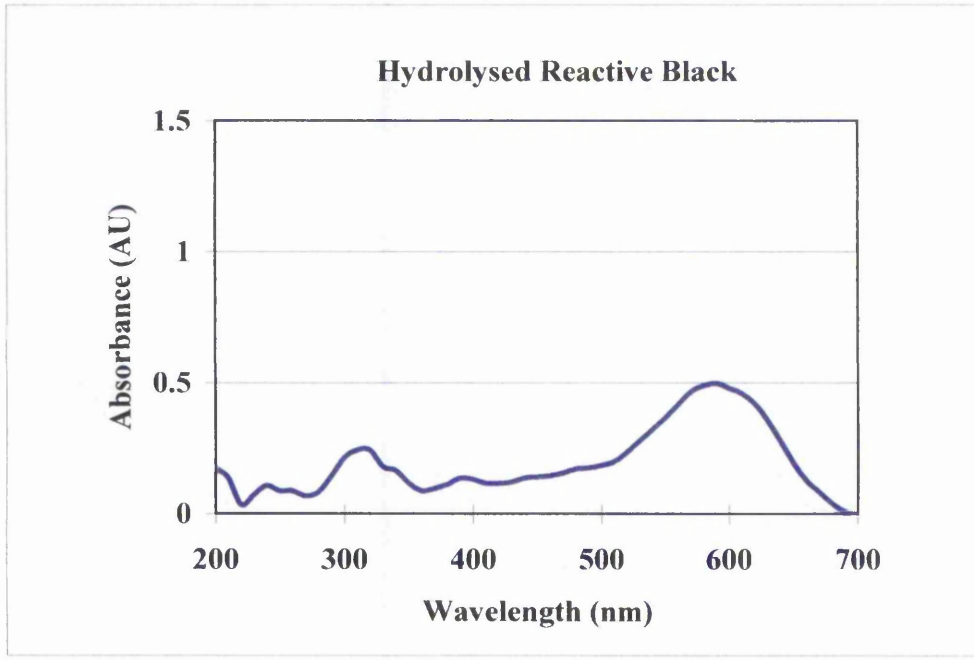


Figure 33. **UV/VIS profiles for (a) Reactive Black parent dye (0.002%w/v) and (b) Reactive Black parent dye decolourised by *E. faecalis* and *C. butyricum* (18h samples diluted 1 in 20).**



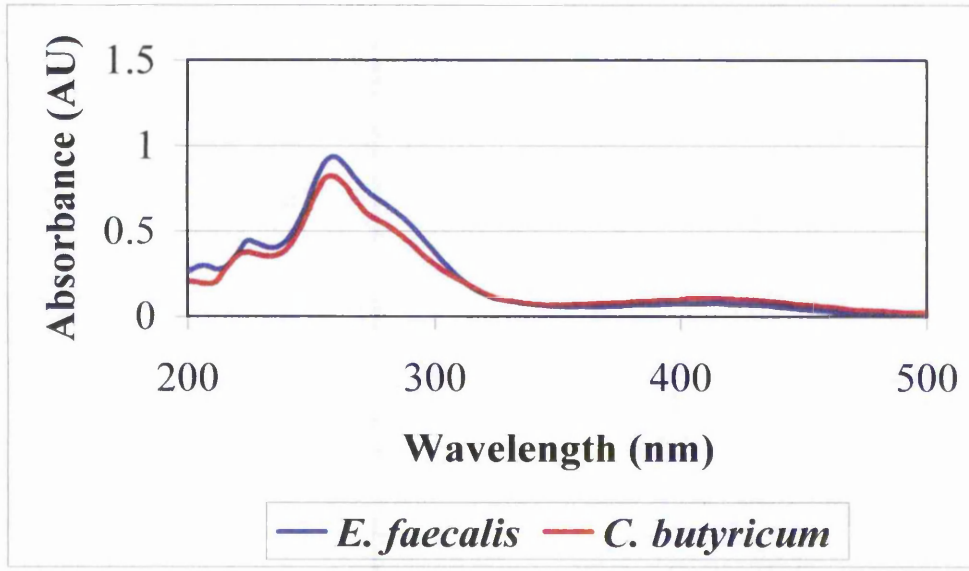
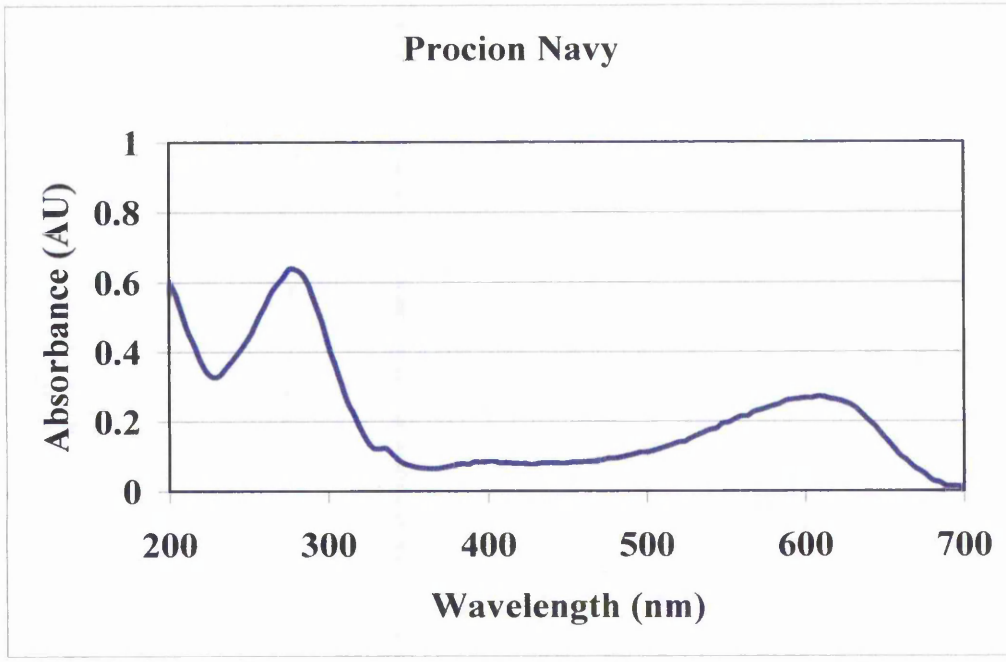


Figure 34. **UV/VIS profiles for (a) hydrolysed Reactive Black (0.002%w/v) and (b) hydrolysed Reactive Black decolourised by *E. faecalis* and *C. butyricum* (18h samples diluted 1 in 20).**



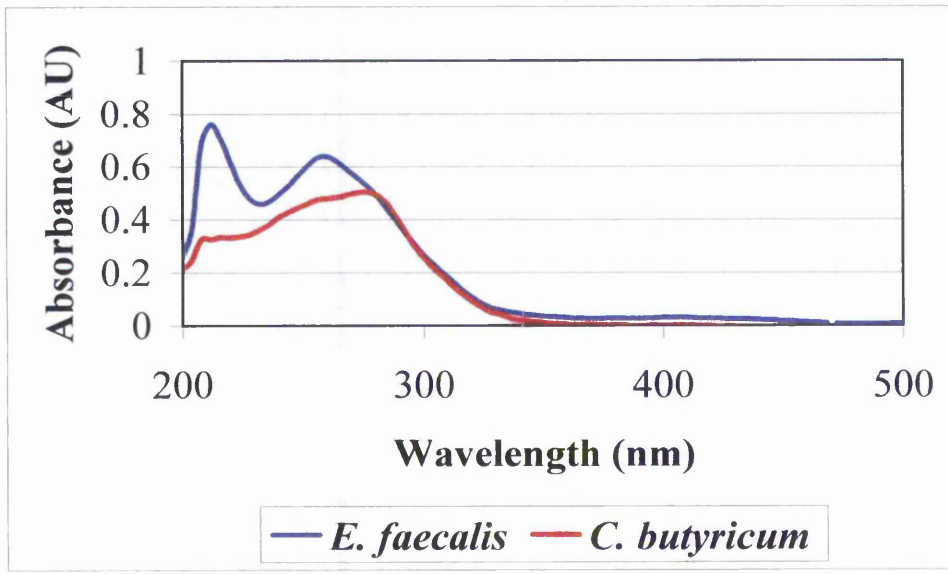
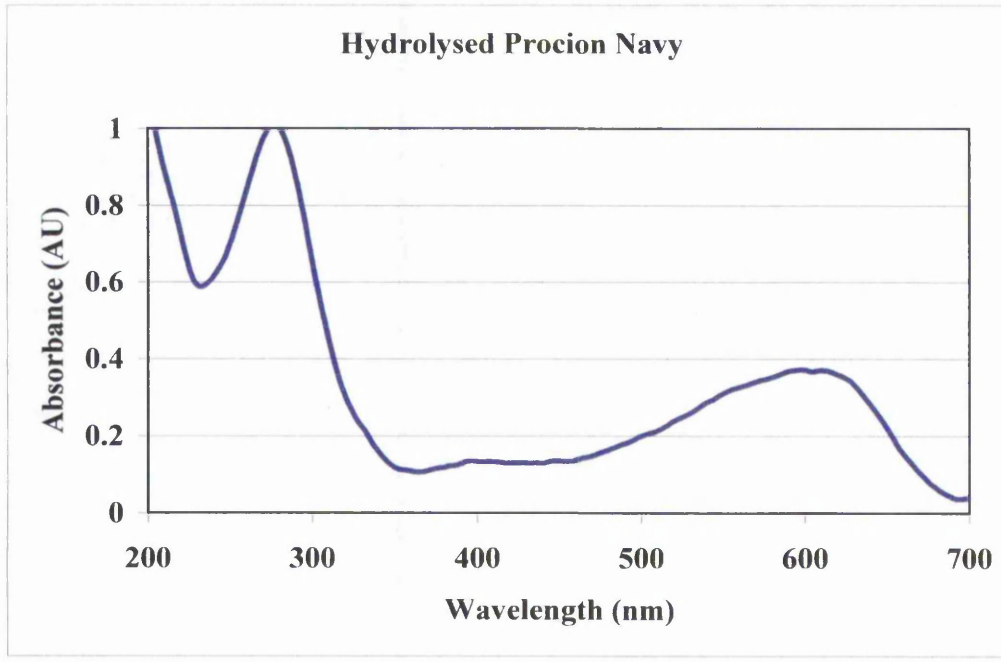


Figure 35. **UV/VIS profiles for (a) Procion Navy parent dye (0.002%w/v) and (b) Procion Navy parent dye decolourised by *E. faecalis* and *C. butyricum* (F5) (18h samples diluted 1 in 50).**



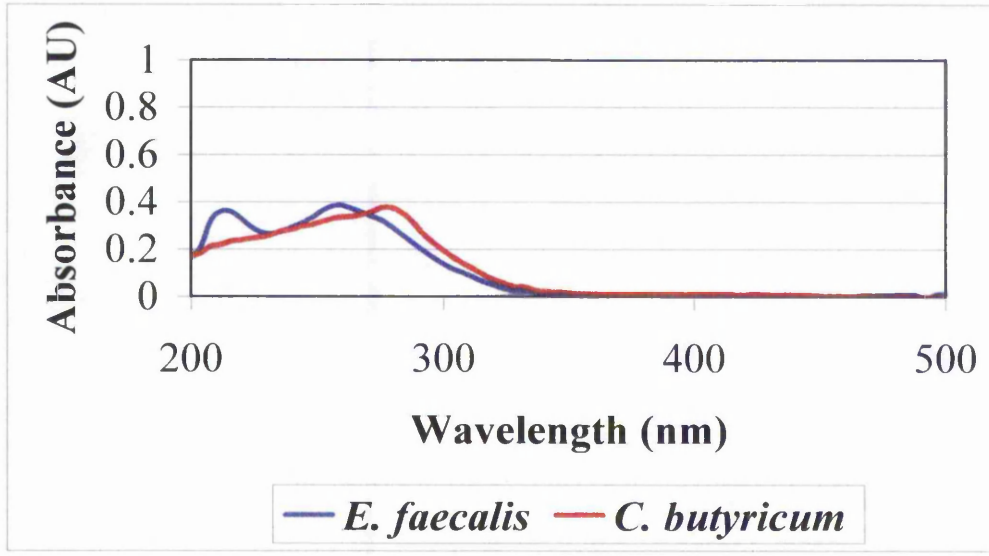
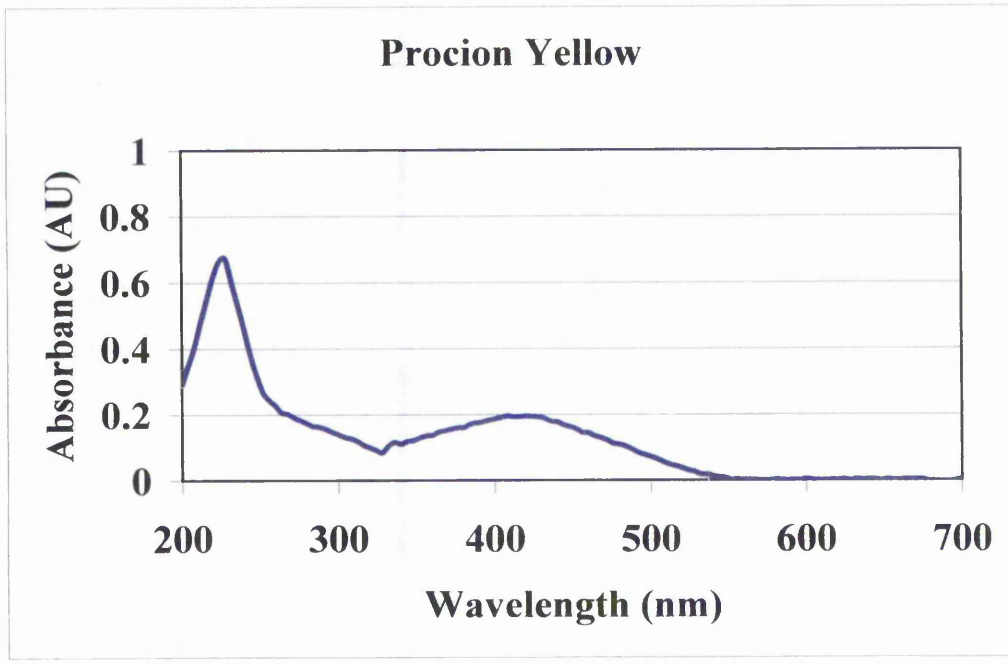


Figure 36. **UV/VIS profiles for (a) hydrolysed Procion Navy (0.002%w/v) and (b) hydrolysed Procion Navy decolourised by *E. faecalis* and *C. butyricum* (F5) (18h samples diluted 1 in 50).**



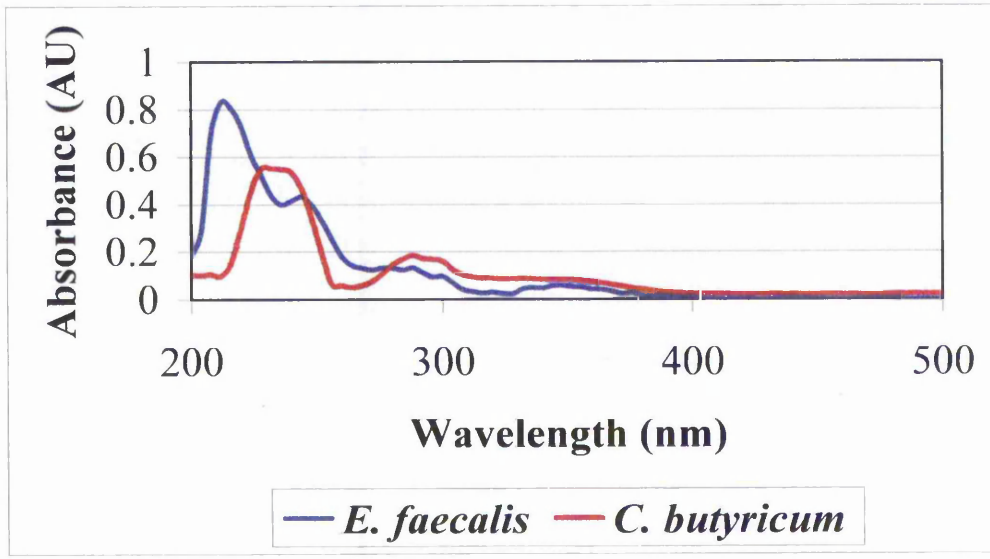
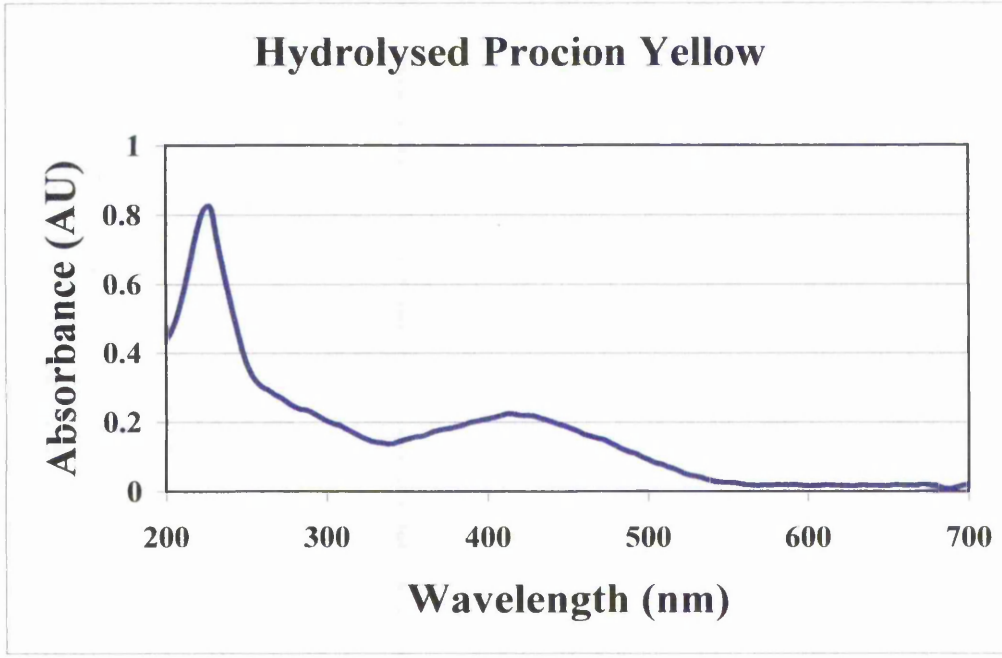


Figure 37. **UV/VIS profiles for (a) Procion Yellow parent dye (0.002%w/v) and (b) Procion Yellow parent dye decolourised by *E. faecalis* and *C. butyricum* (F5) (18h samples diluted 1 in 50).**



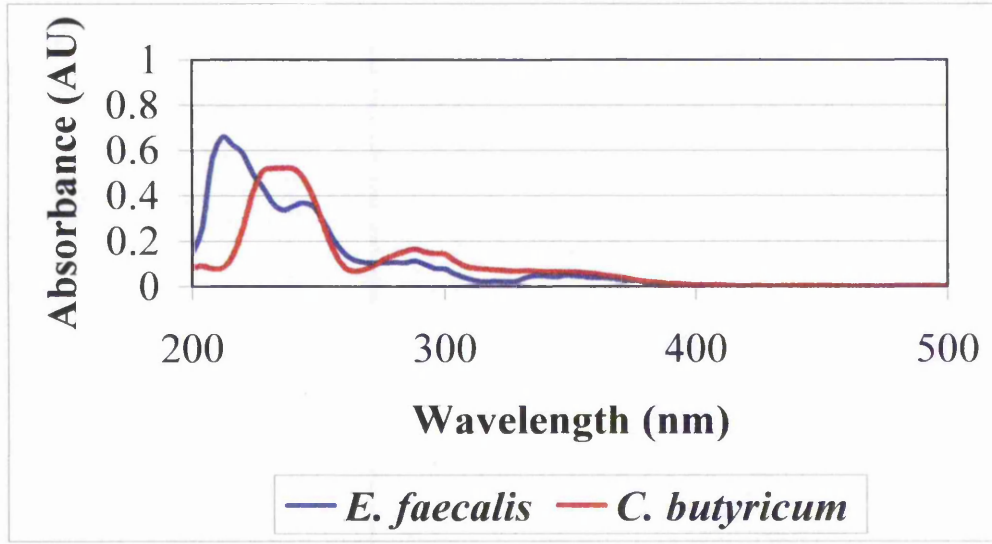
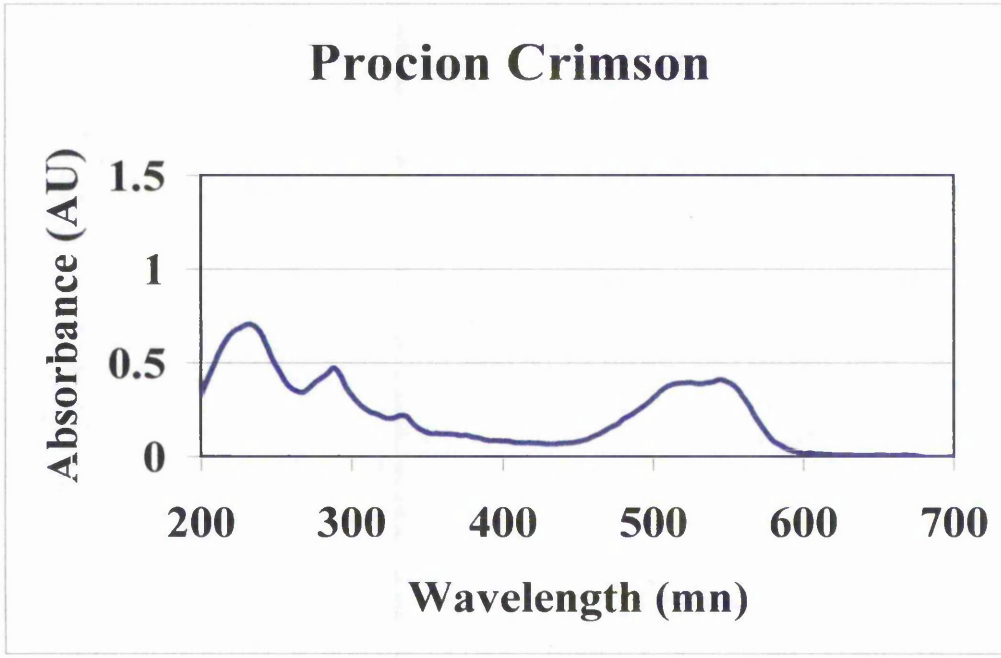


Figure 38. **UV/VIS profiles for (a) hydrolysed Procion Yellow (0.002%w/v) and (b) hydrolysed Procion Yellow decolourised by *E. faecalis* and *C. butyricum* (F5) (18h samples diluted 1 in 50).**



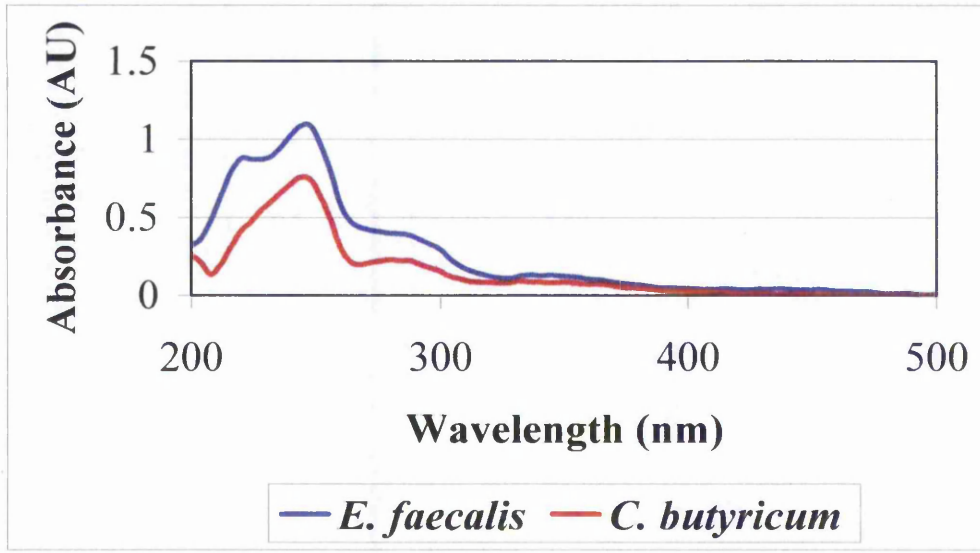
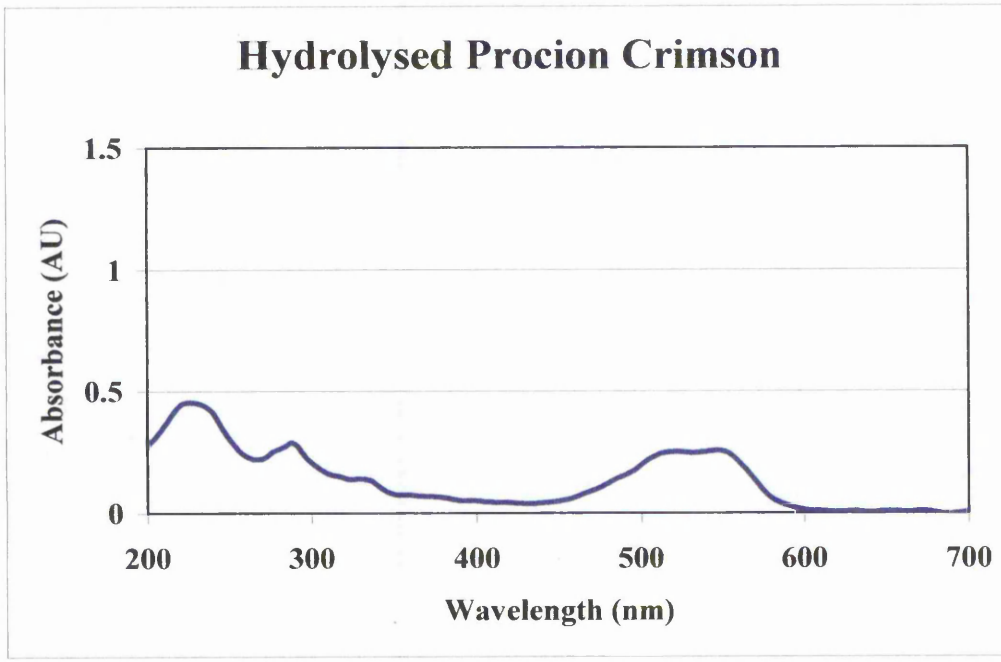


Figure 39. **UV/VIS profiles for (a) Procion Crimson parent dye (0.002%w/v) and (b) Procion Crimson parent dye decolourised by *E. faecalis* and *C. butyricum* (F5) (18h samples diluted 1 in 50).**



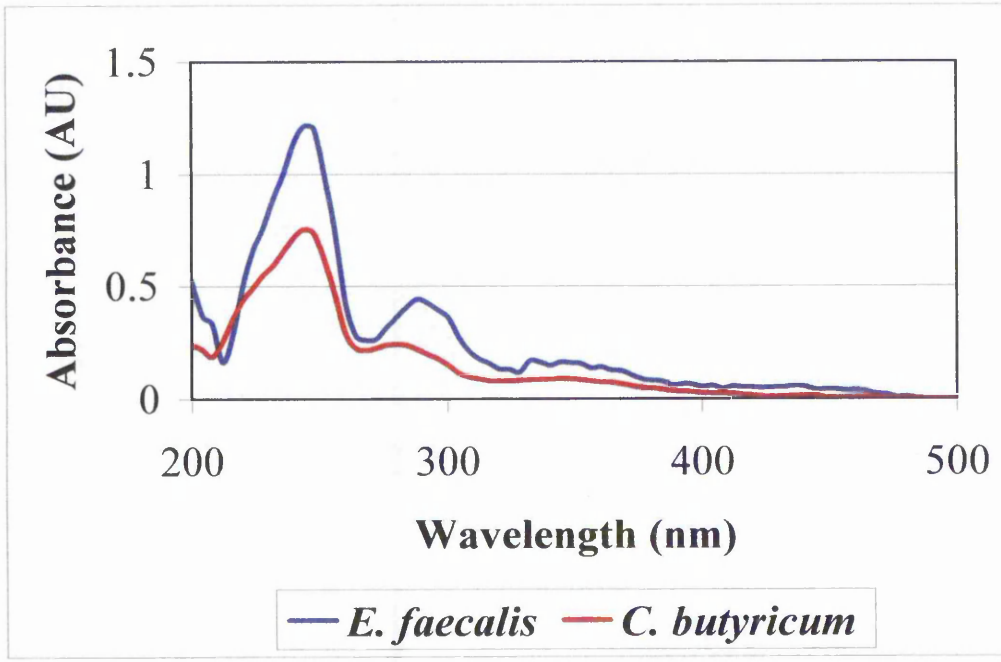


Figure 40. **UV/VIS profiles for (a) hydrolysed Procion Crimson (0.002%w/v) and (b) hydrolysed Procion Crimson decolourised by *E. faecalis* and *C. butyricum* (F5) (18h samples diluted 1 in 50).**

For Procion Navy (Figures 35 and 36) it can be seen that differences are apparent in the UV/VIS profiles for the parent and hydrolysed forms of the dye decolourised by the two strains. For the parent dye and *E. faecalis* two main product peaks are formed in the UV region at 210nm and 260nm with a slight shoulder on the 260nm peak at 280nm. The scan for the parent dye and *C. butyricum* (F5) however shows a broad absorbance over this region rather than defined peaks reaching a maximum at 290nm. The overall profiles for the hydrolysed dye are similar to those for the parent dye but with the broad peak for the *C. butyricum* decolourised sample having a more pronounced maximum at 290nm.

For Procion Yellow (Figures 37 and 38) differences are again apparent in the UV/VIS profiles for the parent and hydrolysed dye decolourised by *E. faecalis* and *C. butyricum* (F5). For the parent dye decolourised by *E. faecalis* four product peaks can be seen in the UV region at 210nm (main peak), 250nm, 290nm and 350nm. For this dye decolourised by *C. butyricum* (F5) the latter two peaks at 290nm and 350nm are seen together with a single broader peak at 240nm. The overall profiles for the hydrolysed dye are the same but with the scan for the *E. faecalis* samples showing a slight shoulder on the 210nm peak.

For Procion Crimson (Figures 39 and 40) the scans for samples decolourised by both strains have the same overall profile with minor differences in the low UV 200-210nm region. Peaks are observed at 240nm, 290nm and 350nm with the 240nm peak displaying a shoulder at 220nm which is more pronounced for *E. faecalis* decolourised parent dye. The profiles obtained for Procion Crimson with both strains resembles the scans for parent and hydrolysed of Procion Yellow with *C. butyricum* (F5). This suggests that Procion Crimson and Procion Yellow may have a common structural basis.

It is apparent from the comparison of the UV/VIS scans of the decolourised dyes that the products of decolourisation for Reactive Black (parent and hydrolysed forms) are the same for *E. faecalis* and *C. butyricum* (F5). For Procion Navy and Procion Yellow differences between the two strains are observed. The differences in the UV/VIS profiles may be due to different breakdown products produced by the two strains, products formed existing in different isomeric states or the dye structure being broken down to a greater extent by one of the organisms. It is thought unlikely that the differences would be due to the dye being incompletely decolourised by a particular

strain as no absorbance was observed in the visible region indicating complete decolourisation of the dye in the assay. The scans for Procion Crimson are similar overall for the two strains and also resemble the Procion Yellow scans for *C. butyricum* suggesting that the two dyes may have a common structural basis. The main disadvantage of UV/VIS scanning was that total absorbance profiles only could be obtained and the information gained with regards to individual decolourisation products present was limited. Comparison of decolourisation products for *E. faecalis* and *C. butyricum* (F5) can be more fully addressed by HPLC separation with diode array detection as detailed in Section 3.11.2.

3.11.2. HPLC.

The UV/VIS profiles of decolourised dyes have indicated that the decolourisation products of Reactive Black and Procion Crimson are the same for *E. faecalis* and *C. butyricum* (F5) whereas for Procion Navy and Procion Yellow they appear to be different. The UV/VIS Spectrophotometry absorbance profiles for individual components are additive according to Beer's law and as such will only give an overall profile for compounds present in a sample which absorbs UV radiation. To confirm the results from the UV/VIS scans selected decolourised samples were analysed by HPLC with diode array detection. The diode array detector was set such that for the main chromatogram detection of peaks was at 260nm and that for each peak detected a UV scan was produced over the range 200nm-370nm.

Chromatograms for decolourised Reactive Black are given in Figures 41 and 42. From the Figures it can be seen that the same two main product peaks (peak 1 around the R_t=7min region and peak 2 at around the R_t=21min region) are formed from the decolourisation of Reactive Black parent dye by both *E. faecalis* and *C. butyricum* (F5). For the *C. butyricum* decolourised Reactive Black (Figure 42) a third peak is seen eluting at R_t=3.4min. This peak was thought to be a *C. butyricum* bacterial metabolite and not a compound originating from the dye as a similar peak was observed in control samples from an assay prepared without dye (Figure 45). For hydrolysed Reactive Black (Figures 43 and 44) two products are again formed by each strain at around R_t=20min and R_t=23min. Each product peak for the *E. faecalis* reduced dye had an identical UV spectra to the corresponding peak for the *C. butyricum* (F5) decolourised samples for both the parent and hydrolysed forms of the dye. These results confirm that

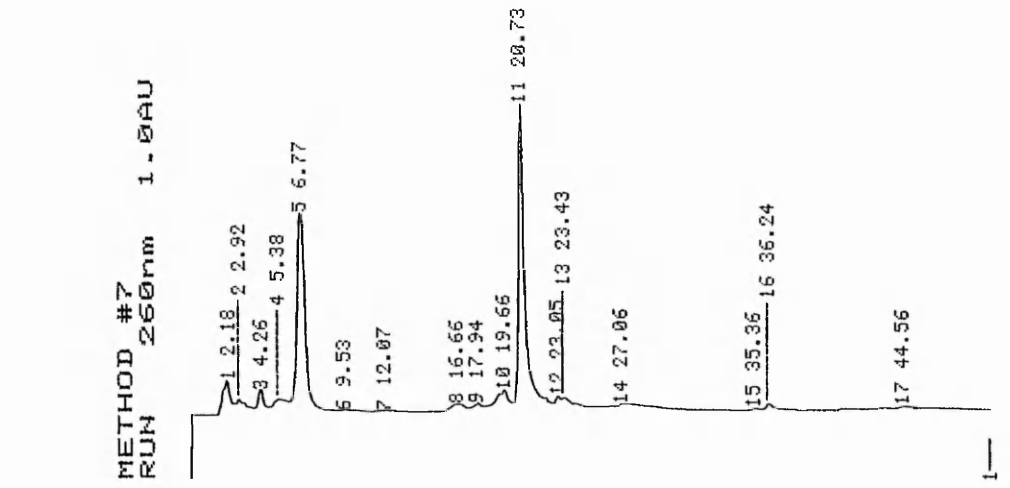
the decolourisation of Reactive Black by *E. faecalis* and *C. butyricum* (F5) yields the same products.

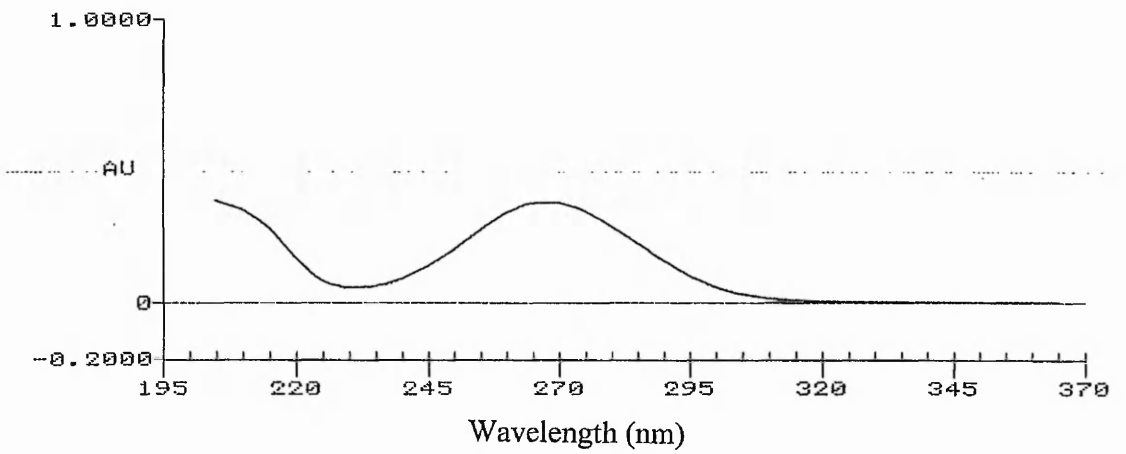
Chromatograms for Procion Navy are given in Figures 46 to 49. For the parent dye (Figures 46 and 47) it can be seen that two main product peaks at R_t=20.5min and R_t=25.5min are common to samples decolourised by both strains. The R_t=3.2min peak for the *C. butyricum* decolourised Procion Navy is present in the chromatogram for control samples as shown in Figure 45. Some differences in the two chromatograms are however apparent. The R_t=25.5min peak in the *C. butyricum* (F5) decolourised sample is double the height of the corresponding peak in the *E. faecalis* decolourised sample with a reduction in height seen for the peak eluting in the R_t=20-20.5min region. The minor product peaks seen at R_t=30min and R_t=30.6min for the *C. butyricum* (F5) decolourised dye are absent in the *E. faecalis* reduced sample. The peaks eluting at R_t=20.5min and R_t=25.5min that are common to samples decolourised by both strains have identical UV spectra which would suggest that the two strains are reducing the dye to the same products. The difference in intensities of the peaks in each chromatogram was not expected as assays for each strain had identical initial dye concentrations and samples were injected directly for HPLC without dilution. The difference in relative amounts of these two products and the absence of peaks in the R_t=30-30.6min region for *E. faecalis* reduced Procion Navy suggest that there are differences in decolourisation products for the two bacterial strains.

For hydrolysed Procion Navy (Figures 48 and 49) the chromatograms show three main products of decolourisation at R_t=20.5min, R_t=21.5min and R_t=25.5min and are the same for both *E. faecalis* and *C. butyricum* (F5) reduced dye. As for the parent dye the peaks for *E. faecalis* reduced hydrolysed dye have identical UV spectra to the corresponding peak in the *C. butyricum* (F5) reduced hydrolysed dye sample. Differences in peak height are again apparent as for the decolourised parent dye with the R_t=21.5min and R_t=25.5min peaks being of greater height in the *C. butyricum* reduced samples.

From the HPLC results it appears that for Procion Navy minor differences are apparent in decolourisation products for the two strains. Differences were observed in the relative amounts of decolourisation products formed although concentration could not be quantified due to the lack of an appropriate analytical standard and for the parent dye decolourised by *C. butyricum* additional product peaks were observed. These variations may account for the differences in UV spectra observed in Figures 35 and 36.

The R_t=20.5min peak from the decolourised Procion Navy has the same retention time and an identical UV spectra as the substituted H-acid component in the decolourised Reactive Black samples as discussed in Section 3.10. This would suggest that Procion Navy had this structural component in common with Reactive Black.





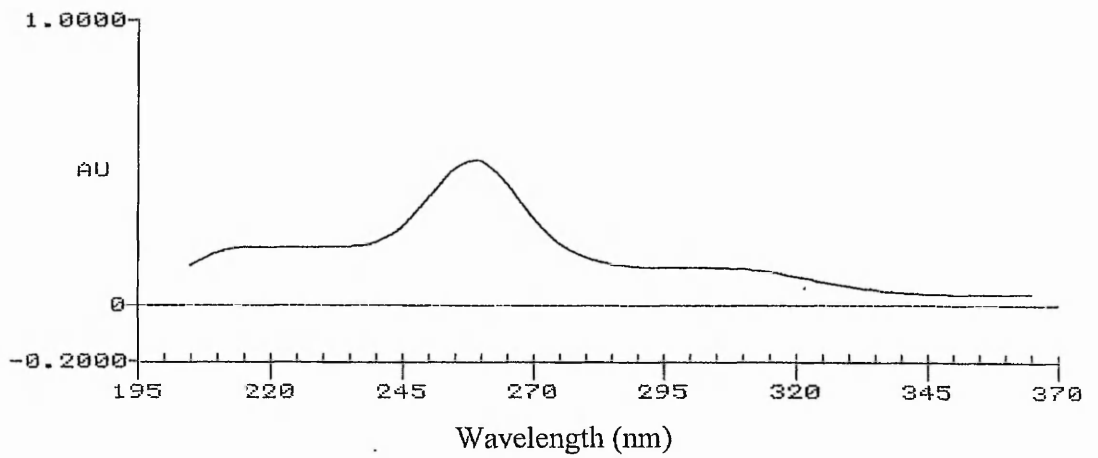
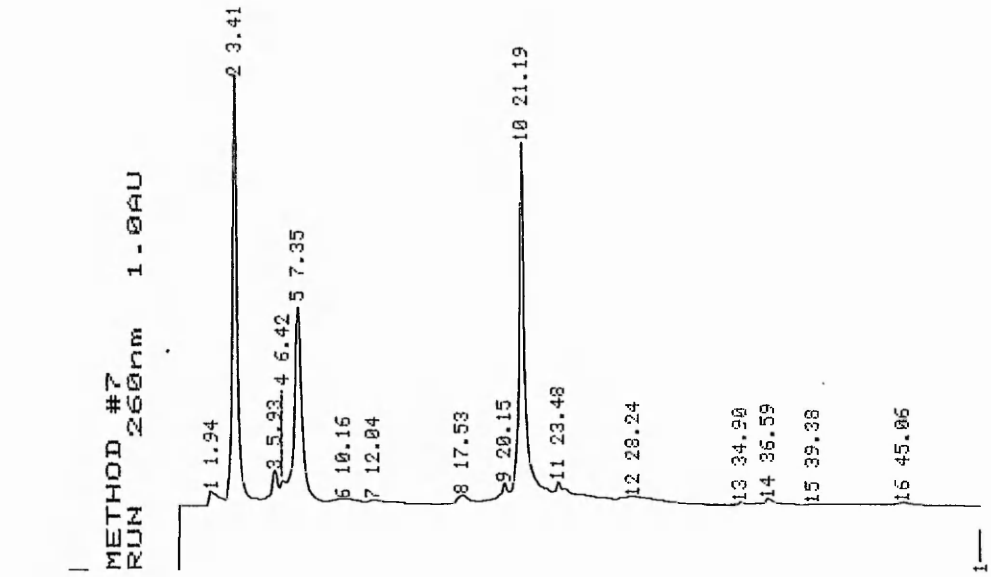
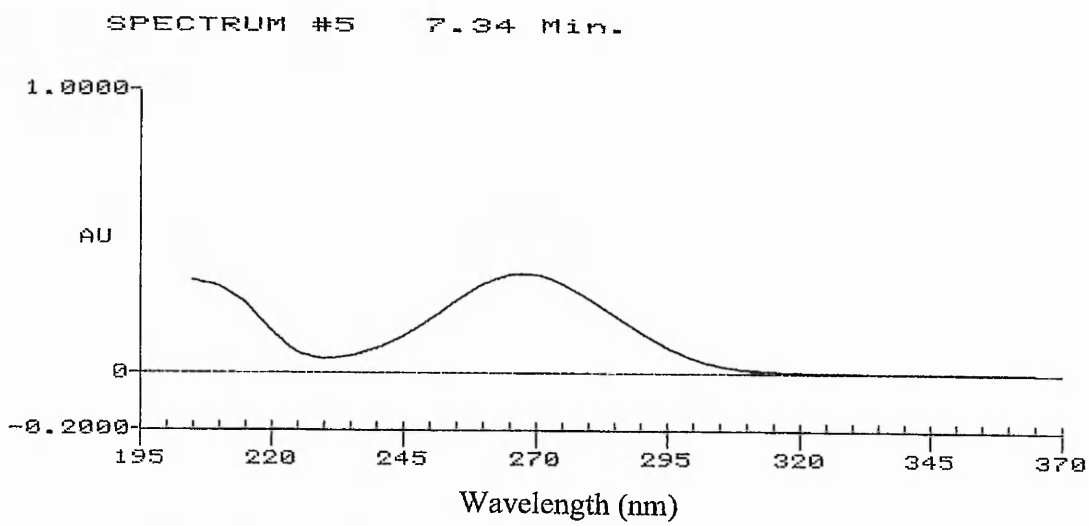


Figure 41. Chromatogram and peak spectra of Reactive Black decolourised by *E. faecalis*. Detection at 260nm. Injection volume 20µl.





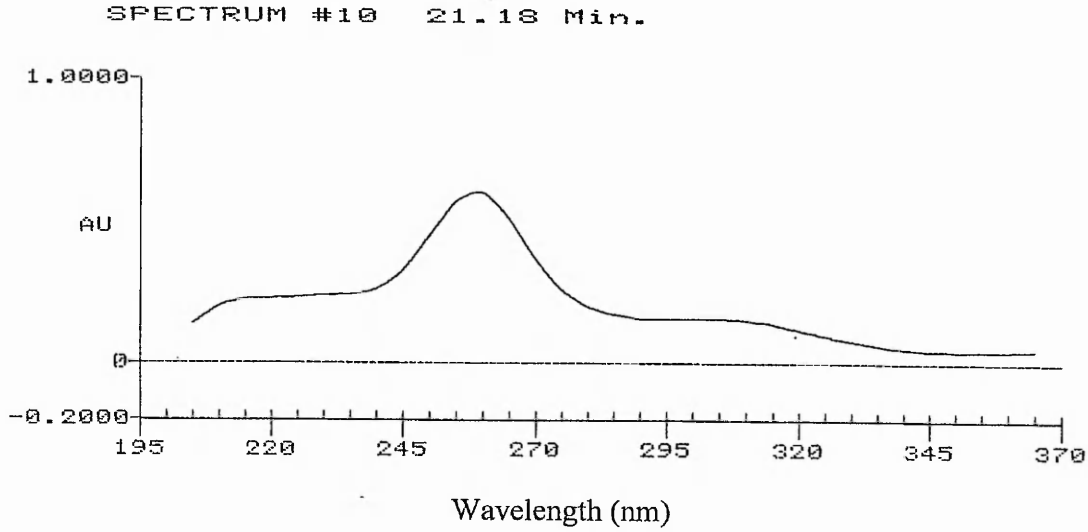
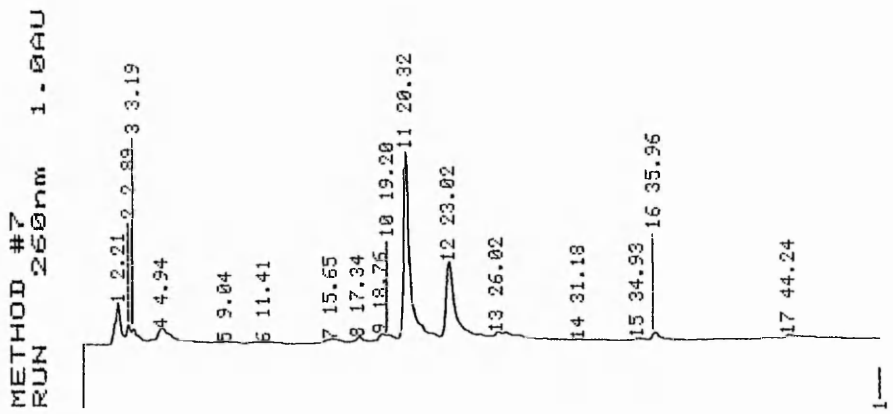
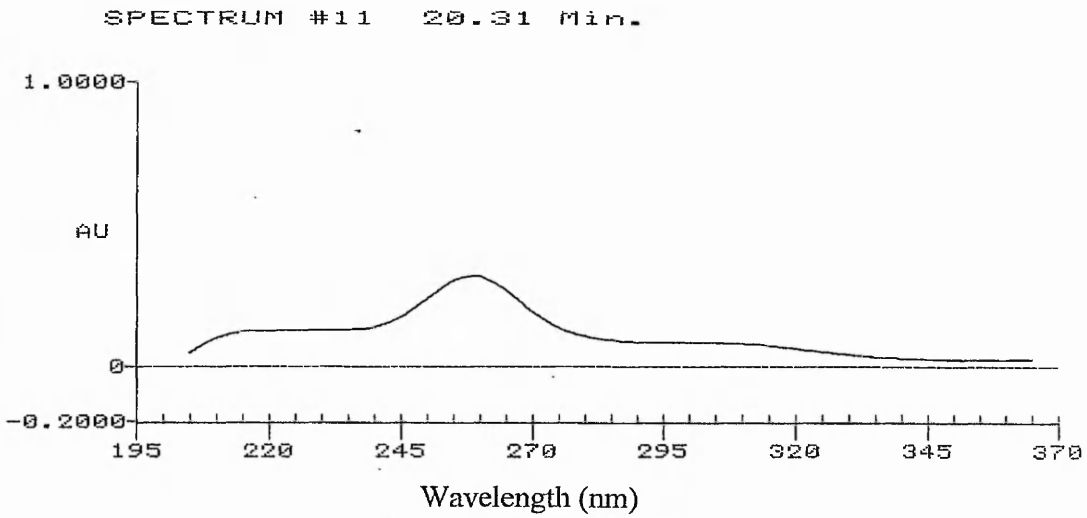


Figure 42. **Chromatogram and peak spectra of Reactive Black decolourised by *C. butyricum* (F5). Detection at 260nm. Injection volume 20µl.**





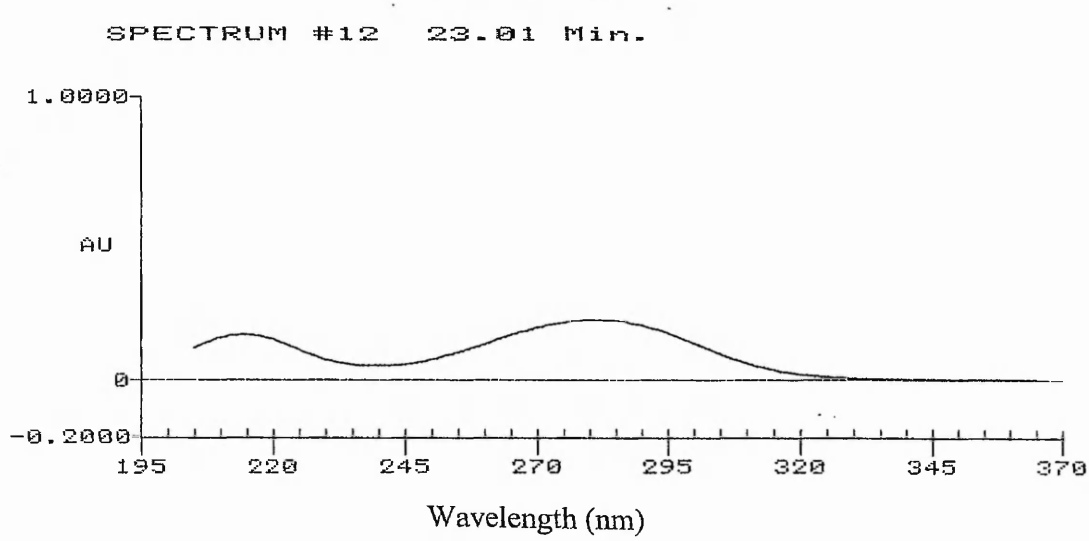
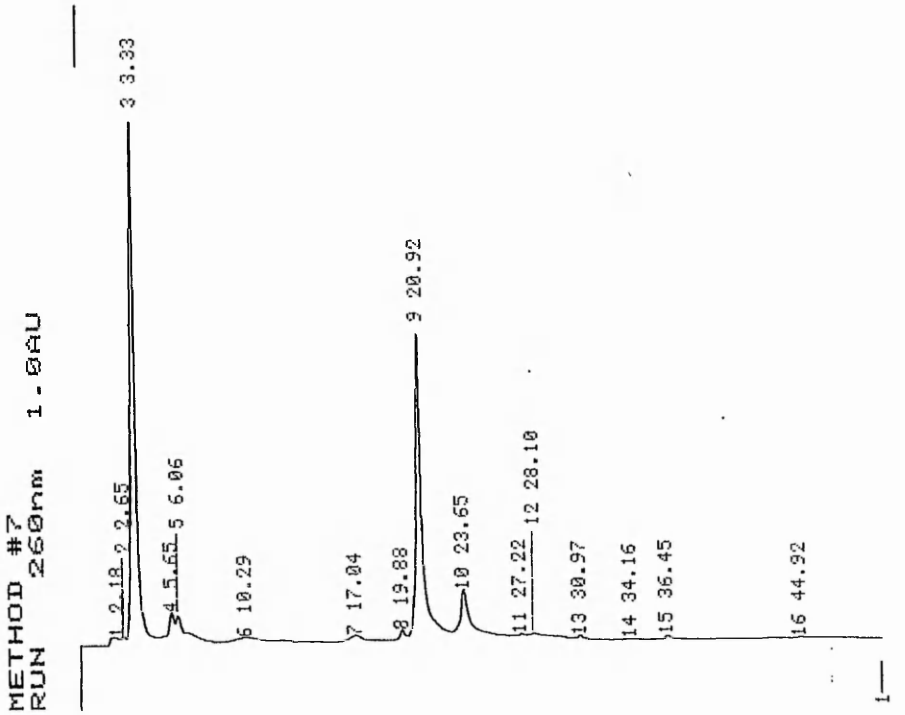
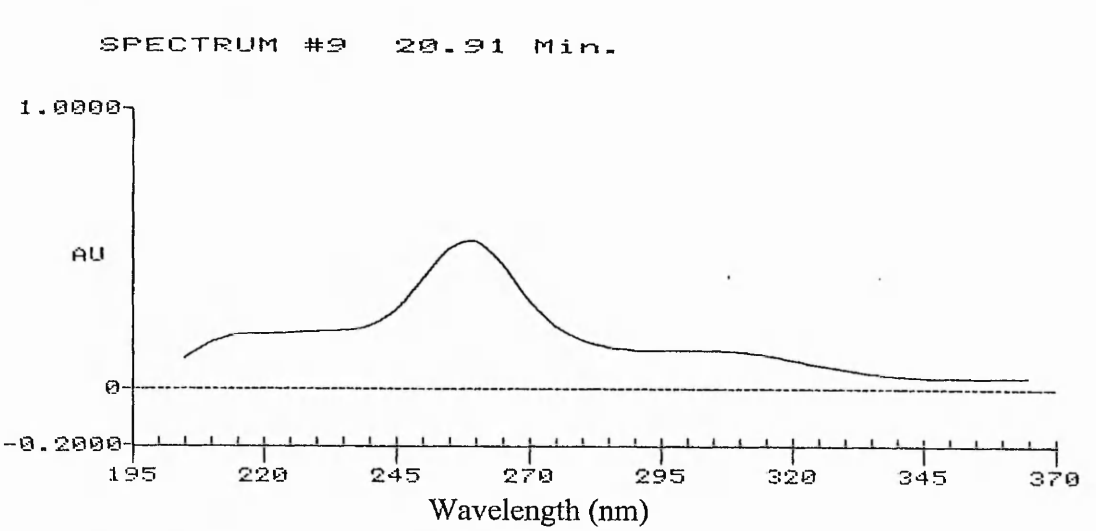


Figure 43. Chromatogram and peak spectra of hydrolysed Reactive Black decolourised by *E. faecalis*. Detection at 260nm. Injection volume 20µl.





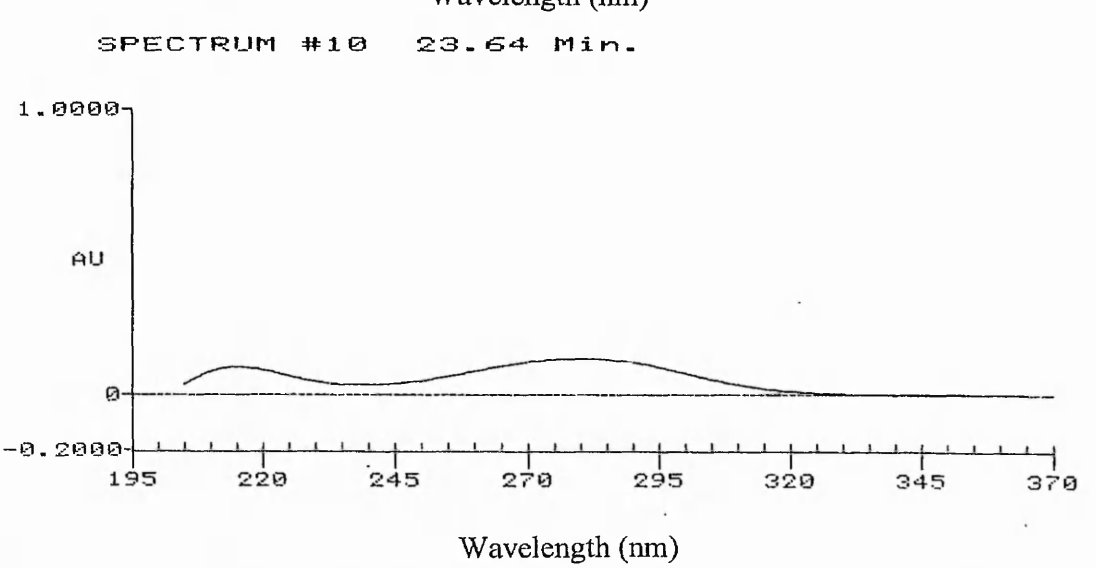
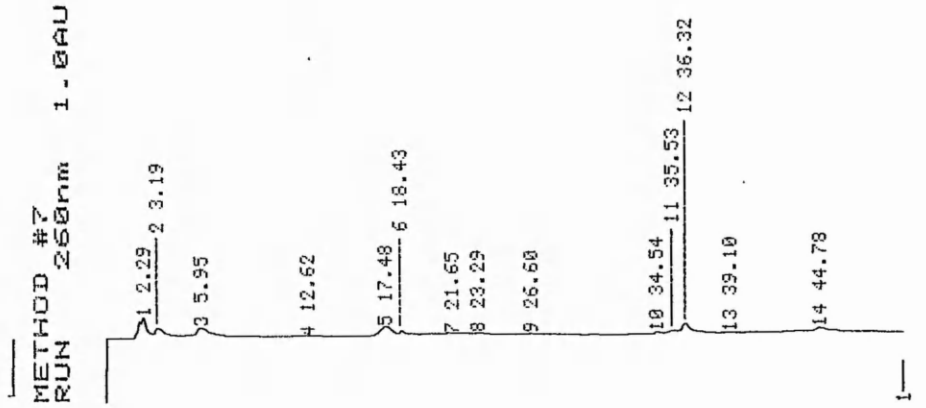


Figure 44. **Chromatogram and peak spectra of hydrolysed Reactive Black decolourised by *C. butyricum* (F5). Detection at 260nm. Injection volume 20μl.**

(a)



(b)

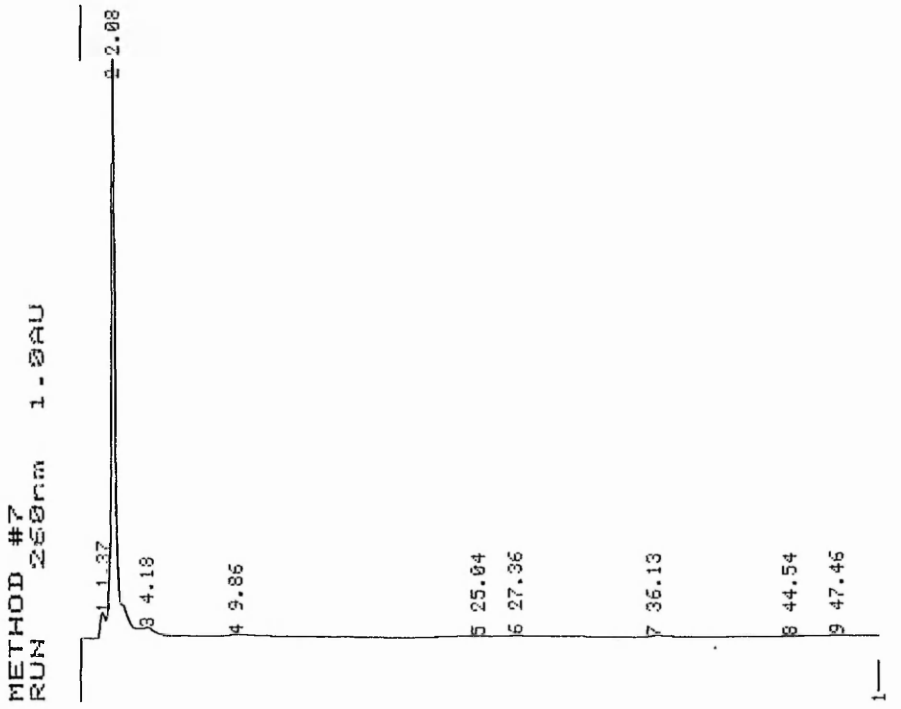
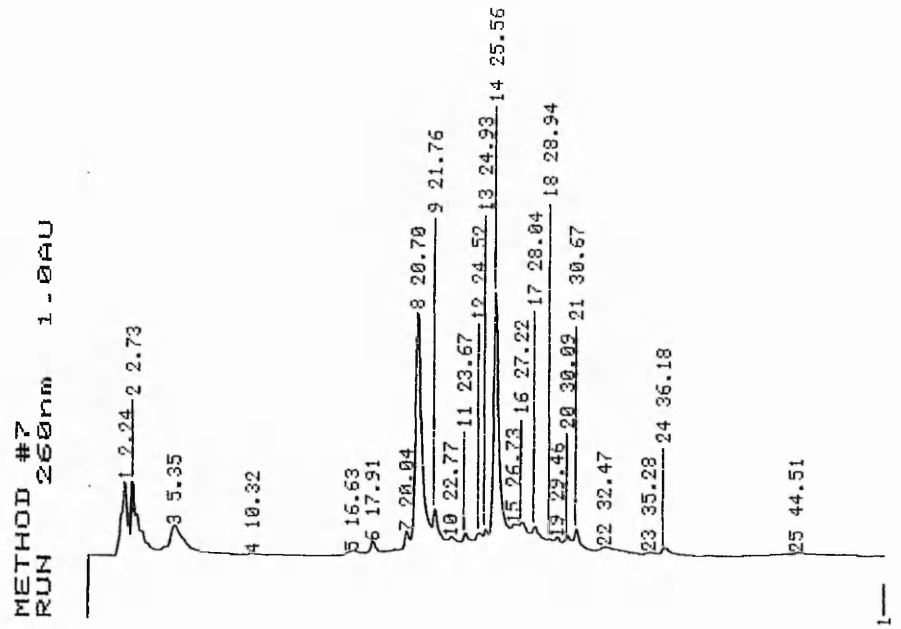
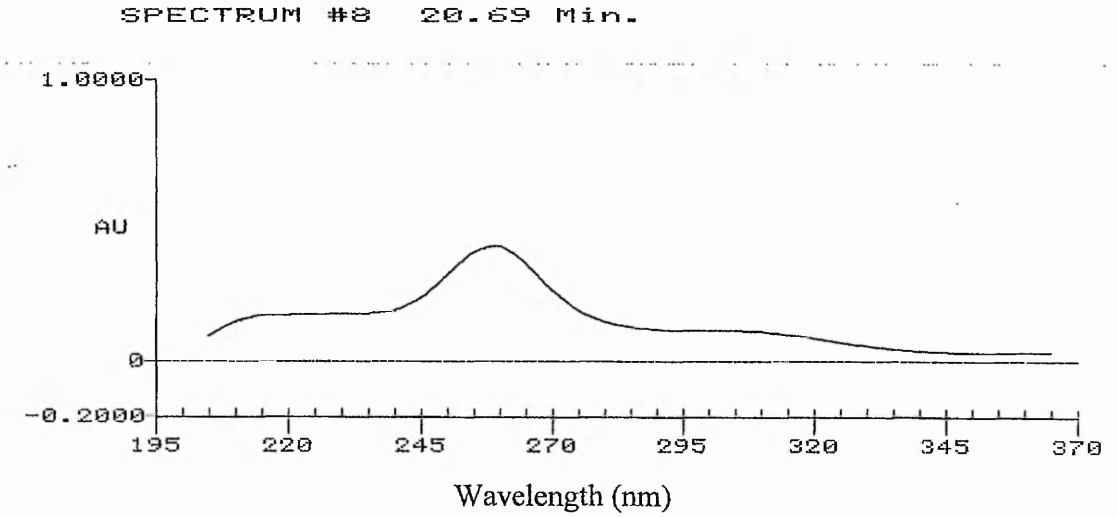


Figure 45. **Chromatogram of samples from control assays prepared without dye for (a) *E. faecalis* and (b) *C. butyricum*. Detection at 260nm. Injection volume 20μl.**





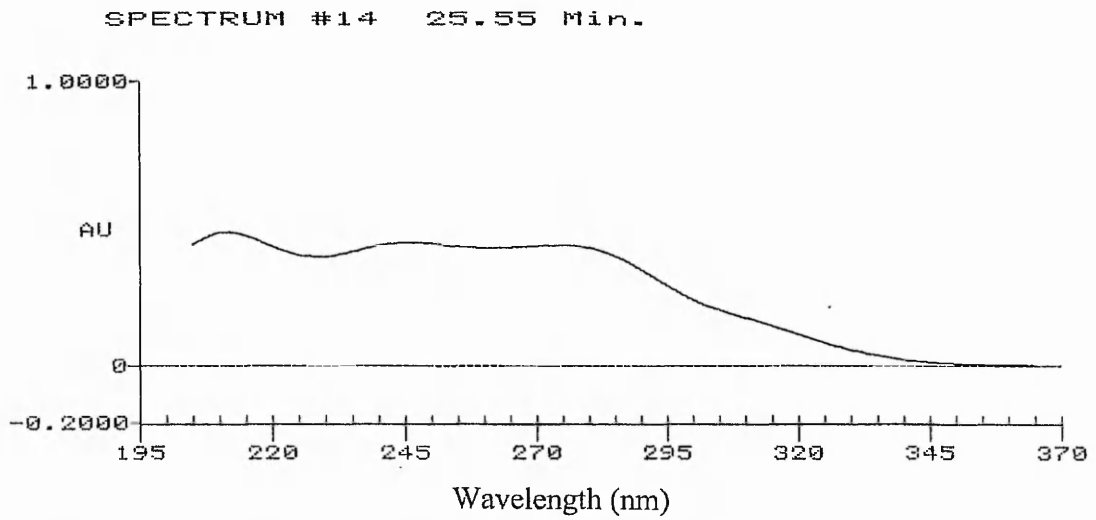


Figure 46. Chromatogram and peak spectra of Procion Navy decolourised by *E. faecalis*. Detection at 260nm. Injection volume 20µl.

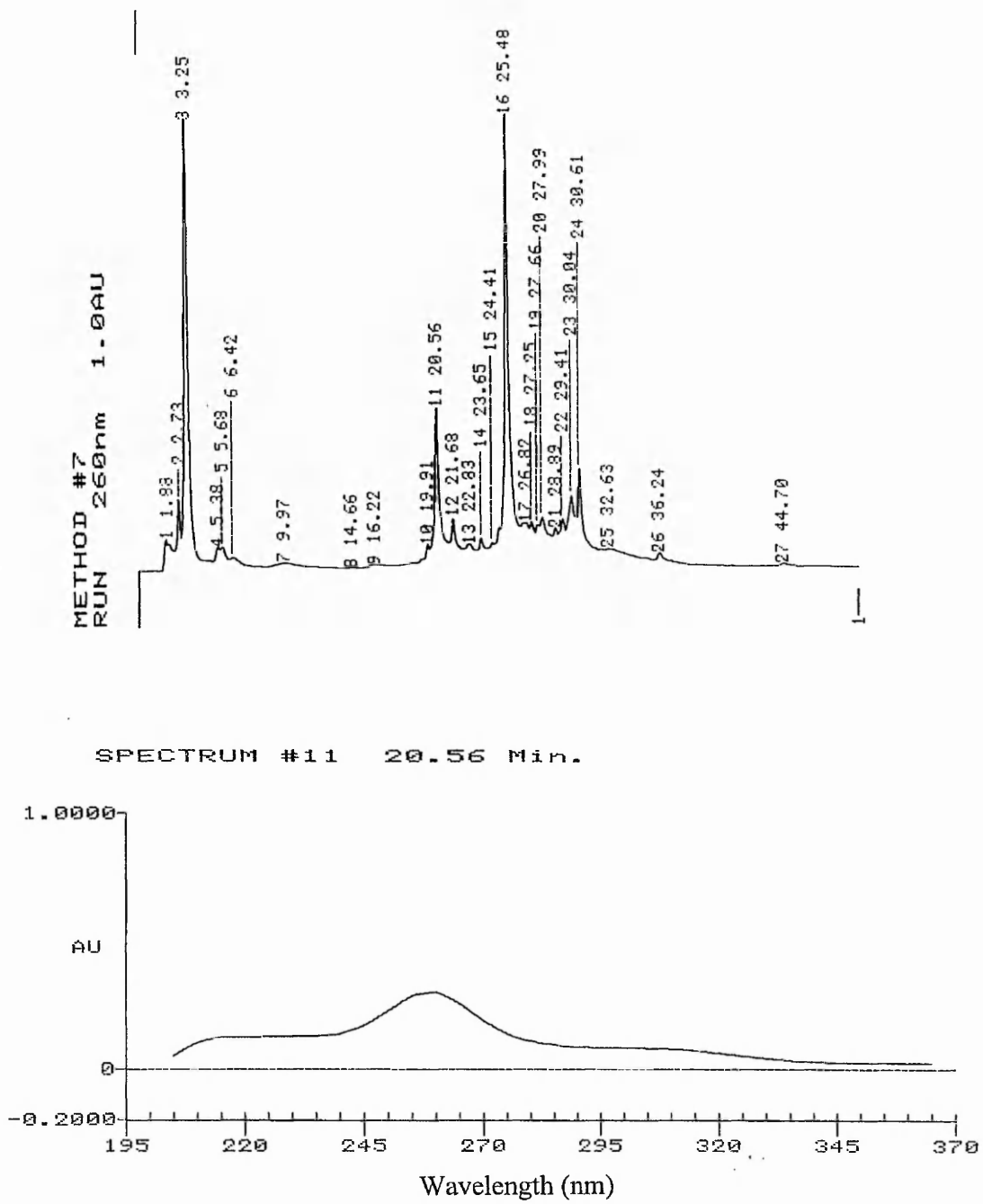
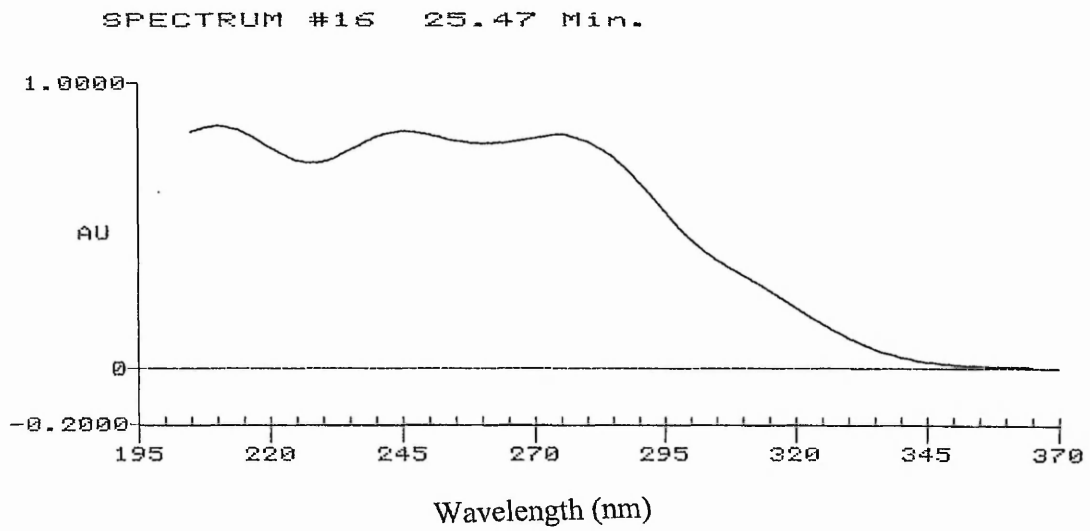


Figure 47. **Chromatogram and peak spectra of Procion Navy decolourised by *C. butyricum* (F5). Detection at 260nm. Injection volume 20μl.**



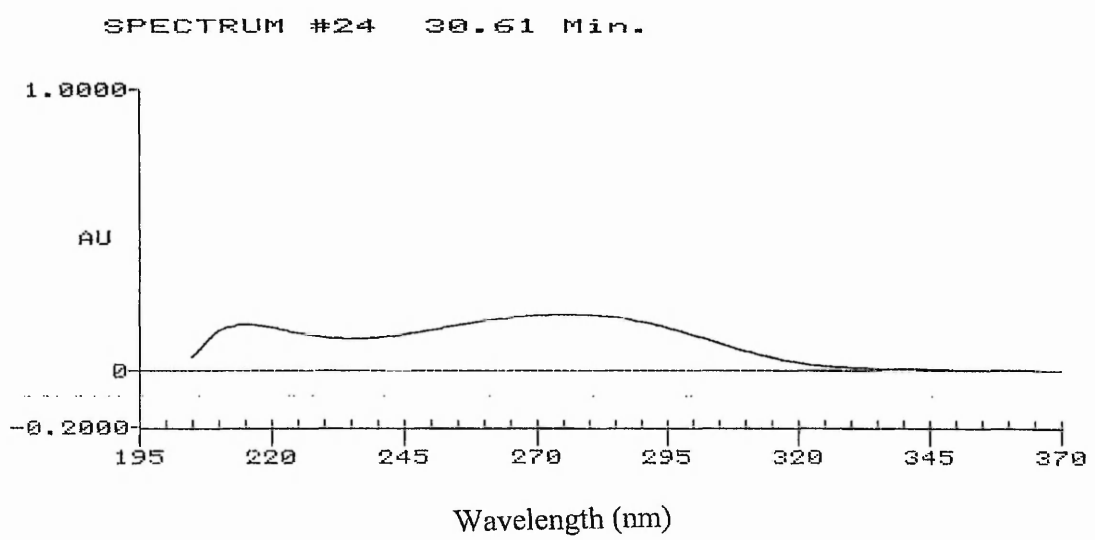


Figure 47. Continued.

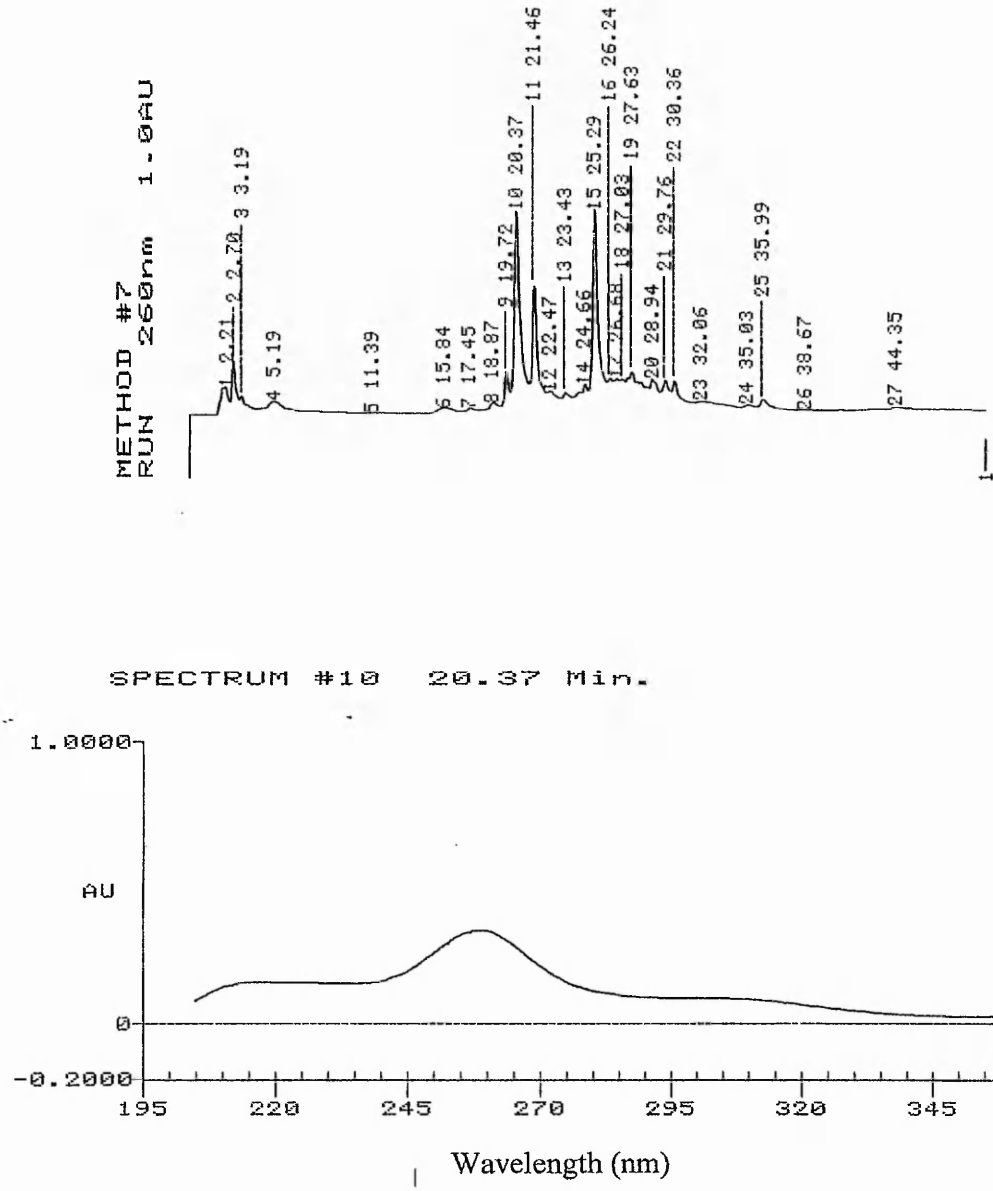
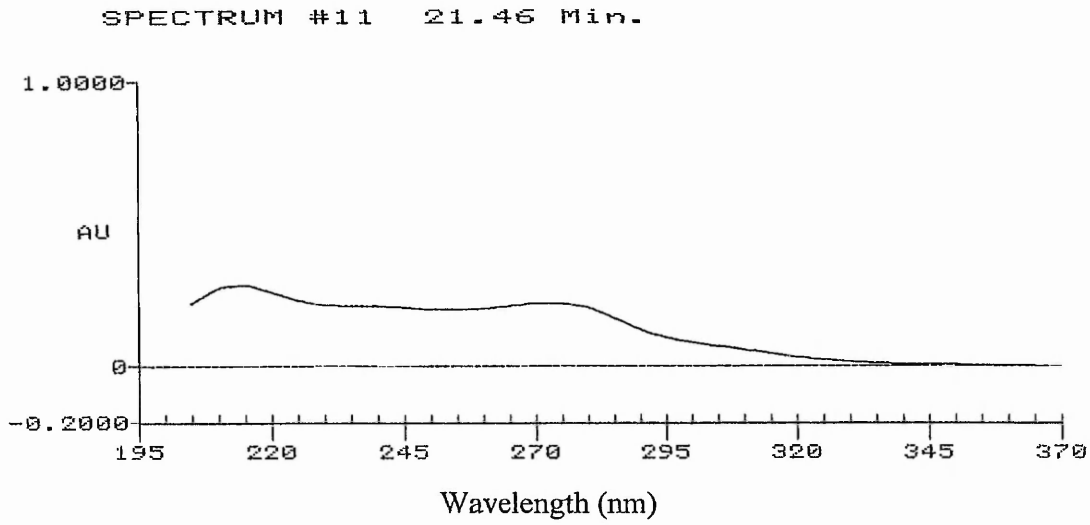


Figure 48. **Chromatogram and peak spectra of hydrolysed Procion Navy decolourised by *E. faecalis*. Detection at 260nm. Injection volume 20μl.**



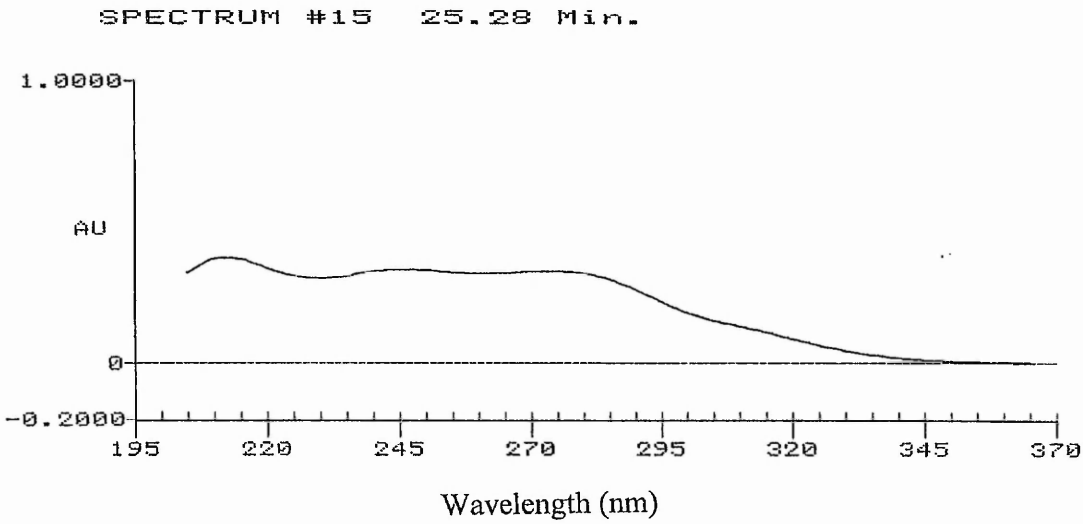


Figure 48. Continued.

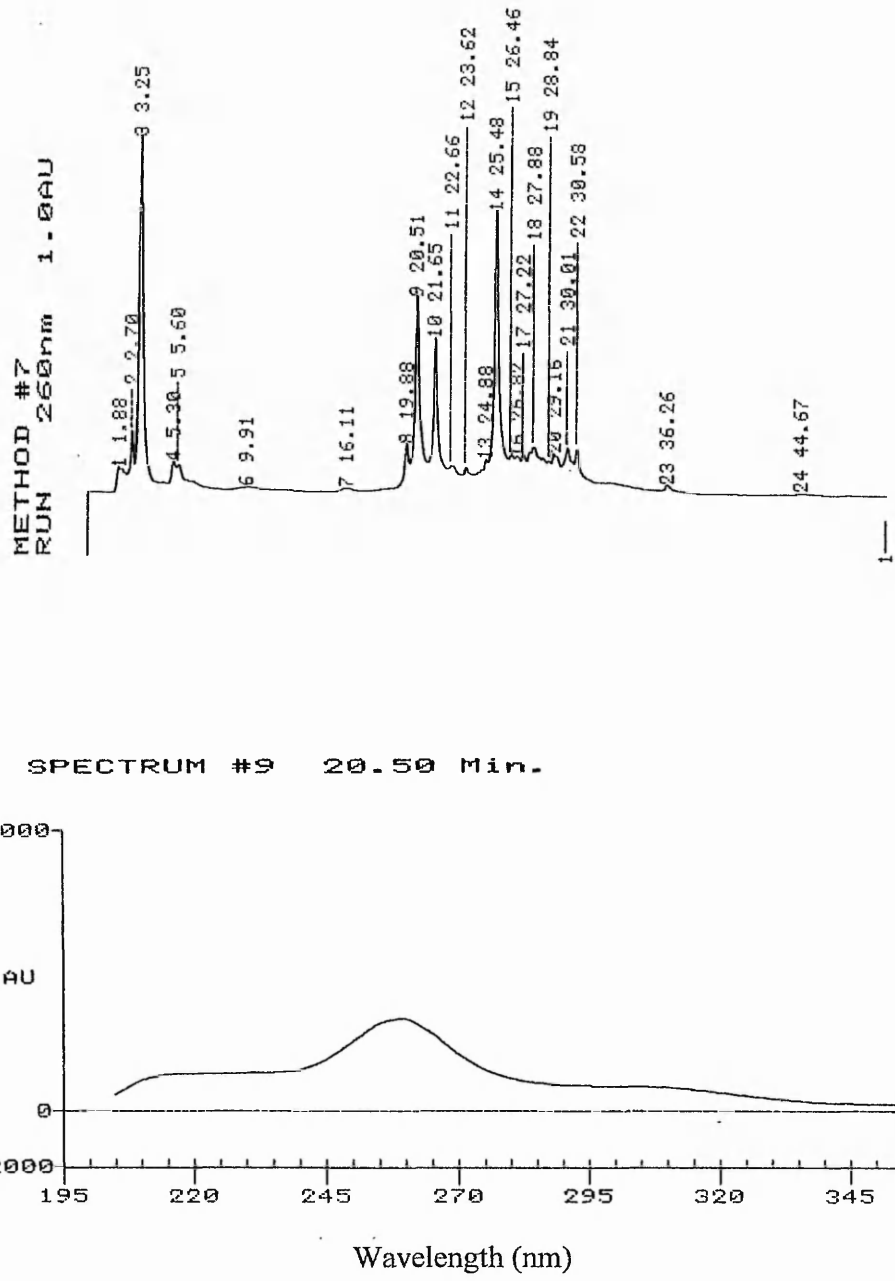
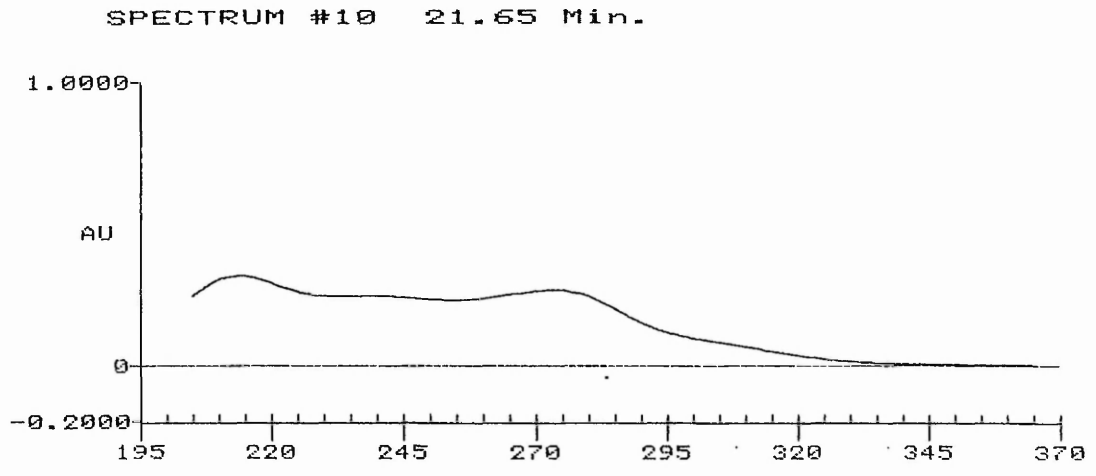


Figure 49. **Chromatogram and peak spectra of hydrolysed Procion Navy decolourised by *C. butyricum* (F5). Detection at 260nm. Injection volume 20μl.**



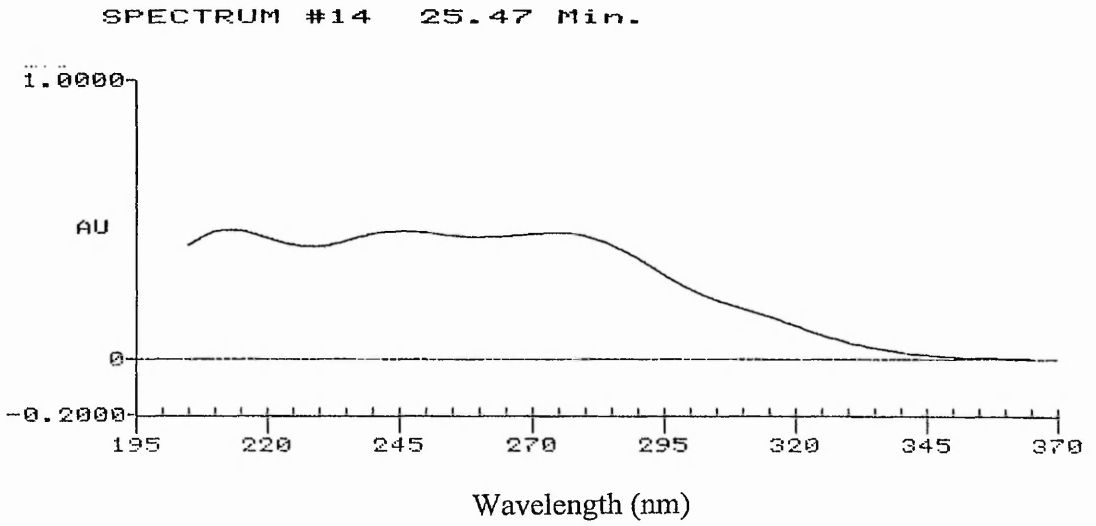


Figure 49. Continued.

3.12. Structural determination of decolourisation products.

The reduction products of the Reactive Black model dye in this study can be predicted from the dye structure (Figure 4) and are thought to be a di-amino H-acid and a vinyl sulphone side chain. It has been shown that for Reactive Black two end products of decolourisation are produced which can be separated by HPLC (Figures 41 to 44). If a quantity of reduced dye can be separated on a semi-preparation scale the products could be purified for further structural analysis such as NMR or mass spectrometry.

A decolourisation assay was prepared with *E. faecalis* and Reactive Black as described in Section 2.8. After decolourisation (120min incubation period) the assay contents were centrifuged to remove the cells and the supernatant reserved for analysis. All sample supernatants were analysed on the day of preparation. The supernatant was injected repeatedly onto the HPLC column as detailed in Section 2.3.2. and the two product fractions were collected from the column exhaust as they eluted. Post collection the fractions were concentrated by running a stream of nitrogen over the surface in order to reduce the total volume. The HPLC method suffered long run times (80min per injection) limiting the quantity of material that could be prepared and difficulties were encountered in reducing the volume of the collected material to concentrate the compound of interest due to the large quantity of phosphate present. Separation of the decolourised products by HPLC was attempted with a lower buffer concentration (10mM phosphate buffer)-methanol gradient and an aqueous-methanol gradient but for both systems chromatography was unstable and reproducible separation could not be achieved. Where fractions were collected these could not be used for mass spectrometry analysis due to the high quantities of phosphate present. An alternative separation technique such as TLC was considered as an option for preparation of decolourisation products being less limited in the quantity of material that could be separated and a solvent system employed that did not involve a buffer salt.

Decolourisation assays were prepared as above for *E. faecalis* and Reactive Black (parent and hydrolysed). Decolourised samples were spotted along the length of a 20x20cm TLC plate as in Section 2.3.3. Post development the plates were air dried and viewed under UV light. The fluorescent sample bands were marked and scraped from the surface for extraction. The compounds of interest were extracted into methanol as this could be evaporated easily to concentrate the test material. A blank portion of TLC plate was developed and treated in the same way as the sample bands to provide a

reference reagent blank. The hR_f values for sample bands were determined and are given in Table 24. Also included are the hR_f values for a sample from a control assay of bacterial cells in buffer without the addition of dye. From Table 24 it can be seen that more than two reduction products are observed in the chromatographed sample which was not as expected from the HPLC results in Section 3.10.

As the numbers of products observed was not as expected the extracted samples were UV scanned against the reagent blank (portion of the TLC plate developed and extracted as for samples) in order to provide additional information. The UV profiles for all bands scanned are given in Appendix 5. It was found that one band in the reduced parent dye sample (hR_f 52, yellow) and two bands in the hydrolysed dye sample (hR_f 51, yellow and hR_f 53, blue) had similar UV profiles to the bands present in the control assay and were thought to be *E. faecalis* metabolites. Only one band from each reduced dye sample (hR_f 76 (green) for the parent dye and hR_f 73 (pink) for the hydrolysed dye) had matching UV spectra. All the remaining bands with the exception of hR_f value 67 (yellow) for reduced hydrolysed Reactive Black had common features in their profiles but were not the same. The UV spectra obtained from the TLC separation did not match those for the reduction products separated by HPLC (Figures 41 to 44). Some similarity was observed however between the UV profile obtained for the H-acid peak (Figure 41) and the profile for the band with hR_f value 67 (yellow) from reduced hydrolysed Reactive Black, both profiles displaying a peak at 260nm.

This contradiction to the HPLC results from Section 3.11. was thought to be due to several reasons. The decolourisation products expected are substituted amines. The amine groups will be basic and as such may have the potential to become protonated to varying extents by interactions with the acidic free silanol groups on the surface of the HPLC column packing and the TLC plate material. In the HPLC system a modifier is present (i.e. the phosphate buffer 100mM, pH6.7) which enables the two product peaks to be detected and reproducibly separated. At this pH and buffer concentration the amine groups may be fully protonated and interactions with the packing material are minimised. This is confirmed by the unstable chromatography observed when trying to separate the reduced dye with a lower concentration of phosphate buffer and the aqueous-methanol gradient as discussed earlier. In the TLC system the modifier is not present and as the basic amine groups may not be fully protonated there is the possibility that these will interact with the acidic silanol groups in the plate material.

Table 24. Decolourisation products of Reactive Black (parent and hydrolysed) separated by TLC and categorised by their hRf values and colours[a].

hR_f category[b]	hR_f values : Reactive Black	Hydrolysed Reactive Black	Control assay[c]
1			46 yellow*
2	52 yellow*	51 yellow*	
3		53 blue*	
4			57 yellow*
5	67 yellow#		
6		69 yellow	
7	70 blue#		
8		73 pink*	
9	76 green*		
10	80 blue#		
11		85 blue#	
12		90 blue#	

KEY
(a) Fluorescent colours observed under UV light.
(b) Category numbers are nominated to represent hR_f values in blocks of similar values.
(c) Assay components: *E. faecalis*, buffer, glucose, no dye.
**UV spectra match
#Similar spectra

This interaction may give rise to multiple products due to the different amine groups interacting with the plate. For the two predicted end products of decolourised Reactive Black there are four amine groups capable of independent interaction (one for the sulphone side chain and three for the H-acid component). This may explain why four products are seen for *E. faecalis* reduced Reactive Black (parent and hydrolysed) rather than two as seen with the HPLC separation. To obtain a separation showing two products a modifier would be needed to protonate the amine groups and stop the silanol group interaction. This would not solve the problem of obtaining pure samples of the decolourised compounds without any salt present. If the differing numbers of products are due to amine interaction it would be expected that UV profile matches would be observed for some of the bands due to the same compounds with differing degrees of protonation migrating to varying extents. The TLC separations did therefore suffer from the same chromatography problems as the HPLC analysis in that a modifier was needed and pure samples of decolourised material could not be obtained for further structural analysis.

If the material removed from the plate did not contain a single component due to co-elution or were contaminated by adjacent sample bands the UV spectra of the bands

may not match and may be different to the spectra of the HPLC separated peaks obtained using the diode array detector. Some matches were observed between bands and others displayed similarities as discussed earlier. The shortest distance measured between consecutive sample bands was 4mm and as such it was thought unlikely that contamination of adjacent sample bands would occur during the physical removal of plate material. It is not known whether the methanol the compounds were extracted into or the TLC solvent used were affecting the structure of the reduction products in any way. TLC separation of unreduced Reactive Black produced multiple bands for both the parent and hydrolysed forms (Table 16, Section 3.1) and although one band in each was found to be prominent other minor bands were observed showing some absorbance at 600nm. The minor bands were thought to be traces of partially coupled dye intermediates and it is not known whether any of the bands observed on the TLC plates for decolourised dye samples were due to these compounds. Another explanation for the difference between HPLC and TLC results may be that the bacteria were producing metabolites which in the HPLC method may be eluting at the solvent front and hence do not interfere with the separation of the dye decolourisation products. In the TLC method separation of these metabolites may be achieved and thus they appear as fluorescent bands on the TLC plate. It has been assumed that the presence of a dye in an assay does not affect the normal metabolism of the bacteria employed. If the dye does cause a production of alternative metabolites then comparison of the control assay bands to decolourised dye bands may not fully distinguish between compounds originating from the dye. It is however thought most likely that the differences between the HPLC and TLC results was due to the amine group interaction with the HPLC column stationary phase and the TLC plate material.

Two other methods (FTIR and scanning fluorimetry as detailed in Sections 2.3.4. and 2.3.5) were employed to fingerprint the fluorescent bands from the TLC plates but both yielded limited information. The FTIR scans were employed in order to gain information about the decolourisation products by determining the functional groups present on their structure. This approach was limited as the only information gained from the scans was that the test compounds were sulphonated aromatic species. This confirms that some of the bands observed on the TLC plates may have the same structure but different hR_f values due to the amine group interaction. The scanning fluorimetry was employed to characterise the bands by their excitation and emission wavelengths. This approach was also abandoned due to difficulty in determining the

wavelengths for some samples due to more than one maxima for excitation and emission being observed.

Chemical reduction of the azo bond was considered to be an alternative way of preparing decolourised material for analysis. This would eliminate the possibility that the contradiction in chromatography results were due to the presence of bacterial metabolites. The chemical reducing agent used was sodium dithionite. The quantity of sodium dithionite required to produce decolourisation of Reactive Black was determined experimentally as given in Section 3.2. Solutions of 0.05% (w/v) Reactive Black and hydrolysed Reactive Black were prepared in distilled deionised water previously degassed with nitrogen. The sodium dithionite (3.9mg/10ml for the parent dye and 3.5mg/10ml for the hydrolysed dye) was added directly to the solutions as a powder and mixed avoiding aeration. The decolourised samples were analysed immediately by TLC as for the biologically reduced samples according to Section 2.3.3. Post development the TLC plates were viewed under UV light and the hR_f values for sample bands determined. The results from the TLC analysis are given in Table 25.

Table 25. Decolourisation products of Reactive Black (parent and hydrolysed) decolourised using sodium dithionite separated by TLC and categorised by their hRf values and colours.

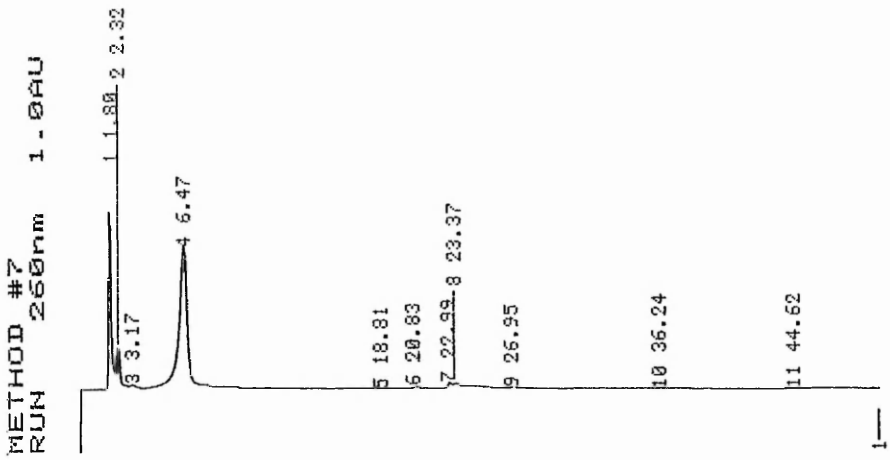
hR_f category	**hR_f values : Reactive Black**	**Hydrolysed Reactive Black**
1		69 orange*
2	71 blue*	
3	74 blue#	
4	80 blue	
5		84 blue#
6		89 blue#

KEY
#* Similar UV spectra.

From Table 25 it can be seen that more than two components are produced from the chemical decolourisation of the dye. Several UV spectra matches were seen for the extracted bands. No UV matches were apparent however between the chemically reduced dye bands and the biologically reduced dye bands in Table 24 although some similarities were observed in terms of hR_f values and the colour fluoresced under UV light. As it was suspected that multiple bands may be present from single chemical

species due to amine group interaction the chemically reduced samples were also analysed by HPLC to determine any similarity to the biologically reduced samples. The resultant chromatograms are given in Figure 50. From Figure 50 it can be seen that only one major product peak is present in the decolourised dye samples for both parent and hydrolysed forms of the dye. In both chromatograms the product peak expected to elute at R_t=20min which has been determined to be the H-acid component of the dye is absent (see Section 3.10.). This suggests the H-acid fragments of the dye structures were attacked by the chemical reducing agent. It is not known whether for the H-acid component tautomerism is energetically favourable leading to formation of an alcohol or an addition compound with the bisulphite present from the sodium dithionite reducing agent. If a bisulphite addition compound were formed the resulting species would be highly polar, highly water-soluble and may not be retained by the HPLC column and thus would elute with the solvent front. This is confirmed by the increased area of the solvent front peak seen for the chemically reduced Reactive Black (parent and hydrolysed) as shown in Figure 50. Due to this finding chemical reduction by sodium dithionite could not be used to prepare decolourised material for structural analysis. The structural determination of dye reduction products was discontinued due to the difficulties encountered in obtaining samples in a matrix suitable for further structural analysis.

(a) Chemically reduced Reactive Black.



(b) Chemically reduced hydrolysed Reactive Black.

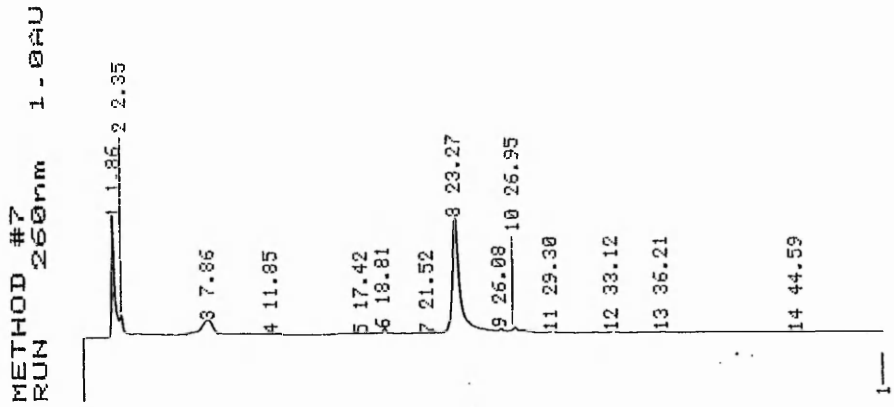


Figure 50. **Chromatograms of chemically reduced Reactive Black (a) parent dye and (b) hydrolysed dye.**

3.13. Toxicity assessment using the Microtox® acute toxicity test.

The toxicity test employed was the Microtox® acute toxicity bacterial luminescence assay. All biologically reduced samples for Microtox® testing were prepared by the author and tests were performed by A. Gottlieb (Department of Life Sciences, The Nottingham Trent University).

Initial toxicity assessment concentrated on Reactive Black (parent and hydrolysed). Samples were prepared and tested according to Section 2.13. Biologically reduced dyes were prepared using *E. faecalis* and *C. butyricum* (F5). Control assays prepared without dye were tested to determine any sample matrix effects and EC_{50} values have been calculated at an equivalent dilution to assays containing dye. Chemically reduced dyes were decolourised with sodium dithionite using the minimum amount of reducing agent required for decolourisation as determined in Section 3.2. For comparison an aqueous solution of sodium dithionite was also tested. Phenol was included in the assessment as a recommended standard for testing the performance of the Microtox® reagent. The Reactive Black intermediate H-acid was also tested for comparative purposes. Resultant EC_{50} values are given in Table 26.

From Table 26 it can be seen that the control assay samples for both *E. faecalis* and *C. butyricum* did show an inhibitory effect on the Microtox® reagent and as such all EC_{50} values for biologically reduced dyes must be considered against this background matrix effect. The control assay consisted of cells, buffer and glucose and as such the observed toxic response was thought to be due to products from the metabolism of glucose or cell lysis compounds. The *E. faecalis* assay prepared with 0.1% glucose (as a control for *E. faecalis* reduced Procion Crimson) had no effect which would imply it is the glucose metabolism in the other control assays responsible for the observed toxic response. It is apparent from Table 26 that for *E. faecalis* reduced Reactive Black the measured EC_{50} values at both 5 and 15min do not differ significantly from that measured for the control assay and that the toxic effect observed can not be said to be due to the dye. For hydrolysed Reactive Black however the 5 and 15min EC_{50} values are lower at 0.15ppm (as compared to 0.7ppm (5min) and 0.8ppm (15min) for the control assay). Hydrolysed Reactive Black was therefore considered to induce a toxic effect on the Microtox® reagent. For *C. butyricum* decolourised Reactive Black it can be seen that for both forms of the dye the measured EC_{50} values differ from that measured for the corresponding control assay being 1.7ppm for the parent dye and 0.25ppm for the

hydrolysed dye (at 5min) compared to 7.9ppm measured for the control. For *C. butyricum* reduced dyes therefore it appeared that both forms of Reactive Black displayed toxic effects to the Microtox® reagent. The EC_{50} value for biologically reduced Reactive Black at 1.7ppm (5min) would not be observed for the *E. faecalis* reduced samples due to the background matrix having a lower EC_{50} than this. For both strains therefore it is apparent that the hydrolysed Reactive Black has a lower EC_{50} value than the parent Reactive Black and therefore is considered to be more toxic to the Microtox® reagent. This can also be deduced from the EC_{50} values measured for chemically reduced dye samples. Sodium dithionite decolourised dyes had EC_{50} values (5min) of 4.9ppm for the parent dye and 0.23ppm for the hydrolysed dye and as such the hydrolysed dye is again shown to have a greater toxic effect. For the two forms of the dye the equivalent top concentration of sodium dithionite in the Microtox® test for chemically reduced samples was 39ppm for the parent form and 35ppm for the hydrolysed form. Both measured EC_{50} values for the chemically reduced dye samples are below the background level of 34.9ppm for a solution of sodium dithionite and therefore the observed toxicity can be said to be due to the reduced dyes.

In this toxicity assessment the compounds of interest (i.e. the biologically reduced dyes) are not isolated but are present in a mixture of chemicals and as such any EC_{50} value measured will be the EC_{50} of whichever compound in the mixture displays the greatest inhibitory effect on the Microtox® reagent. The concentrations producing the 50% inhibition measured can be considered against control samples prepared in the same way but without the addition of dye, which gives an indication of toxic response, but in doing this it is necessary to make several assumptions. It is assumed that the presence of a dye in a bacterial assay does not interfere with the normal metabolism of the bacterial culture employed. Compounds present that may originate from the bacterial cells (lysis products) or glucose metabolites are assumed to be the same in assays with and without dye. As it has been proposed that decolourisation occurs due to dyes acting as alternative electron acceptors it is not known whether this is a valid assumption. It has also been assumed that the toxic response observed is due to a single compound in the mixture and that synergistic effects are absent. The mean measured EC_{50} for phenol, tested as a single isolated compound, was 18.7ppm which is higher than the measured values for both chemically and biologically reduced Reactive Black. Phenol would generally be considered to be an undesirable environmental chemical capable of producing toxic effects and therefore it is necessary to determine whether the EC_{50}

values measured for the reduced Reactive Black are a true indication of toxic effects or an enhanced toxic response due to sample matrix effects.

Table 26. Toxicity assessment of reduced Reactive Black (parent and hydrolysed). EC_{50} values for the Microtox® test.

Dye	Reduction / compound	EC_{50} (ppm)			
		5min	**SD**	**15min**	**SD**
Parent	*E. faecalis* (n=4)	**0.7**	0.1	**0.7**	0.1
	C. butyricum (n=4)	**1.7**	0.1	**2.0**	0.4
	Chemical (n=3)	**4.9**	1.5	**5.7**	2.6
	H-acid fraction	Not tested.			
	Vinyl sulphone fraction	No effect at concentration tested (2.73)*			
Hydrolysed	*E. faecalis* (n=5)	**0.15**	0.02	**0.15**	0.02
	C. butyricum (n=5)	**0.25**	0.05	**0.24**	0.03
	Chemical (n=3)	**0.23**	0.09	**0.19**	0.07
	H-acid fraction	No effect at concentration tested (2.18)*			
	Vinyl sulphone fraction (n=4)	**0.34**	0.16	**0.44**	0.21
Control assay[a]	*E. faecalis*: 1% glucose (n=3)	**0.7**	0.2	**0.8**	0.2
	E. faecalis: 0.1% glucose	No effect			
	C. butyricum (n=3)	**7.9**	1.6	**6.2**	1.7
Sodium dithionite		**34.9**	6.3	**33.9**	18.6
H-acid		**49.7**	13.4	**49.5**	6.6
Phenol		**18.7**	2.8	**24.5**	7.2

KEY

* (top concentration tested) no effect.

n Number of measurements.

a Assay components: Cells, buffer, glucose, no dye. EC_{50} values represent the equivalent dilution as if the assay had contained dye.

In an attempt to isolate the components of biologically reduced Reactive Black (parent and hydrolysed) for Microtox® testing, samples of reduced dye were repeatedly injected onto the HPLC and component peaks collected as they eluted from the column. The identity of each component was assumed to be as deduced in Section 3.10. The concentration of each component was estimated according to Section 2.13.1. Samples were tested as in Section 2.13.2. Resultant EC_{50} values are given in Table 26. From the Table it can be seen that the H-acid fraction from hydrolysed Reactive Black did not produce a toxic effect at the concentration tested. Difficulties were encountered with attempts to concentrate the collected fractions due to the high amount of phosphate from the HPLC mobile phase as discussed in Section 3.12. The volume of sample that could be tested in the Microtox® test could not be increased to increase the concentration of material tested. Testing of a solution of the commercially available Reactive Black intermediate H-acid gave an EC_{50} value of 49.7ppm. Although it was recognised that the structure of this compound would differ from the H-acid fraction isolated from the decolourised dye sample by 2 amino groups, due to the higher EC_{50} obtained it was assumed that the H-acid fraction was not responsible for the toxic effect observed. This was confirmed by the subsequent results for the vinyl sulphone dye fractions. The H-acid fraction for the parent dye was assumed to be the same structure as that in the hydrolysed dye and so was not tested. The vinyl sulphone dye fraction for the hydrolysed dye only produced a toxic effect with a measured EC_{50} value of 0.34ppm (the two side chains differing in the terminal group being a sulphonate group for the parent dye and a hydroxy group for the hydrolysed dye). Testing of the HPLC mobile phase with the same composition as the time of elution for each fraction did not produce a toxic effect. It was concluded therefore that the hydrolysed Reactive Black displayed a greater toxic effect than the parent dye and that the toxicity was primarily due to the vinyl sulphone side chain.

Toxicity assessment of biologically reduced dyes was repeated for the Procion dyes with *E. faecalis* and *C. butyricum* and the resultant EC_{50} values are given in Table 27. From the Table it can be seen that the EC_{50} values measured for the reduced Procion dyes are of the same order as those determined for the control assays and in some cases are higher. For Procion Navy and Procion Yellow the EC_{50} values for the hydrolysed form of the dyes are lower than for the parent form and are also slightly lower than the corresponding control backgrounds but do not differ by more than one standard deviation of the control measurement. No comment can therefore be made as to the

toxic effects of the Procion dyes. For Procion Crimson decolourised by *E. faecalis* the control assay (0.1% glucose) had no effect and so the measured values for Procion Crimson may be more of a true reflection of the toxic effect of the dye's degradation compounds. For *C. butyricum* reduced Procion Crimson the EC_{50} values measured are slightly higher than the corresponding control assay. Any toxic effect produced from the decolourised dye may therefore be masked by the effects of the sample matrix.

It has been shown in the literature that some dyes that have been considered to be non-toxic or non-carcinogenic yield toxic or carcinogenic metabolites upon reduction. It has been demonstrated that the decolourisation products of Reactive Black produce inhibition of the Microtox® test reagent with greater toxic effects displayed by the hydrolysed dye. Due to difficulties encountered with sample matrix effects no comment can be made as to the toxic potential of the Procion dyes. For comparison the toxicity of the unreduced dyes was investigated. Tests were performed according to Section 2.13 and the colour correction procedure applied according to Section 2.13.3. Resultant EC_{50} values are given in Table 28. The values in the Table represent single measurements due to time constraints. From the Table it can be seen that there is a difference in EC_{50} with and without the colour correction applied. For Reactive Black it can be seen that the effect of colour correction is greatest for the parent dye with EC_{50} (5min) values changing from 27.5 and 17.8ppm to 92.0 and 38.0ppm after the correction had been applied. For the hydrolysed dye the change in EC_{50} values was not as pronounced as for the parent dye changing from 11.4 and 9.9ppm to 17.9 and 12.8ppm. This would be expected as the lower the EC_{50} value for a coloured compound the less effect the colour will have due to the higher dilution of the sample in the measurement. For the Procion dyes the colour correction changed the test results significantly in that no toxic effect was observed on the Microtox® reagent for any of the dyes at the top concentration tested (100ppm). The apparent EC_{50} for the dyes obtained without the colour correction may be an artifact of the dye absorbance at 490nm (the wavelength at which the Microtox® test reagent emits light) with the differences in apparent values due to differences in absorbance of the dyes at this wavelength. These observations are however based on discreet measurements and the problem of testing coloured compounds needs to be investigated further.

From the limited study of the unreduced dyes it appears that the toxicity of Reactive Black decolourisation products was higher than the unreduced dye. The unreduced hydrolysed Reactive Black appeared to exhibit a greater toxic response than unreduced

parent Reactive Black. As the two structures are thought to differ only in the terminal group of the vinyl sulphone side chain this confirms the findings of tests on the decolourised dye fractions. The terminal hydroxyl group on the vinyl sulphone side chain enhanced toxicity and hence the decolourisation products of the hydrolysed dye produced greater inhibition of the Microtox® test reagent than the decolourisation products of the parent dye.

Table 27. Toxicity assessment of reduced Procion dyes (parent and hydrolysed). EC_{50} values for the Microtox® test.

Reduction	Dye	EC_{50} (ppm)			
		5min	**SD**	**15min**	**SD**
E. faecalis (n=4)	Procion Navy	**0.5**	0.2	**0.6**	0.2
	Hydrolysed Procion Navy	**0.5**	0.1	**0.5**	0.1
	Procion Crimson	**25.1**	7.2	No effect (100)*	
	Hydrolysed Procion Crimson	**20.3**	4.0	**24.5**	3.5
	Procion Yellow	**1.0**	0.2	**1.0**	0.3
	Hydrolysed Procion Yellow	**0.5**	0.3	**0.5**	0.3
C. butyricum (n=4)	Procion Navy	**9.4**	0.6	**7.5**	0.6
	Hydrolysed Procion Navy	**6.3**	2.8	**6.9**	3.1
	Procion Crimson	**8.1**	0.7	**7.3**	0.9
	Hydrolysed Procion Crimson	**9.1**	1.0	**7.2**	1.2
	Procion Yellow	**7.8**	1.7	**8.0**	1.0
	Hydrolysed Procion Yellow	**6.5**	1.3	**4.5**	1.0
Control assay[a] (n=3)	*E. faecalis*: 1% glucose	**0.7**	0.2	**0.8**	0.2
	E. faecalis: 0.1% glucose	No effect			
	C. butyricum	**7.9**	1.6	**6.2**	1.7

KEY

* (top concentration tested) no effect.

n Number of measurements.

a Assay components: Cells, buffer, glucose, no dye. EC_{50} values represent the equivalent dilution as if the assay had contained dye.

Table 28. Preliminary toxicity assessment of unreduced dyes (parent and hydrolysed) with and without colour correction. EC_{50} values for the Microtox® test.

Dye	EC_{50} (ppm)			
	Uncorrected		Corrected	
	5min	15min	5min	15min
Reactive Black	27.5	30.5	92.0	81.7
	17.8	16.4	38.0	32.0
Hydrolysed Reactive Black	11.4	11.1	17.9	16.0
	9.9	8.8	12.8	11.1
Procion Navy	17.8	16.9	No effect at concentration tested (100)*	
Hydrolysed Procion Navy	25.0	30.7		
Procion Crimson	13.8	13.1	No effect at concentration tested (100)*	
Hydrolysed Procion Crimson	11.3	10.4		
Procion Yellow	22.6	21.0	No effect at concentration tested (100)*	
Hydrolysed Procion Yellow	34.0	34.3		

KEY
* top concentration tested.

4. DISCUSSION.

4.1. Dye choice, isolation of decolourising bacteria and screening.

There has been to date a somewhat random approach in the choice of dyes and organisms used for decolourisation studies. This is perhaps due to the vast number of dyes available and the wide range of organisms reputed to have decolourising ability. Organisms studied include both pure and mixed cultures of bacteria and fungi together with complex microbial consortia such as anaerobic digester sludge. A review of the literature (Section 1.6) identified 5 main approaches used previously for development of decolourisation systems. The approach taken in this study was to isolate specific bacterial strains capable of decolourising a range of textile dyes currently in use.

The number of dyes commercially available has been estimated to be in the thousands. Due to the very large numbers of dyes available and their diverse chemical composition the most practical approach to identify which dyes to include in this study was consultation with the local textile finishing industry. Following consultation with two dyehouses 18 dyes were identified as being representative of those generally in use. These 18 dyes were from various classes including reactive, disperse, acid and direct. Four of the 18 were from the reactive dyeing class and were used almost exclusively for the dyeing of cotton by one of the dyehouses consulted. Of these four, one was a vinyl sulphone (Reactive Black 5) with the other three being chorotriazine dyes (Procion Navy H-EXL, Procion Crimson H-EXL and Procion Yellow H-EXL). Use of all 18 dyes was considered desirable for decolourisation screening experiments but impractical for in-depth studies. Nomination of Reactive Black 5 as a model dye and inclusion of the other three reactive dyes in some studies was considered to be an appropriate compromise.

Isolation of decolourising bacteria was undertaken through plating serial dilutions of textile effluent samples from one of the local dyehouses onto solid media containing target dyes similar to the methods employed by Zhou and Zimmerman (1993) and Coughlin *et al.* (1997). Initially five of the 18 dyes were used, including the model dye Reactive Black, due to their chemical structures being known. Only Reactive Black and Erionyl Navy Blue (Acid Blue) had an appropriate depth of colour when mixed with the agar medium to allow the clear zones surrounding decolourising

colonies to be easily identified (Figure 12, Section 3.3). Isolations were performed with both aerobic and anaerobic incubation and typical counts for dye decolourisers were 3 x 10^6 cfu/ml effluent for the aerobic plates and 2 x 10^2 cfu/ml effluent for the anaerobic plates. These figures imply that in the textile effluent samples used for isolation aerobic decolourising strains predominate. The textile effluent used as the isolation source was obtained from an effluent holding tank undergoing a constant cycle of filling and discharge. The environment within the tank therefore provided enrichment conditions for both aerobic and anaerobic organisms capable of growth in the presence of dyestuffs and associated auxiliary chemicals. Although the aerobic decolourisers appeared to predominate it is doubtful that the decolourisation observed for the aerobically incubated plates was truly aerobic. The centre of these colonies however may be sufficiently anaerobic to produce azo cleavage.

Eleven aerobic decolourising strains were isolated in this study and were identified as *Shewanella putrefaciens* (four strains), *Aeromonas masoucida, Pseudomonas capacia, Pseudomonas vesicularis, Pseudomonas chloraphis* and *Sphingomonas paucimobilis* (Section 3.3.1). Inconclusive identification results were obtained for two isolates but as both displayed red pigmentation these were deemed inappropriate for decolourisation studies. Pseudomonads capable of decolourisation have been isolated by several authors for example Idaka *et al.* (1987), Yang *et al.* (1991), Dykes *et al.* (1994) and Jian *et al.* (1994). Accounts have also been given of pseudomonads capable of total dye degradation (decolourisation and mineralisation) (Kulla, 1981; Kulla *et al.*,1983 and Haug *et al.*, 1991). The degradative capacity of *Pseudomonas* spp. towards aromatic compounds has been extensively researched and a summary is given in Table 7, Section 1.6. Hu (1992) and Jian *et al.* (1994) have used *Aeromonas* strains previously for decolourisation and an account of decolourisation by *Sphingomonas* sp. has been given by Coughlin *et al.* (1997). To this author's knowledge *Shewanella putrefaciens* has not been previously reported as a strain utilised for dye decolourisation. During the purification and identification process problems were encountered with a number of the aerobic isolates in that strains became difficult to culture and/or lost the ability to decolourise Reactive Black dye plates. It was thought possible that the media used for isolation and identification was too nutrient rich for these strains which may indicate the ability to decolourise dyes for these strains could be plasmid based. An essential requirement

for growth may have also been present in the original textile effluent which was absent in the laboratory media. Of the aerobic isolates only the four *S. putrefaciens* strains remained viable and retained the ability to decolourise Reactive Black dye plates.

Twelve bacterial strains were purified from the anaerobically incubated Reactive Black dye plates (Section 3.3.2). Six strains were found to be identical with respect to colony morphology and biochemical profile and were identified as *Clostridium butyricum /beijerinckii.* The other six strains had identical colony morphology with five of the six identified as *Clostridium butyricum /beijerinckii* (within the five two different biochemical profiles were observed). The remaining sixth isolate was identified as *Clostridium inocuum.* Rafii and Cerniglia (1990a), Knapp and Newby (1995) and Semde *et al.* (1998) have also reported clostridia strains capable of dye decolourisation. No problems were encountered with viability or loss of decolourising ability with the anaerobes and decolourisation zones produced by these organisms on the Reactive Black plates were clearer and more defined than the zones observed for the aerobic strains.

Investigation of the range of dyes decolourised by a selection of the isolated strains was undertaken in a similar manner to the isolation experiment using agar plates containing all 18 dyes identified as being representative of those in use by local industry (Table 19, Section 3.4). For comparison it was decided to include *Enterococcus faecalis* in the study, as this strain is known to produce azo fission (Gingell and Walker, 1971; Walker and Ryan, 1971 and Sweeney, 1995). In general the environmental isolates decolourised a wider range of dyes than the laboratory strain *E. faecalis* possibly due to tolerance to the presence of dyestuffs attained prior to isolation. *E. faecalis* decolourised 8 of the 18 dyes although weak zones only were observed for 6. The *S. putrefaciens* V1 and V2 decolourised 10 and 13 dyes respectively (with weak zones for 1 and 3 respectively). The clostridia strains were the most efficient with regards to the range of dyes decolourised with *C. butyricum* (N1) decolourising 15 dyes (2 weakly), *C. butyricum* (F3) and (F5) decolourising 16 dyes and *C. inocuum* (F6) decolourising 17 dyes (2 weakly). The decolourisation zones were more defined and easier to see on the anaerobic culture plates. This may be due the strict anaerobic conditions maintained during growth. Two dyes, Procion Crimson and Solophenyl Black, were decolourised by all six isolates but not

E. faecalis however it was shown in subsequent experiments that *E. faecalis* was capable of decolourising Procion Crimson under different experimental conditions. This aspect will be discussed in Section 4.2.

Accumulation of dyes by several strains was observed. For four selected anaerobes accumulation was seen for 5 of the 18 dyes (Erionyl Navy Blue, Indosol Blue, Indosol Rubinole, Indosol Yellow and Solophenyl Navy), all of which with the exception of Erionyl Navy Blue (Acid Blue) were of the direct dyeing class. This may suggest that adsorption could be a function of dye type. Similar accumulation of these dyes was observed for *E. faecalis* although some adsorption was also seen for Erionyl Yellow (Acid Orange) with this strain. Adsorption of three dyes was observed for the two aerobic isolates: Dispersol Black (a blend), Erionyl Yellow (Acid Orange) and Foron Black (azo: class unknown). No accumulation of the reactive dyes (Reactive Black and the three Procion dyes) was observed with any strain.

Hitz *et al.* (1978) studied the bio-elimination of various dyes from different classes and determined that the extent to which dyes are adsorbed varies between the dye classes and has no structural correlation. Acid dyes were found to have low adsorption due to their high solubility that appears to depend on their degree of sulphonation. Reactive dyes had a very low degree of adsorption apparently unaffected by the degree of sulphonation or ease of hydrolysis. Basic dyes had typically high levels of adsorption and disperse dyes were in the high to medium range. Direct dyes had a high degree of adsorption unrelated to the number of sulphonic acid groups. In agreement with Hitz *et al.* (1978) it was found in this study that the anaerobes and *E. faecalis* adsorbed four of the five direct dyes tested and no adsorption was observed for the four reactive dyes with any strain. None of the direct dyes were adsorbed by the aerobic strains however indicating that bio-elimination may depend on organism type in addition to dye class. Contrary to the findings of Hitz *et al.* (1978) the two acid dyes used in this study were both adsorbed by selected strains with Erionyl Navy Blue (Acid Blue) adsorbed by the anaerobes, Erionyl Yellow (Acid Orange) adsorbed by the aerobes with *E. faecalis* adsorbing both. This apparent specificity between strains again indicates that organism type may also be a factor in bio-elimination. In contrast to the findings of this study and those of Hitz *et al.* (1978) removal of reactive dyes by adsorption has

been reported. Zhou and Zimmerman (1993) found that of 5 reactive dyes tested 3 were removed by adsorption and only 2 copper complex dyes were degraded by actinomycete strains. Hu (1992) tested the adsorption capacity of *Aeromonas* cells for 11 reactive dyes and found removal efficiencies ranged from 12.9% to 94.3% with 8 of the 11 dyes tested however being removed less than 33%.

No two isolates produced identical results for all 18 dyes tested indicating a degree of specificity (Table 19, Section 3.4). Five dyes were not decolourised by either of the *S. putrefaciens* strains (Procion Navy, Dispersol Blue, Serilene Yellow, Serilene Yellow Brown and Foron Black). Dispersol Blue was the only dye not decolourised by the anaerobes and was the only dye not decolourised by any strain tested. Disperse dyes however are not generally considered to be a major cause of coloured effluents as they have high fixation rates (for polyester dyeing) of 90-100% (Easton, 1995) and are likely to be removed in conventional biological treatment processes by adsorption to biomass (Pierce, 1994).

Decolourisation screening of fungal strains from the Department of Life Sciences culture collection was undertaken for comparative purposes in the same manner as for the bacterial isolates. Of the 18 representative dyes tested two strains decolourised 17 dyes (*C. versicolor* and *C. cerebella*), one decolourised 16 dyes (*P. versicolor*) and one (*P. placenta*) decolourised 5 dyes only (Table 20, Section 3.4.2). No accumulation of dye was observed for any fungal strain. Only one dye, Serilene Yellow, was not visually decolourised by any strain. For three of the dyes (Erionyl Navy Blue, Indosol Blue and Indosol Rubinole), coloured products were observed indicating partial bond breakage within the dye structures. This was not observed for any of the bacterial strains.

4.2. The chemical and biological reduction of selected dyes.

One of the reasons why reactive dyes are considered to be a particular problem is their existence in textile effluents in a highly soluble hydrolysed form. It has been suggested however that for the vinyl sulphone type of reactive dyes both the vinyl sulphone reactive form and the hydrolysed form of the dye would be present in an effluent and that it is the vinyl sulphone that predominates (Camp and Sturrock, 1990). In this case the well-documented hydrolysis reaction competing with the covalent bonding of dye to fibre would not be significant. The model dye for this

study, Reactive Black, is a dye of this type. Ganesh *et al.* (1994) studied the decolourisation of the vinyl sulphone and hydrolysed form of Reactive Black in batch reactors. Under aerobic conditions the vinyl sulphone form of the dye was reduced by more than 50% in two digesters during the first day of operation with sorption of the dye to the sludge deemed to be the primary dye removal mechanism. For the hydrolysed form of Reactive Black however no colour removal was observed and no dye appeared to be sorbed to biomass. The findings of Camp and Sturrock (1990) have not been confirmed and as it has been shown by Ganesh *et al.*, (1994) that the vinyl sulphone form of Reactive Black can be removed in aerobic treatment systems it was decided to concentrate on the parent and hydrolysed forms of Reactive Black.

The chemical and biological decolourisation of four reactive dyes in their parent and hydrolysed forms was investigated (Sections 3.2., 3.6. and 3.7). Chemical decolourisation was achieved using sodium dithionite as reducing agent. Initial studies concentrated on the chemical decolourisation of Reactive Black. It was assumed that the quantity of reducing agent required for decolourisation could be predicted from knowledge of dye structure assuming that dye decolourisation proceeded via azo bond cleavage alone. As the structures of the three Procion dyes were unknown calculation of the minimum weight of reducing agent required for decolourisation of a known weight of dye was determined by titration of dye solutions against a solution of sodium dithionite under various conditions. For Reactive Black the decolourisation was deemed to be pH dependent with greater quantities of reducing agent required with increasing pH (Table 17, Section 3.2). The buffered systems at pH7 and pH10 were inhibitory to decolourisation with only partial bond breakage observed at pH10. In an unbuffered solution at this pH a greatly increased quantity of sodium dithionite was required for decolourisation with a fall in pH to pH 4 at the end of the titration.

Reduction of a dye solution with sodium dithionite would normally be undertaken in alkaline aqueous solution (Gasparic, 1977) however it appeared that for Reactive Black acidic conditions were more conducive to decolourisation. Michelsen (1992) has also noted increased efficiency of sodium dithionite at reduced pH in a comparison of chemical reducing agents for the treatment of Navy 106 wash water. Increased efficiency of the chemical reduction at pH3 may be due to the higher

availability of hydrogen atoms at this pH for reduction of the azo bond to the corresponding amines or that in acid conditions the structure of Reactive Black may be rendered more amenable to decolourisation. The latter effect may be explained by the observation of Walker and Ryan (1971) that rate of reduction depends on molecular parameters influencing the ease with which electrons are accepted by the azo group which in turn is influenced by electron density in this region. It was postulated that for the dye Red 10B and its analogues the free amino group at position 1 of the naphthalene ring exists in hydrogen bonded form with the acidic naphthol group at position 8. When the amino group was substituted with a less basic group such as an acetyl group hydrogen bonding is favoured between the naphthol group and the azo nitrogen as illustrated in Figure 51. For Reactive Black it may be possible that in aqueous conditions the basic amino group at position 8 of the naphthalene ring would also form a hydrogen bond with the acidic naphthol group at position 1 (see Figure 4). In acid conditions the basic amino group may be fully protonated with the naphthol group forming a hydrogen bond with the azo nitrogen. This would result in a lowering of electron density in the azo region making the bond more susceptible to reduction.

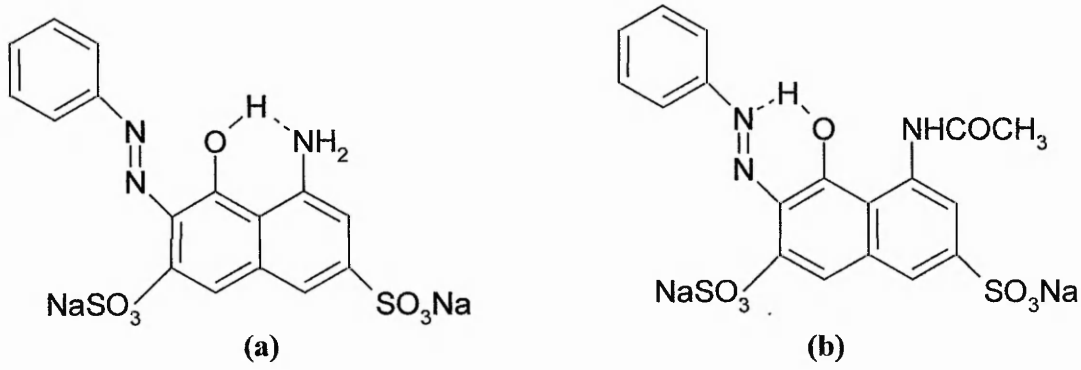


Figure 51. Hydrogen bonding in Red 10B (a) and Red 2G (b). Reproduced from Walker and Ryan (1971).

It was noted that during addition of sodium dithionite to Reactive Black a colour shift from dark blue to purple was observed indicating asymmetric breakage of the

two azo bonds present in the dye structure (Figure 7). Similar colour shifts were observed for Procion Navy (Figure 9) and Procion Crimson (Figure 10) indicating the presence of multiple azo bonds being broken sequentially. For Procion Yellow the dye solution changed from yellow to colourless (Figure 11) and was thought to be monoazo or of such a structure that multiple bonds would be broken simultaneously.

In unbuffered aqueous solution the amount of reducing agent compared to that for Reactive Black was higher for Procion Navy and Procion Crimson (Table 18, Section 3.2). A similar amount was consumed by Procion Yellow for decolourisation. As was observed for Reactive Black, buffered conditions at pH7 were inhibitory to decolourisation. Of the three Procion dyes only Procion Yellow decolourised at this pH with partial bond breakage observed for the other two Procion dyes suggesting again that Procion Yellow could be monoazo. Contrary to the findings for Reactive Black the amount of reducing agent required at pH3 increased for Procion Crimson and Procion Yellow with no real difference observed for Procion Navy. The general increase in reducing agent required for the Procion dyes could be due to a difference in purity, number of azo groups or the presence of other reducible groups on the dye structure.

Accounts given in the literature on decolourisation of Procion dyes include the decolourisation of Reactive Red 120 by Sangaleti *et al.* (1995) and Reactive Red 141 by Carliell *et al.* (1994, 1995) and O'Neill *et al.* (2000). The structures given for each dye show that two azo groups are present. It is thought likely that the Procion dyes used in this study would be of a similar structure. The increased amount of reducing agent needed cannot therefore be attributed purely to increased numbers of azo groups. The purity of Procion Crimson has been estimated to be >75% whereas the purities of Procion Navy and Procion Yellow are unknown. The purity of Reactive Black is estimated to be in the region of 55%. Although Procion Crimson would consume more reducing agent due to its higher purity it is also thought unlikely that this could account for the amount needed to produce decolourisation (more than 4 times that for Reactive Black in aqueous unbuffered solution).

Carliell *et al.* (1994, 1995) proposed that four products were obtained upon reduction of Reactive Red 141 under anaerobic conditions and that decolourisation

proceeded via azo bond cleavage and breakage of the amine linkages between the chromophore and the reactive group and within the reactive group itself (see Figure 2). As Procion Crimson may have a similar structure the reduction of this dye could proceed in a common manner and the increased quantity of sodium dithionite required for decolourisation may be due to other groups in the dye structure, such as the amine linkages, undergoing reduction. It was thought that for Reactive Black the azo groups were rendered more amenable to reduction in acid conditions. For Procion Crimson and Procion Yellow a greater amount of reducing agent was required at this pH. It is not known whether the azo groups in these dyes or the other reducible groups on the dye molecules would also be rendered more susceptible to reduction at this pH.

In unbuffered aqueous solution and buffered at pH3 the amount of reducing agent required by the parent and hydrolysed form of Reactive Black were similar. At pH7 however the hydrolysed dye required more than twice the amount of sodium dithionite to produce decolourisation. The only difference in structure between the two forms of the dye is thought to be the terminal groups on the vinyl sulphone side chains, being a sulphonate for the parent dye and a hydroxy group for the hydrolysed dye. A similar effect was observed for the Procion dyes and in aqueous unbuffered solution and at pH7 the amount of reducing agent required for decolourisation (or partial decolourisation at pH7) was higher for the hydrolysed dye. The difference in structure between the two forms of a Procion dye was thought to be the group on the triazine ring, being a chloro for the parent dyes and a hydroxy group for the hydrolysed dyes. For the hydrolysed dyes it is not known whether sodium dithionite would be consumed in solution by groups cleaved from the dyes during hydrolysis (i.e. sulphonate group for Reactive Black and the released chlorine for the Procion dyes) and hence the quantity required for decolourisation is increased. At pH3 these groups may be fully protonated and hence the effect is not observed at this pH.

Preliminary experiments for biological reduction were undertaken to determine the effect of glucose concentration on decolourisation, variation in rate between batches of cells and linearity of rate with cell density. Biological reduction of test dyes was required for two reasons: production of decolourised material for structural analysis and measurement of decolourisation rate. For production of decolourised material the effect of glucose concentration on decolourisation was investigated using *E.*

faecalis and Reactive Black (Section 3.5.2). Initial assays were prepared using five glucose concentrations ranging from 1% to 0.05%. Visual differences in decolourisation for each glucose concentration were observed. At glucose concentrations <0.1% assays appeared visually decolourised with a thin blue ring around the surface. Following centrifugation to remove the cells however the supernatants appeared blue. It was thought that this could be due to a redox effect. Under the low redox conditions produced within the assay the dye molecule may be forced into the partially reduced hydrazo form which reverts back when the solution is disturbed. This would explain the blue ring seen around the surface of the assay tube where the solution was exposed to oxygen.

The 0.2% glucose assay supernatant was purple indicating that the dye may be partially reduced either by only some molecules having complete azo reduction or by asymmetric azo bond fission within a dye molecule. The colour of this assay was similar to the first colour shift observed in the titrations of Reactive Black with sodium dithionite as discussed above. The 0.5% and 1.0% glucose assay samples remained green/yellow after centrifugation and decolourisation in these assays was considered to be complete. A brown layer observed at the surface of the assays was thought to be due to oxidation of the aromatic amines produced by decolourisation, a similar effect being observed by Knapp and Newby (1995) in samples of decolourised stilbene effluent. It was decided to utilise a glucose concentration of 1% for production of decolourised material.

In a check on decolourisation of the three Procion dyes with 1% glucose it was found however that decolourisation did not occur for Procion Crimson. Subsequently, the assays with a range of glucose concentrations (as used for Reactive Black) were prepared for Procion Crimson (Section 3.5.2.). It was found that decolourisation was observed only at <0.1% glucose indicating that the higher glucose concentrations were inhibitory to decolourisation of Procion Crimson by *E. faecalis*. Chung *et al.* (1978b) noted glucose to be inhibitory to the decolourisation of Tartrazine by *B. thetaiotaomicron* although no biochemical explanation for this was apparent. Sweeney (1995) also observed inhibition by glucose of the azoreductase activity of *B. thetaiotaomicron* in the decolourisation of Amaranth, Sunset Yellow and Carmoisine. Conversely it was found that the presence of glucose enhanced the azoreductase activity of *E. faecalis* towards the same dyes.

Knapp and Newby (1995) in their study on treatment of stilbene effluent also noted that higher concentrations of glucose were inhibitory to decolourisation.

In this study it was found that the level of glucose in assays for the production of decolourised material had an effect on the extent to which decolourisation proceeded. For complete decolourisation of Reactive Black a glucose concentration >0.5% was required whereas for Procion Crimson glucose >0.1% was found to be inhibitory. As it was intended to standardise all rate measurement assays the effect of glucose concentration on initial decolourisation rate was determined (Section 3.5.2). No significant difference was determined between 0.1% and 1% glucose assays with regards to initial rates for Reactive Black. At these glucose concentrations the initial decolourisation rate for Procion Crimson was much lower than that for Reactive Black with no significant difference observed between the two levels of glucose. Rate measurements were standardised on 1% glucose.

It was deemed impractical to work on a number of isolates for detailed studies into decolourisation therefore choice of one isolate was deemed necessary (Section 3.5.3). Problems were encountered with the aerobic *S. putrefaciens* strains in that compact cell pellets were not formed during centrifugation making preparation of decolourisation assays difficult. The assays appeared to be visually decolourised but when samples were removed and centrifuged the sample supernatants were blue. These problems were not encountered with any of the anaerobic strains. One of the anaerobic strains, *C. butyricum* (F5) was chosen for further study.

For *E. faecalis* and *C. butyricum* (F5) it was found that no significant difference was observed in initial rates measured with three different batches of cells and hence results from different batches of cells could be compared directly (Section 3.5.4). The initial decolourisation rate was found to be linear with cell density (Figure 16, Section 3.5.5) indicating that decolourisation was due to biological activity and that cell density could be varied as necessary in order to obtain measurable rates. The form of the curve for *E. faecalis* and hydrolysed Reactive Black (Figure 15, Section 3.5.4) suggested that the decolourisation reaction followed first order kinetics. This was confirmed by a plot of ln[dye concentration] versus time producing a straight line. The decolourisation reaction was observed to consist of two phases. The first was an initial linear reduction in dye concentration followed by a second phase exponential type decay. The exponential decay curve indicated that the

decolourisation reaction was proceeding to an end point thought to be due to an inhibition of activity by accumulation of decolourisation end products. This is in agreement with observations by Wuhrmann *et al.* (1980) that for some compounds the rate of decolourisation decreases more rapidly than predicted by a first order reaction when a large percentage of dye has already been reduced. It was assumed that the toxicity of degradation products accumulating in the medium was responsible for this effect. Carliell (1993) noted that measured rates of decolourisation for Reactive Red 141 followed first order kinetics and were inversely proportional to initial dye concentration also thought to be due to an inhibitive concentration of degradation products. In contrast Semde *et al.* (1998) found decolourisation rate to be linear (zero order) with no change in rate observed for decolourisation of Amaranth by *C. perfringens* when the initial concentration of the dyes was doubled.

For *C. butyricum* (F5) and Reactive Black the reaction was observed to have three phases (Figure 15, Section 3.5.4). The initial linear reduction in dye concentration followed by the exponential type decay were both present as for *E. faecalis.* In addition to these an initial lag phase was observed. The time of the lag phase before decolourisation commenced varied with cell density, i.e. the lower the cell density the longer the lag time before decolourisation commenced. It was thought that the lag time was due to a definitive period of time required for the production of reducing equivalents by *C. butyricum* from the glucose in the assay. This would explain the increase in lag time with reduction in cell density.

Initial decolourisation rates for whole cell assays with *E. faecalis* ranged from 0.8 to 51.7mg dye/h/g dry cell wt (with glucose) and 0.9 to 12.5mg dye/h/g dry cell wt (without glucose) for the three Procion dyes and Reactive Black (Table 21, Section 3.6). The decolourisation rates for Reactive Black (both parent and hydrolysed) were higher than for all 3 Procion dyes with and without glucose. The initial decolourisation rate observed for hydrolysed Reactive Black was faster than the initial rate measured for the parent dye, in the presence of glucose being more than twice the magnitude (51.7 and 21.2 mg dye/h/g dry cell wt respectively). This was thought to be due to differences in the degree of sulphonation for the two forms of the dye.

Hydrolysis of Reactive Black results in a loss of two sulphonate groups from the two vinyl sulphone side chains. As the sulphonate groups confer a high degree of water solubility to the dye structure their presence may inhibit passage of a dye molecule through the cell membrane of *E. faecalis*. The hydrolysed dye, being less sulphonated, may pass through the cell membrane faster and hence be decolourised at a faster rate. This may be partially explained by measurement of initial decolourisation rates for cell free extracts as discussed below.

It has been noted by Brilon *et al.* (1981a) that the degradation of naphthalene sulphonic acids is initiated by oxygenolytic displacement of the sulphonic acid groups and as such de-sulphonation is strictly an aerobic reaction. It has been shown in this study that for Reactive Black the decolourisation rate is higher for the hydrolysed dye thought to be due to differences in the degree of sulphonation for the two forms of this dye attained via hydrolysis. It would not be expected however in view of the study by Brilon *et al.* (1981a) that further desulphonation of the dye molecule would occur in the anaerobic conditions required for azo reduction and hence increase the decolourisation rate further.

In the absence of glucose the difference in rate for the parent and hydrolysed forms of Reactive Black was also observed but was less pronounced (8.9 and 12.5 mg dye/h/g dry cell wt respectively). In the absence of glucose, production of reducing equivalents for dye decolourisation relies on residual substrates within the cell which may not be as readily available as an external energy source which may explain why the rates without glucose are lower. Other accounts given of enhanced decolourisation rates in the presence of glucose include Haug *et al.*, (1991), Carliell *et al.* (1995) and Sweeney (1995). Some studies have also noted decolourisation occurring only in the presence of an external energy source (Nigam *et al.*, 1996 and Coughlin *et al.*, 1997).

A distinction in initial decolourisation rate between the parent and hydrolysed forms of the Procion dyes with whole cells of *E. faecalis* was not as clear as for Reactive Black. It is thought that the difference in sulphonation of the two forms of Reactive Black was responsible for the different initial rates observed. Hydrolysis of the Procion dyes is thought to alter the groups present on the triazine ring and would not be expected to result in loss of sulphonate groups from the dye molecule. As such it may be expected that the initial decolourisation rates of the parent and hydrolysed

Procion dyes would be similar. The numbers of sulphonate groups on the Procion dye structures is unknown and so it is not known whether the overall lower rates for the three Procions are due to a slower passage across the cell membrane due to their presence. The dyes may also have structures such that steric effects may hinder access to the azo bonds. The structures of Procion dyes given Sangaleti *et al.* (1995) and Carliell *et al.* (1994, 1995) of Reactive Red 120 and Reactive Red 141 show these dyes to have 6 and 8 sulphonate groups respectively. If the Procion dyes used in this study are of a similar structure to those given in the literature then the lower initial decolourisation rates observed may be due in part to the higher numbers of sulphonate groups present for this type of dye.

For *C. butyricum* (F5) whole cell assays the initial decolourisation rates for Reactive Black were again higher than for the Procion dyes (Table 22, Section 3.6). Measured rates ranged from 2.3 to 102.7mg dye/h/mg dry cell wt (with glucose) and 1.1 to 49.9mg dye/h/mg dry cell wt (without glucose). The rate for the hydrolysed form of Reactive Black was again much higher than the parent dye in the presence of glucose (102.7 and 59.0 mg dye/h/g dry cell wt respectively) possibly due to the mechanism discussed above. Again no clear distinction was present between the parent and hydrolysed forms of the Procion dyes.

In general the initial decolourisation rates with *C. butyricum* (F5) were higher than with *E. faecalis* although these cannot be compared directly due to the assays being performed at different temperatures (30°C for *C. butyricum* and 37°C for *E. faecalis*). The overall trend appeared to be the same as for *E. faecalis* with the initial decolourisation rates for whole cell assays descending in the order Reactive Black>Procion Yellow>Procion Navy>Procion Crimson.

Beydilli *et al.* (1998) studied the anaerobic biodegradability of 6 reactive dyes in their parent and hydrolysed forms. Decolourisation was found to occur at a faster rate in the presence of an external carbon source and for Yellow 17 no significant difference in rate was observed for the parent and hydrolysed form of the dye. For Red 2 however the hydrolysed dye exhibited a faster decolourisation rate than the parent dye. These findings are contradictory to the observations from this study as Reactive Yellow 17 is a monoazo vinyl sulphonyl dye (of a similar type as Reactive Black) and as such would be expected to lose one sulphonate group upon hydrolysis.

It may be expected therefore that the decolourisation rate of the hydrolysed form of this dye would be higher than for the parent form. Reactive Red 2 is a monoazo dichlorotriazinyl dye (of a similar type to the Procion dyes) which would not be expected to lose any sulphonate groups upon hydrolysis and as such it may be expected that the decolourisation rates for the parent and hydrolysed forms of the dye would be comparable.

The difference in initial decolourisation rate for the parent and hydrolysed forms of Reactive Black observed in this study was thought to be due to differences in the number of sulphonic acid groups on the dye structure. Measurement of initial decolourisation rates in cell free assay removes this rate-limiting factor and thus may allow a better comparison of decolourisation rate for the two forms of Reactive Black and the Procion dyes. For *E. faecalis* cell free extracts a similar overall pattern to the whole cell experiments was observed in that initial rates decreased in the order Reactive Black>Procion Yellow>Procion Navy>Procion Crimson. Measured rates for the textile dyes ranged from 4.5 to 72.7mg dye/h/g protein. In contrast to the whole cell assays the parent form of Reactive Black was observed to have a higher initial rate of decolourisation than the hydrolysed dye (Table 23, Section 3.7) being 72.7 and 54.1mg dye/h/g protein respectively. This confirmed the theory that the increased number of sulphonate groups on the parent dye may be inhibiting transport across the cell membrane resulting in a lower reduction rate for the parent dye in whole cell assays.

For *C. butyricum* cell free extracts Reactive Black was decolourised at comparable rates to the three Procion dyes. In contrast, the decolourisation rate for Reactive Black in whole cell assays with this strain was much higher than for the Procion dyes. Measured rates for the textile dyes ranged from 4.3 to 41.8mg dye/h/g protein (Table 23, Section 3.7) with rates decreasing in the order Procion Yellow>Reactive Black>Procion Navy>Procion Crimson. For Procion Yellow and Procion Crimson a difference in rate between the two forms of the dyes was apparent with higher rates observed for the hydrolysed dyes. For Procion Navy no real difference was apparent and for Reactive Black the hydrolysed dye had a slightly higher decolourisation rate. Comparable reduction rates for the four dyes in cell free assays suggest that cell permeability may again be an important factor in decolourisation of the four dyes by whole cells of *C. butyricum*.

Direct comparison of whole cell rates and cell free extract rates cannot be made as calculations have been made on the basis of dry cell weight and protein respectively. An estimation of the protein content of total cell mass at 50% (Green, 1991) gives an indication of the difference in rates between the two sets of assays as one unit of protein is equivalent to two units of dry cell mass. Decolourisation rate has been shown to be linear with cell density (Figure 16, Section 3.5.5) and as such a doubling of the measured whole cell rates would give an estimation of whole cell rate based on protein. Estimation of results in this way shows that rates for the Procion dyes are generally higher in cell free assays than in whole cell assays for *E. faecalis* which may be due to elimination of the membrane transport rate-limiting factor as discussed above. For *C. butyricum* the decolourisation rate for Reactive Black in cell free assay was lower than with whole cells of the same strain. The reduced rate for Reactive Black in cell free assay may be due to this dye having a toxic effect on the *C. butyricum* cell extract. The role of this dye in toxicity will be discussed in Section 4.4.

Increased decolourisation rate in cell free assays compared to whole cell experiments has also been observed by Wuhrmann *et al.* (1980). In this study the parent form of Reactive Black decolourised by *E. faecalis* had an increased rate in cell free measurement (from 42.4 (estimated) to 72.7mg dye/h/g protein) whereas for the hydrolysed form the rate was much reduced (from 108.2 (estimated) to 54.1mg dye/h/g protein). The increase in rate for the parent form in cell free assay can be explained by differences in the degree of sulphonation for the two forms of the dye. It was observed previously that the overall form of the decolourisation curves suggests that the decolourisation reaction proceeded to an end point induced by an accumulation of toxic products. The reduced decolourisation rate for the hydrolysed dye may be due to this form of the dye or its reduction products having a toxic effect on the crude cell free extract.

Comparison of data from this study for chemical and biological reduction of the four reactive dyes revealed a correlation between initial decolourisation rate for whole cell assays with glucose and the redox potential at decolourisation for the chemical system at pH3 (Figure 20, Section 3.8.2). Measured correlation coefficients were r^2=0.97 for *E. faecalis* and r^2=0.92 for *C. butyricum* (F5). The redox potential at decolourisation was also found to correlate (r^2=0.94) with the ratio of reducing agent

(to that for Reactive Black) required producing decolourisation at the same pH (Figure 8, Section 3.2). It was observed that the initial decolourisation rate for the biological system decreased as the redox potential at which decolourisation occurred for the chemical system became more negative and the more chemical reducing agent was required to decolourise the dye under test. This may indicate that the chemical and biological decolourisation systems investigated proceed via the same mechanism. These relationships have the potential to be exploited to predict biological decolourisation rates from simple chemical experiments although for confirmation this would have to be investigated further with a wider range of dyes.

The redox potential at decolourisation for Reactive Black decolourised by *E. faecalis* was determined for both the parent and hydrolysed forms of the dye (Section 3.8.1). Decolourisation did not occur with the initial drop in redox potential observed indicating that it was not the fall in redox potential alone that produced decolourisation of the dye (Figure 18). This was confirmed by the observation that decolourisation occurred for Sunset Yellow in a similar assay with *E. faecalis* before the redox potential had fallen by an appreciable extent (Figure 19). For the four reactive dyes in this study it is not known whether the redox potential at decolourisation in a biological system is related to initial decolourisation rate however this could be tested via a series of decolourisation assays as used for Reactive Black. Bragger *et al.* (1997) observed a decrease in decolourisation rate as redox potential became more negative. Semde *et al.* (1998) observed that for a mixture of dyes decolourisation occurred preferentially in the order of smallest negative redox potential.

4.3. Azoreductase and the mechanism of decolourisation.

Electrophoretic separation of a crude cell free extract of *E. faecalis* yielded two sites of decolourisation (Figure 21, Section 3.9) on activity stained gels with typical R_f values of 0.72 (site 1) and 1.0 (site 2) on 10% gels. No decolourisation was observed at either site in the absence of NADH. Protein bands at site 1 were visible following silver staining whereas at site 2 no protein bands could be detected by this technique (Figure 23, Section 3.9). The molecular weight of the azoreductase at site 1 was determined to be 114.4 kDa. Decolourisation at site 2 was thought to be due to a chemical entity present in the crude cell extract. Decolourisation occurred at site 2 in the absence of tracking dye and therefore could not be attributed to

interaction of bromophenol blue in the sample loading buffer and the staining dye or cell extract components. Decolourisation did not occur at either site with boiled cell extracts indicating thermal instability of both decolourising entities. Both decolourising species retained activity during oxygen exposure.

A summary of isolated bacterial azoreductases has been given in Table 9, Section 1.7. The molecular weight of *E. faecalis* azoreductase determined in this study is in the middle of the range of values quotes by other authors. The azoreductase of *E. faecalis* was isolated from the soluble fraction of sonicated cells indicating an intra-cellular association in agreement with Idaka *et al.* (1987) and Dykes *et al.* (1994).

Detection of the azoreductase activity of *E. faecalis* was obtained by activity staining in the open laboratory indicating this activity was oxygen insensitive as was found for the *Pseudomonas* and *Klebsiella* strains used by Zimmerman *et al.* (1982, 1984) and Dykes *et al.* (1994). In contrast the azoreductases of a range of anaerobic bacteria isolated by Rafii and Cerniglia (1990a) were irreversibly inactivated by oxygen. Rafii and Cerniglia (1990a) also found that for the species of anaerobic bacteria investigated the addition of co-factors was not necessary for the appearance of decolourised bands on activity stained gels but did speed their development. Contrary to this finding the two decolourisation sites obtained for *E. faecalis* in this study could not be visualised without the addition of NADH. Rafii and Cerniglia (1990a, 1993a) proposed that for *C. perfringens* azoreductase co-migrated with dehydrogenase and that azoreductase and nitroreductase were due to the same enzyme. It is not known whether this is the case for *E. faecalis* although this could be tested by a repeat of the electrophoretic separations with activity staining for dehydrogenase with a tetrazolium salt and nitroreductase with 4-nitrobenzoic acid.

Zimmerman *et al.* (1982, 1884), Rafii and Cerniglia (1990a) and Dykes *et al.* (1994) all reported the isolation of a single azoreductase site for the bacterial strains tested (Table 9, Section 1.7). In this study two decolourisation sites were visualised on activity stained gels for *E. faecalis*, one found to have a molecular weight of 114.4 kDa and the other thought to be due to a chemical entity present in the crude cell free extract. Accounts have been given by Ghosh *et al.* (1992, 1993) of the presence of two azoreductases for *Sh. dysenteriae* and *E. coli* determined to be due to specific proteins. Two azoreductases have also been isolated for *C. cuvata* by Kakuta *et al.* (1998).

In this study azoreductase activity could not be obtained on an activity stained gel for cell free extracts of *C. butyricum* (F5). Several reasons have been proposed for this as discussed in Section 3.9. For the anaerobic strains tested by Rafii and Cerniglia (1990a) azoreductase activity was determined to be in spent culture supernatants and irreversibly inactivated by oxygen. In this study the soluble fraction of broken cells was used which was known to have azoreductase activity as measurable decolourisation rates were obtained with the extracts in cell free assay (Table 23, Section 3.7). It was therefore thought unlikely that azoreductase activity was not detected due to its presence in a different portion of the cell extract preparation. Inactivation by oxygen was a possible explanation for the lack of activity despite precautions taken to avoid this. Another possible reason for the lack of activity could be that the cell extract was not concentrated enough to give a visual reduction of the dye on a gel due to the limited amount of material that could be loaded. Precipitation of proteins from cell free preparations and repeating the experiments could test this. It was not known whether the azoreductase of *C. butyricum* (F5) required a co-factor present in the cell extract that may not have been available after separation.

In this study formation of the hydrazo intermediate of Reactive Black by *S. putrefaciens* and *E. faecalis* (when assays contained <0.1% glucose) has been discussed in Section 4.2. For both strains whole cell assays with Reactive Black appeared visually decolourised, changing back to the original blue colour upon centrifugation to remove the bacterial cells indicating that decolourisation of the dye was not complete and reduction of the dye to the hydrazo form only was likely.

Confirmation of intermediate formation and asymmetric azo bond breakage during decolourisation was achieved in this study by HPLC separation of timed samples from decolourisation assays with *E. faecalis* and Reactive Black (parent and hydrolysed). For the parent form of Reactive Black (Figure 31, Section 3.10) changes to the dye structure were apparent within the first minute of the decolourisation assay. Comparison of chromatograms for a solution of unreduced dye (Figure 30) and the 0min sample from the assay (Figure 31) indicated the formation of two decolourisation products eluting with R_t=4.7min and R_t=23.5min. By 5min from the start of the assay the unreduced dye peak was absent from the chromatogram indicating changes to one or both azo bonds in the dye structure.

Both bonds could not be completely cleaved at this time as samples appeared blue/purple. Chromatograms for subsequent timed samples indicated that the peak at R_t=4.7min was one of the final products (later identified to be the vinyl sulphone side chain as discussed in Section 3.10) which continued to increase in area to a maximum at complete decolourisation. The product peak at R_t=23.5min was not persistent as its formation proceeded to a maximum followed by reduction in area concomitant with the formation of the second final product peak eluting at R_t=21min (identified to be the di-amino H-acid fraction of the dye). As was predicted from the structure of Reactive Black (Figure 4) two decolourisation products were formed confirming reduction by azo cleavage.

For hydrolysed Reactive Black (Figure 32) three intermediates were formed during decolourisation which were also not persistent with two final products formed eluting at R_t=21min and R_t=23min. By elimination, the peak at R_t=21min was assumed to be the di-amino H-acid fraction and the peak at R_t=23min was thought to be due to the hydrolysed vinyl sulphone side chain. Repetition of these experiments yielded similar chromatograms indicating that in duplicate assays the decolourisation of Reactive Black proceeds via the same mechanism. In agreement with this study Oxspring *et al.* (1996) has shown the production of two main product peaks from decolourisation of Reactive Black using capilliary electrophoresis.

If degradation of a dye proceeds via azo bond cleavage it may be expected that identical end products would be produced for a particular dye when decolourised by different bacterial strains. Confirmation of this was attempted in this study by comparison of UV/VIS spectra (Figures 33-40, Section 3.11.1) for Reactive Black and the three Procion dyes (parent and hydrolysed) decolourised by *E. faecalis* and *C. butyricum* (F5). It was found that for Reactive Black and Procion Crimson the UV/VIS profiles for samples decolourised by both strains were similar indicating that the same decolourisation products were produced for these dyes by *E. faecalis* and *C. butyricum* (F5). For Procion Navy and Procion Yellow the UV/VIS spectra of decolourised samples showed differences for each bacterial strain. As the UV/VIS spectra obtained gave only the overall profiles for the decolourised dye samples confirmation of these results was sought via HPLC analysis for Reactive Black and Procion Navy.

For Reactive Black, the chromatograms (Figures 41-44, Section 3.11.2) for samples decolourised by *E. faecalis* and *C. butyricum* (F5) were similar indicating that these two strains did produce the same decolourisation products for this dye. For Procion Navy (Figures 46-49, Section 3.11.2) some differences in decolourisation products for each bacterial strain were apparent. Two product peaks observed were common to samples decolourised by both strains although differences in the relative amounts detected were apparent. Two further product peaks were observed for *C. butyricum* (F5) reduced dye samples that were absent from the *E. faecalis* assay chromatograms. It has been proposed by Carliell *et al.* (1994, 1995) that decolourisation of the Procion dye Reactive Red 141 proceeded via azo bond cleavage and breakage of the amine linkages between the chromophore and the reactive group and within the reactive group. It would be expected that Procion Navy would have a similar structure to Reactive Red 141. If this were the case one of the bacterial strains tested may break down the dye structure further than cleavage of the azo bond. Breakage of amine linkages in other parts of the dye molecule may explain the differences observed for Procion Navy.

4.4. Products of decolourisation and toxicity assessment.

It has been demonstrated in this study that decolourisation of Reactive Black does proceed via azo bond cleavage to form two main products, a di-amino H-acid and a vinyl sulphone side chain. It has been shown however that for some dyes other parts of the structure may also be attacked (Carliell *et al.*, 1994 and 1995) and that different bacterial strains may produce different decolourisation products (as shown for Procion Navy in this study). It was attempted in this study to develop methods for the identification of dye reduction products using the model dye Reactive Black decolourised by *E. faecalis* and subsequently apply those methods to the analysis of other dye decolourisation products.

The problems encountered with this analysis have been discussed in Section 3.12. Initial inspection of TLC separations of decolourised samples yielded more than two reduction products for the parent and hydrolysed forms of Reactive Black which was not as expected from the HPLC results. Several common products were observed with respect to hR_f values and colour fluoresced under UV light for the reduced parent and hydrolysed dye (Table 24, Section 3.12). From the HPLC analysis only one common fraction (i.e. the di-amino H-acid) from these two forms of Reactive

Black was expected. UV/VIS scans of sample bands extracted from the TLC plates revealed that some bands having similar hR_f values and fluorescent colours did not have the same UV spectra. The most likely explanation for this was thought to be each amine group on the dye reduction products independently interacting with the free silanol groups on the TLC plate surface and thus giving rise to multiple bands on the TLC plate. Samples of dye reduction products could not be obtained in an appropriate matrix for further structural analysis. Discontinuation of this work was justified in that the structure of a dye decolourisation product would only be important if that product were found to display toxicity.

The likely environmental concentration of a dye has been predicted to be in the region of 1ppm by Easton (1995) and between 1 and 5ppm by Hobbs (1989) with worst case scenario concentrations of up to 1555ppm. Pierce (1994) has suggested that the discharge from a model dyehouse dyeing with reactive dyes will be on average 60ppm. Hobbs (1989) has proposed that a dye could be considered to be unlikely to cause harm if a factor of 100 exists between the likely environmental concentration and the concentration at which a dye has been shown to produce adverse effects. If the environmental concentration of a dye were 1ppm then the dye would be considered unlikely to cause harm if toxic effects were only observed above a concentration of 100ppm (equivalent to 100mg/l). Brown *et al.* (1981), Carliell *et al.* (1995) and Beydilli *et al.* (1998) all observed toxicity of textile dyes above 100mg/l only. This may lead to the conclusion that dyes would not be a major toxic problem and that acceptance as an aesthetic problem alone (Easton, 1995) may be justified.

Concern has been expressed in view of extensive research undertaken on the benzidine based dyes that certain dyes may not produce a toxic response when tested but may yield toxic metabolites upon reduction (see Section 1.4.2). Chung and Cerniglia (1992) stated that toxicity testing of intact dye molecules should not be considered to encompass all potential dangers. Were dyes to undergo reductive cleavage post release into the environment or in a decolourisation treatment system producing aromatic amines there would be a need for an assessment of the toxicity of the dye breakdown products in addition to the toxicity of the original dye.

In this study it has been demonstrated that toxic effects are apparent for the decolourisation products of Reactive Black in the Microtox® test at concentrations

(original dye concentration reduced) in the region of 1.7-4.9ppm for the parent dye and 0.15-0.25ppm for the hydrolysed dye (Table 26, Section 3.13). These concentrations are of the same order as the likely concentration of a dye released into the environment and as such are of concern. It was apparent from this study that the sample matrix had an important influence on the final test result in that a toxic response observed is assumed to be due to the most toxic component within the mixture. Control assays of cells and glucose in phosphate buffer produced EC_{50} (5min) values of 0.7ppm for *E. faecalis* and 7.9ppm for *C. butyricum* (F5) based on an equivalent concentration as if the assays had contained dye. All measured EC_{50} values for biologically reduced dye samples therefore had to be assessed taking into consideration the EC_{50} value obtained for the control assays. Assessment of data in this way indicated that the EC_{50} values obtained for the Procion dyes (Table 27, Section 3.13) were not significantly different to those for the control assays and therefore no comment can be made as to their toxicity in this test.

Friedman *et al.* (1980) noted that in a study of 28 commercial grade textile dyes it was not known if the mutagenic response observed for nine of the dyes in the Ames test was due to the dyes themselves or impurities in the preparations carried over from their manufacture. The inability of an anaerobic culture to decolourise the printing dye Reactive Yellow 95 was attributed to inhibitory or toxic components present in the commercial preparation carried over from manufacture (Carliell *et al.*, 1994 and 1995). The toxicity observed for Reactive Black in this study was determined to be due to the dye itself and not an inherent impurity as HPLC fractionation of the decolourisation assay components and subsequent Microtox® testing yielded an EC_{50} value for the hydrolysed vinyl sulphone side chain of 0.34ppm. A sample of HPLC mobile phase at the same composition as the isolated dye fraction did not produce an inhibitory effect. No effect was observed at the top concentration tested (2.18ppm) for the di-amino H-acid fraction common to both forms of Reactive Black. It was recognised that the H-acid fraction from the dye and the commercially available H-acid differed by two amino groups. The lesser inhibitory effect of the di-amino H-acid fraction of the dye may be expected as a much higher EC_{50} value for the commercial preparation of H-acid (49.7ppm at 5min) was obtained. The parent form of Reactive Black decolourised by *C. butyricum* (F5) was found to have an EC_{50} value less than the control assay for this

bacterial strain. This was not apparent from testing of the fractionated dye products due to difficulties encountered with concentration of collected material due to the large amounts of phosphate present.

The increased inhibitory effect of the hydrolysed vinyl sulphone side chain may result from an increased rate of passage across the cell membrane of *V. fischeri* (Microtox® reagent) due to the loss of sulphonate groups during the hydrolysis process. In agreement with this theory, Ogawa *et al.* (1981) found that Acid Orange 10 (a di-sulphonated dye) inhibited cell division of *B. subtilis* less than Acid Orange 12 (a mono-sulphonated dye). The role of sulphonation as a rate-limiting factor to membrane transport has already been discussed in Section 4.2. Determination of the hydrolysed vinyl sulphone side chain as a source of toxicity may explain why the decolourisation rate of the hydrolysed form of Reactive Black is lower than the parent form in cell free assays for *E. faecalis* whereas the reverse was noted for whole cell experiments as discussed in Section 4.2. The reduced decolourisation rate of hydrolysed Reactive Black may be due to inhibition of the crude cell free extract by production of the side chain toxic metabolite from reduction of the dye. This may also explain the lower decolourisation rates observed for Reactive Black in cell free assays for *C. butyricum.*

It has been assumed in this study that the inhibition of *V. fischeri* (Microtox® reagent) and hence measured EC_{50} values are due to the most toxic component in a mixture and that synergistic effects are not significant. It has been shown in this study that for biologically reduced dyes the control assays displayed apparent toxicity and data has been assessed in view of this background matrix effect. It has been demonstrated however by Haniffa and Selvan (1991b) that for the toxicity assessment of bleaching and dyeing effluents using fish test species synergistic effects were apparent as combining the wastes produced a mixed effluent 1.14 times more toxic than the dyeing effluent alone. The results obtained for decolourised samples of hydrolysed Reactive Black in this study were confirmed by isolation of single components through HPLC fractionation and toxicity testing of those fractions. This aspect should however be considered in testing of other samples in similar matrices using these techniques.

To determine whether the products of decolourisation differed in toxicity to the intact dye molecules unreduced dyes were also tested and were found to have higher

EC_{50} values than the decolourised dyes indicating that toxicity increased with azo reduction (Table 28, Section 3.13). A major drawback of the Microtox® acute toxicity test was the inability to test coloured compounds directly as the test relied on light emission from the test bacterium (*V. fischeri*) at 490nm, a wavelength at which most, if not all, dyes will absorb. Application of a colour correction procedure allowed adjustment of results to take into account the apparent toxicity created by the reduction in light detected from the test bacterium due to absorption of that light by the test dye. Non-adjustment of data created a false increase in percentage inhibitory effect on light levels as the concentration of the dye increased towards the top of the dilution series (i.e. more coloured).

After colour correction the EC_{50} (5min) values for unreduced Reactive Black were determined to be in the region of 38-92ppm and 13-18ppm for the parent and hydrolysed forms of the dye respectively (Table 28, Section 3.13). Non-correction of data gave values in the region 18-28ppm and 9-11ppm (at 5min) for the unreduced parent and hydrolysed form of the dye respectively. Enhanced toxicity for the hydrolysed form of the dye was again apparent possibly due to reduction in degree of sulphonation increasing the rate of transport across the cell membrane of the test bacterium as discussed earlier. The resultant EC_{50} values for the unreduced hydrolysed Reactive Black were comparable to the values measured for phenol (18.7ppm (5min) and 24.5ppm (15min)). The EC_{50} values obtained for phenol were in agreement with those reported by Ghosh and Doctor (1992).

For the Procion dyes colour corrected data indicated these dyes had no toxic effect at the top concentration tested (100ppm). Although it is recognised that this aspect was investigated to a limited extent this finding is in agreement with the studies by Brown *et al.* (1981), Carliell *et al.* (1995) and Beydilli *et al.* (1998) in which toxicity was only observed above 100mg/l for a variety of textile dyes. For reasons discussed previously no comment can be made on whether there is an increase in toxicity due to azo reduction of these dyes.

It has been stated that the reductive cleavage of azo linkages is probably the most toxicologically important metabolic reaction of azo compounds (Chung *et al.*, 1992). Brown and DeVito (1993) have proposed several mechanisms of dye toxicity. Azo reduction can activate toxicity by production of toxic aromatic amines as observed for benzidine based dyes (Morgan *et al.*, 1994) and the food dyes Amaranth and

Sunset Yellow (Sweeney *et al.*, 1994) or it can produce a detoxification reaction as observed for phenylazoaniline dyes (Rosenkrantz and Klopman, 1989). This study has demonstrated that for the textile dye Reactive Black 5 azo reduction results in an increase in toxicity for the Microtox® test with enhanced toxicity observed for the decolourisation products of the hydrolysed form of the dye. Fractionation of dye decolourisation products confirmed that the hydrolysed vinyl sulphone side chain was a source of toxicity in the Microtox® test.

Two accounts are given in the literature on the use of the Microtox® test for toxicity assessment of textile effluents (Rutherford *et al.*, 1993 and Svenson *et al.*, 1996). No comments were given as to the role of dyes in observed toxicity in either study as both relied on GC-MS for identification of toxic components and it has been proposed that this technique discriminates against non-volatile species such as dye molecules (Games and Hites, 1977).

Several investigations have been undertaken to evaluate interspecies correlation between the Microtox® test and toxicity to other organisms. Blum and Speece (1991) determined the correlation to be good (r^2=0.70 to 0.82) between the Microtox® test, three bacterial groups (aerobic heterotrophs, *Nitrosomonas* and methanogens) and toxicity to fathead minnows for 50 to 100 chemicals (chlorinated aliphatic hydrocarbons, substituted benzenes and phenols). Rosenkrantz *et al.* (1993) determined that the Microtox® test could be used as an indicator of potential toxicity to fish and mammals as significant commonalities were found between structural determinants associated with the Microtox® test and those associated with toxicity to fish and mammals. Chang *et al.* (1981) reported correlation coefficients for the Microtox® test of 0.9 with rat toxicity and 1.0 with fish toxicity for selected compounds including benzene, toluene and phenol. DeZwart and Slooff (1983) have recommended use of the Microtox® test as a prescreening tool in the hazard assessment of chemicals following comparison of the toxicity of 15 chemicals to 20 different test species.

The relative sensitivity of the Microtox® test has been compared to *Daphnia*, rainbow trout and fathead minnow by Munkittrick *et al.* (1991) for a range of pure compounds and industrial effluents. It was determined that although differences were apparent in the relative sensitivity of the Microtox® test and other acute

lethality tests the Microtox® assay was the best available choice for the rapid assessment of toxicity for diverse environmental samples.

The lack of data in the literature for Microtox® assessment of dyes and textile effluents makes comparison of the results from this study with the literature difficult. Studies on the use of the test for other types of compounds however indicate the results from the toxicity tests in this study can be regarded as a fair assessment of the inhibitory effects of the dyes and their reduction products tested.

4.5. Conclusions.

The aims and objectives of this study have been partially achieved. The isolation of a variety of bacterial strains from textile effluent capable of decolourising a range of textile dyes has been demonstrated. A decolourisation assay has been developed and used to decolourise four reactive dyes (Reactive Black, Procion Navy, Procion Crimson and Procion Yellow) in both their parent and hydrolysed forms. The environmental isolate *C. butyricum* (F5) was shown to decolourise the four reactive dyes at higher initial rates than the laboratory strain *E. faecalis*. The degree of sulphonation has been highlighted as a primary rate-limiting factor in decolourisation. For Reactive Black in particular, sulphonation was important as the hydrolysed form of this dye was decolourised at a higher rate than its parent form in whole cell assay. In contrast, the reverse was true for decolourisation by cell-free extracts. *E. faecalis* azoreductase was isolated from cell free extracts by gel electrophoresis and found to have a molecular weight of 114.4kDa. *C. butyricum* azoreductase could not be isolated using the same techniques. For Reactive Black, a dye of known structure, decolourisation has been shown to proceed via an intermediate to form two products. The same reduction products were observed when the dye was decolourised by two different bacterial strains. For Procion Navy, structure unknown, it has been shown that different bacterial strains may give rise to different decolourisation products. The reduction products of Reactive Black have been shown to have toxic implications as the hydrolysed vinyl sulphone side chain was identified as a source of toxicity in the Microtox® test.

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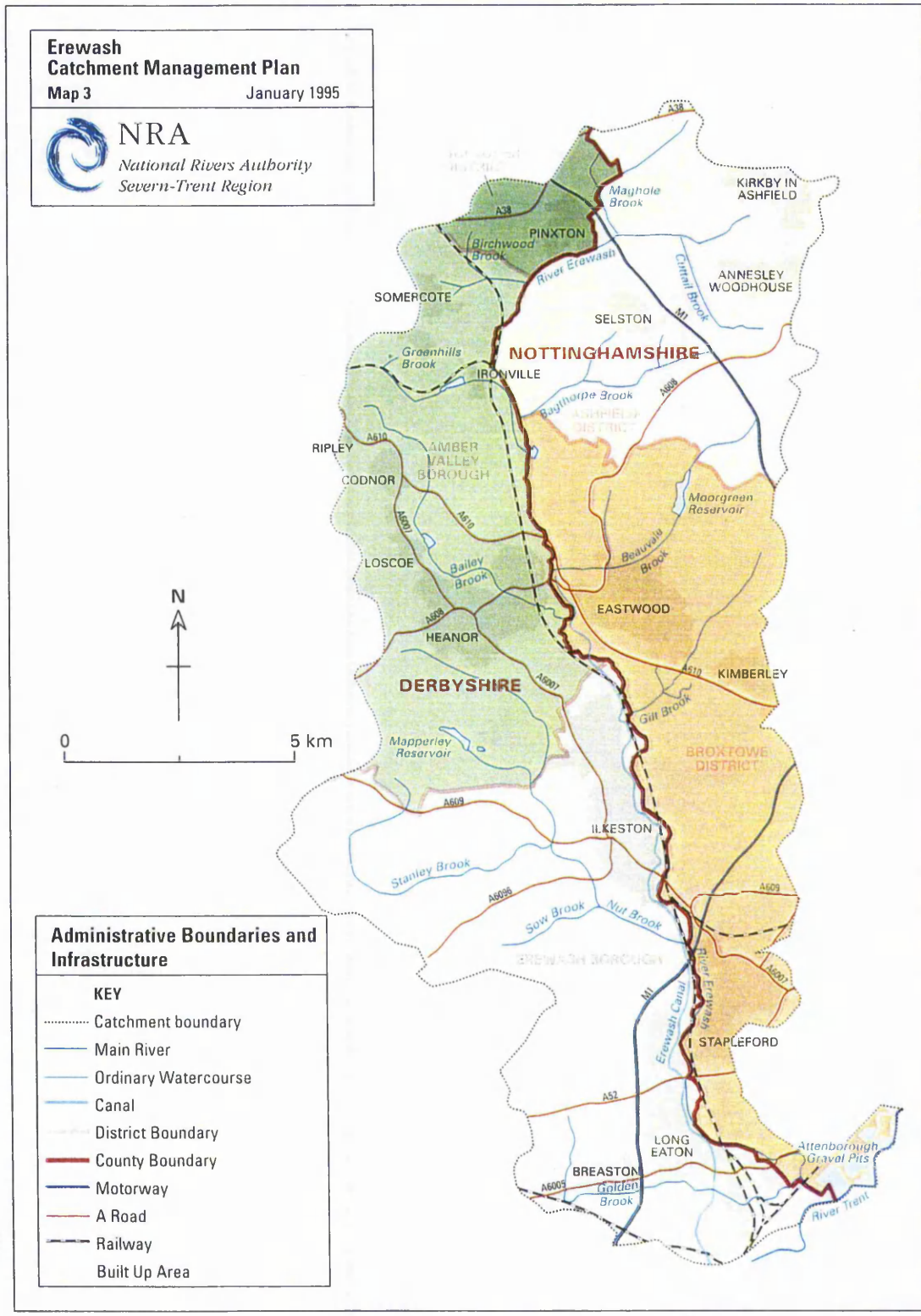
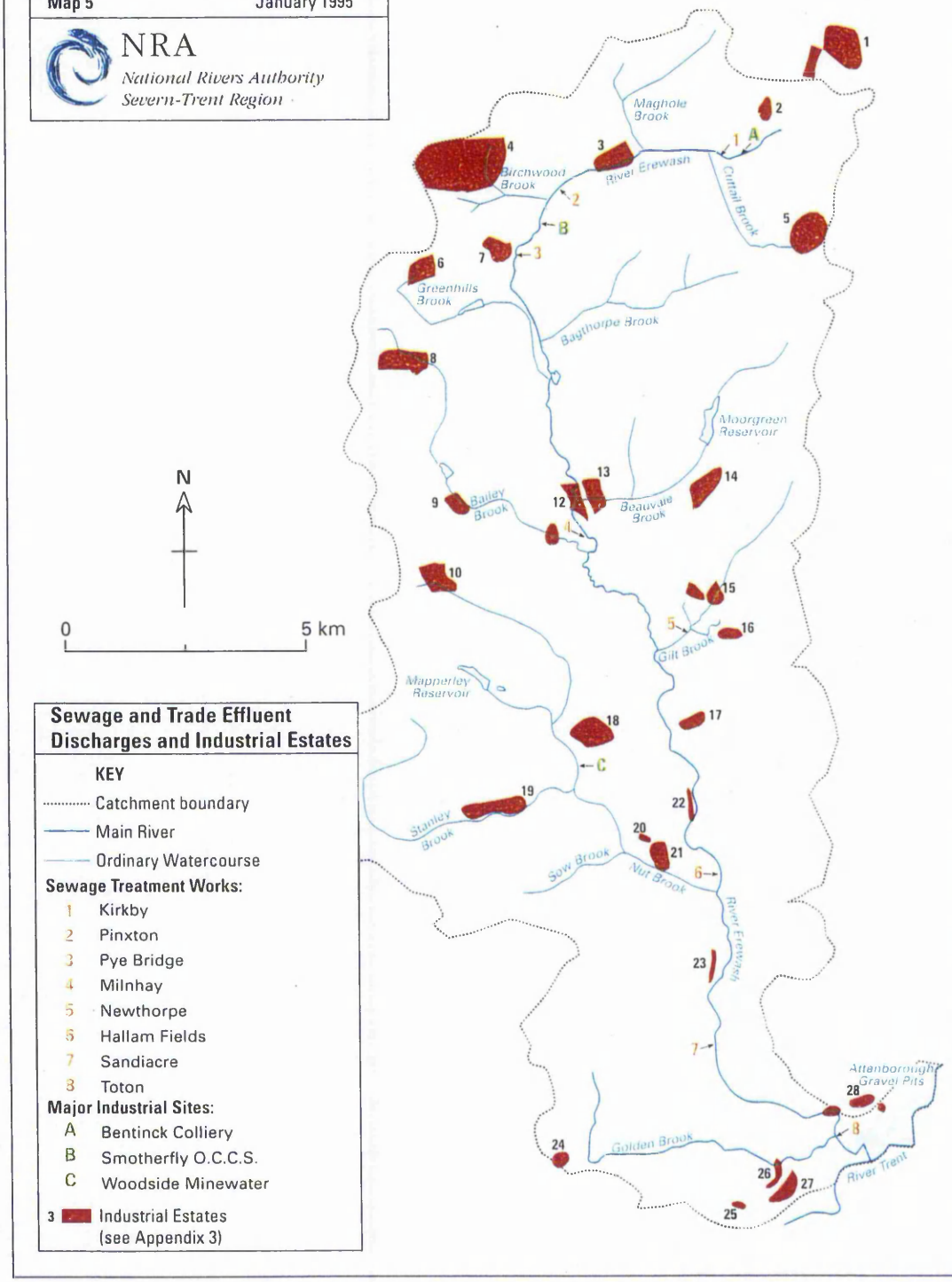
Appendix 1.

Maps of the Erewash Valley as discussed in Section 1.3. Reproduced from the River Erewash catchment management plan consultation report, The National Rivers Authority, Severn Trent Region, January 1995.


Erewash
Catchment Management Plan
Map 3
January 1995
NRA
National Rivers Authority
Severn-Trent Region
KIRKBY IN ASHFIELD
PINXTON
SOMERCOTE
ANNESLEY WOODHOUSE
SELSTON
NOTTINGHAMSHIRE
IRONVILLE
RIPLEY
CODNOR
LOSCOE
EASTWOOD
HEANOR
KIMBERLEY
DERBYSHIRE
ILKESTON
STAPLEFORD
LONG EATON
BREASTON
Maghole Brook
Birchwood Brook
River Erewash
Cinderhill Brook
Greenhills Brook
Baytnorpe Brook
Moorgreen Reservoir
Beauvale Brook
Bailey Brook
Gill Brook
Mapperley Reservoir
Stanley Brook
Sow Brook
Nut Brook
Erewash Canal
Attenborough Gravel Pits
Golden Brook
River Trent
AMBER VALLEY BOROUGH
BROXTOWE DISTRICT
N
0
5 km
Administrative Boundaries and Infrastructure
KEY
Catchment boundary
Main River
Ordinary Watercourse
Canal
District Boundary
County Boundary
Motorway
A Road
Railway
Built Up Area

Erewash
Catchment Management Plan
Map 5 January 1995

NRA
National Rivers Authority
Severn-Trent Region



Sewage and Trade Effluent Discharges and Industrial Estates

KEY

- Catchment boundary
- Main River
- Ordinary Watercourse

Sewage Treatment Works:

1. Kirkby
2. Pinxton
3. Pye Bridge
4. Milnhay
5. Newthorpe
6. Hallam Fields
7. Sandiacre
8. Toton

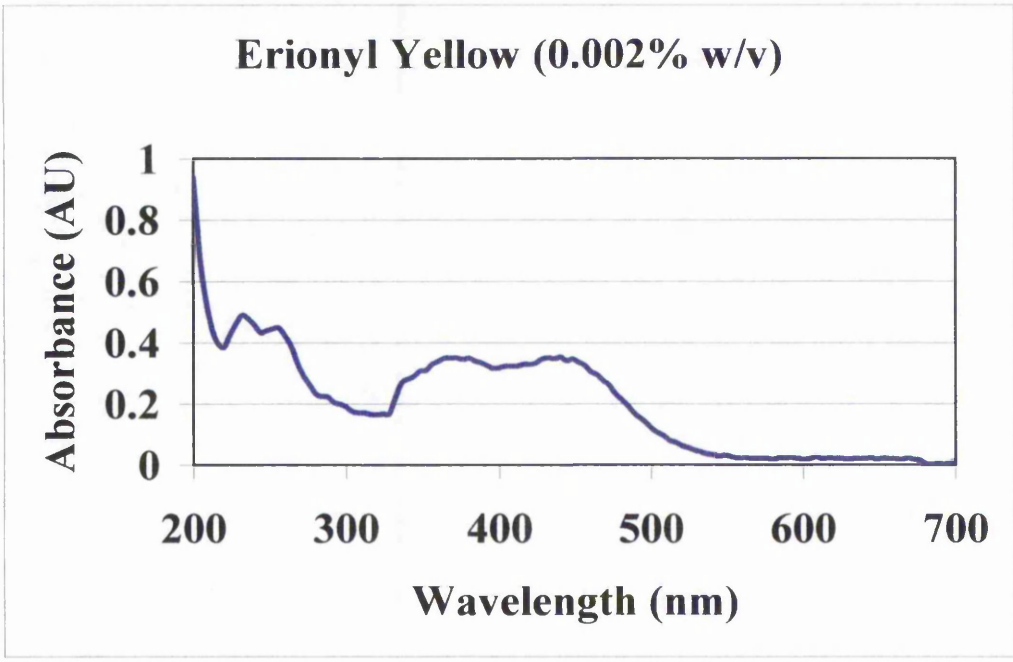
Major Industrial Sites:

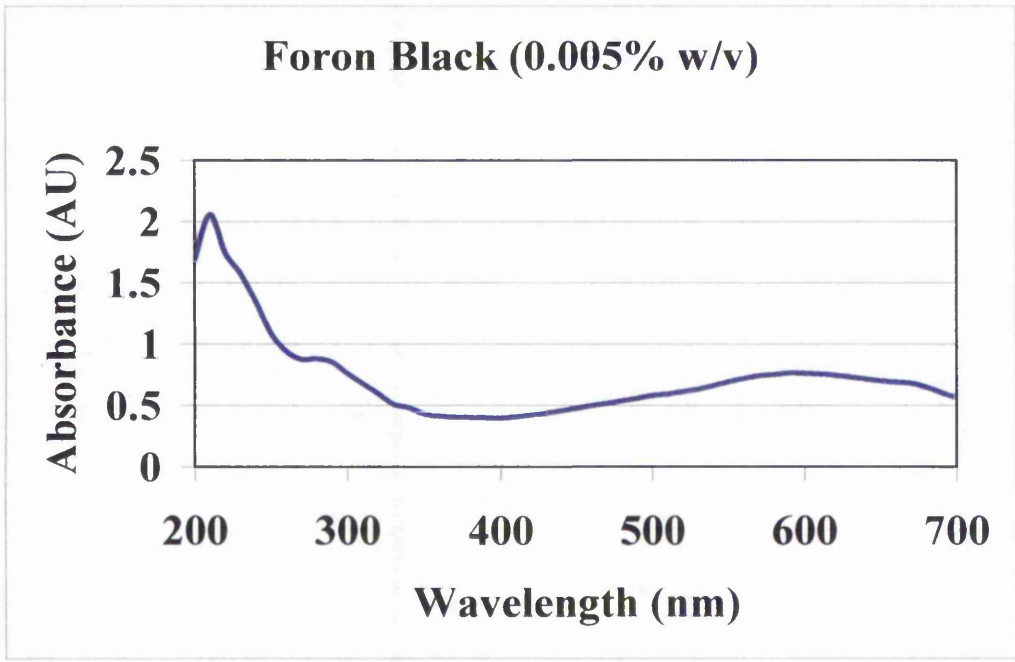
A. Bentinck Colliery
B. Smotherfly O.C.C.S.
C. Woodside Minewater

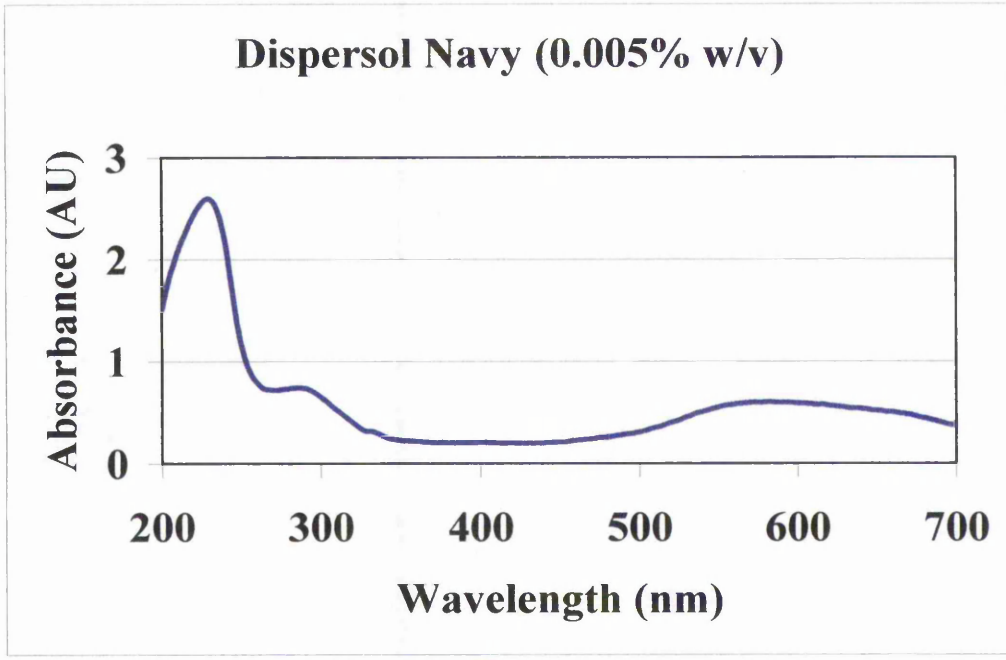
3 Industrial Estates (see Appendix 3)

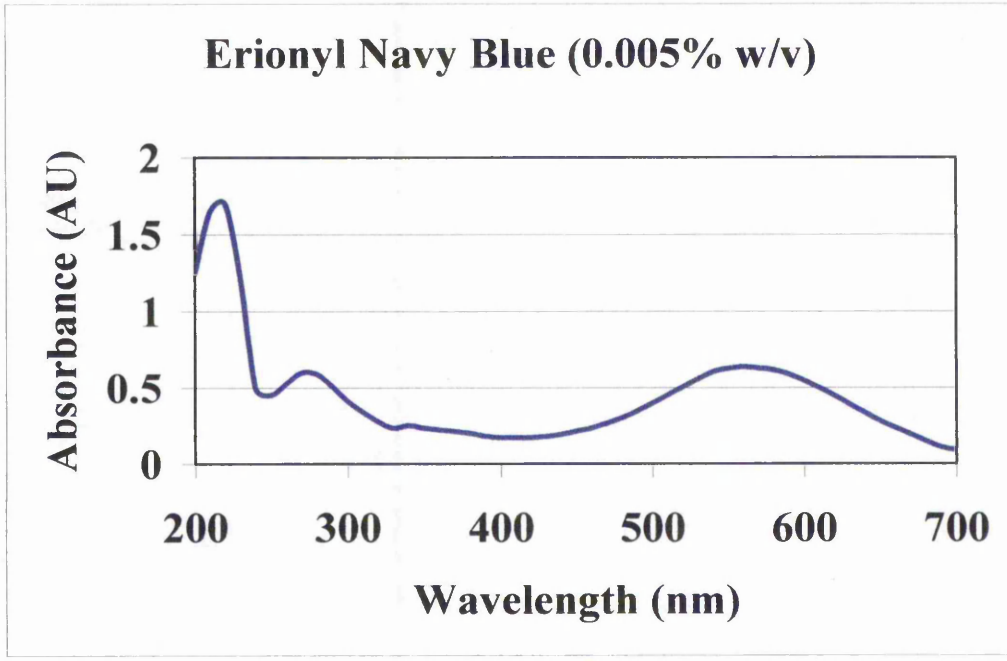
Appendix 2.

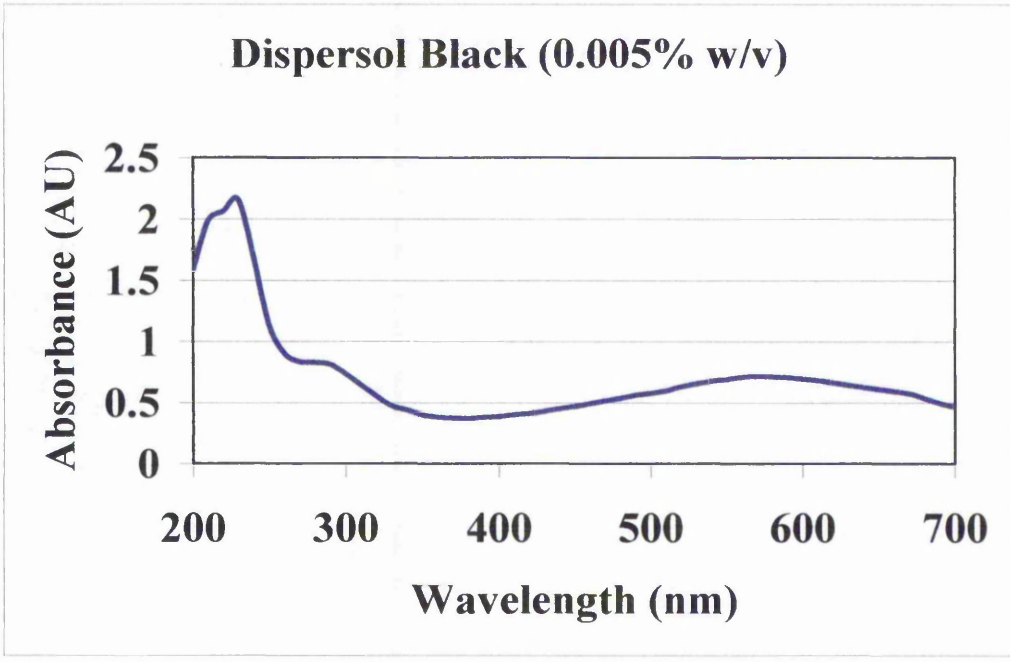
UV/VIS profiles of the 18 representative textile dyes (Table 15) as discussed in Section 3.1. Absorbance – concentration calibration curves for Reactive Black and the three Procion dyes as discussed in Section 3.5.1. Absorbance – concentration calibration curves for Reactive Black at 490nm as used for colour correction procedure for Microtox® testing as discussed in Section 2.13.3.

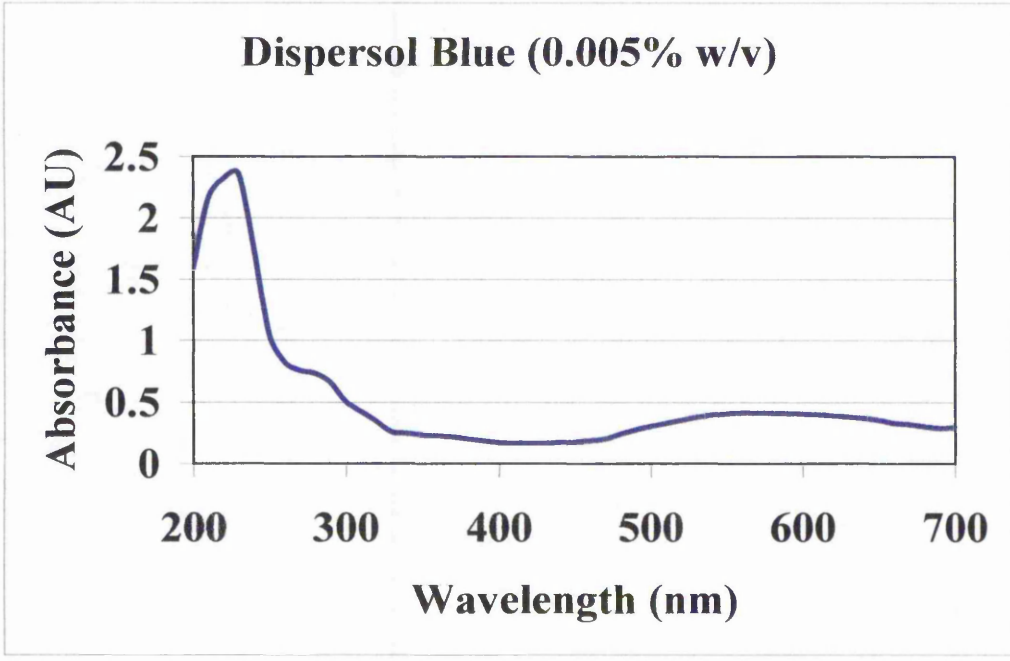

Erionyl Yellow (0.002% w/v)
Absorbance (AU)
1
0.8
0.6
0.4
0.2
0
200
300
400
500
600
700
Wavelength (nm)

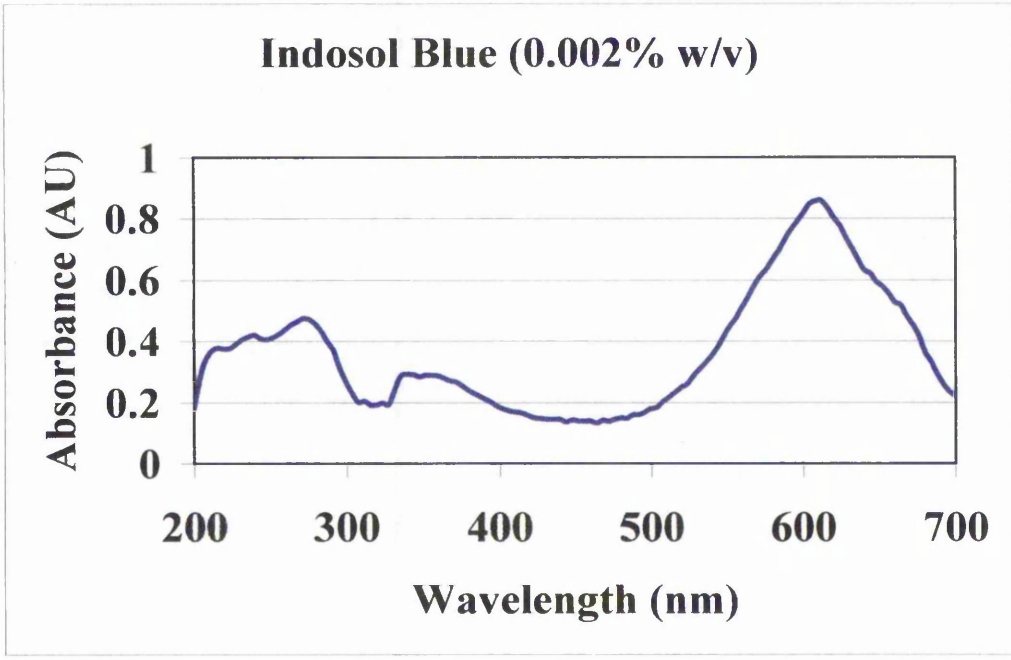

Foron Black (0.005% w/v)
Absorbance (AU)
2.5
2
1.5
1
0.5
0
200
300
400
500
600
700
Wavelength (nm)

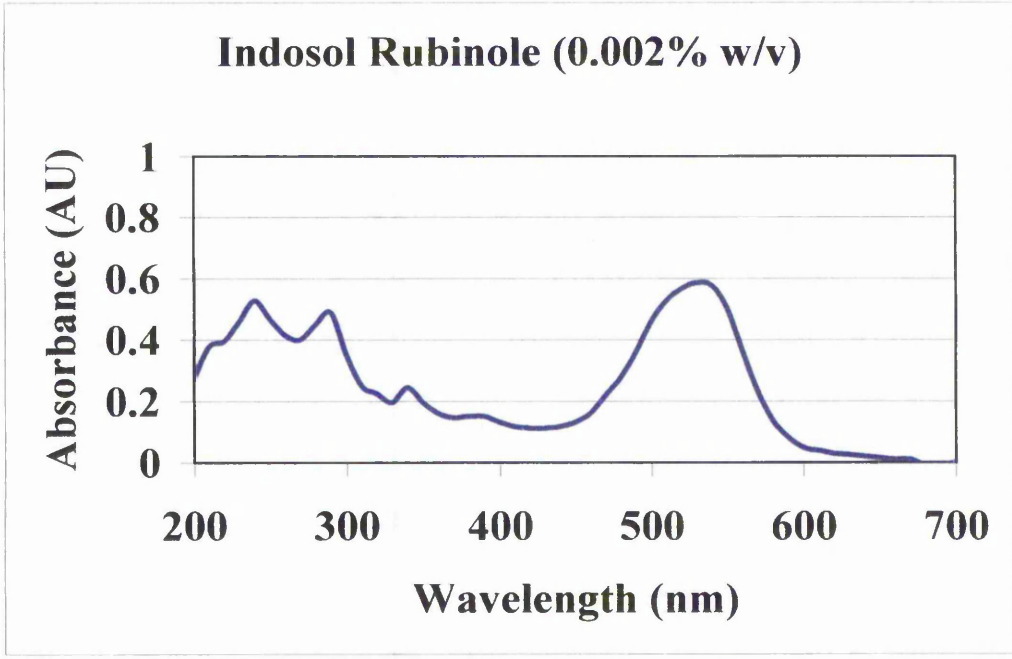

Dispersol Navy (0.005% w/v)
Absorbance (AU)
3
2
1
0
200
300
400
500
600
700
Wavelength (nm)

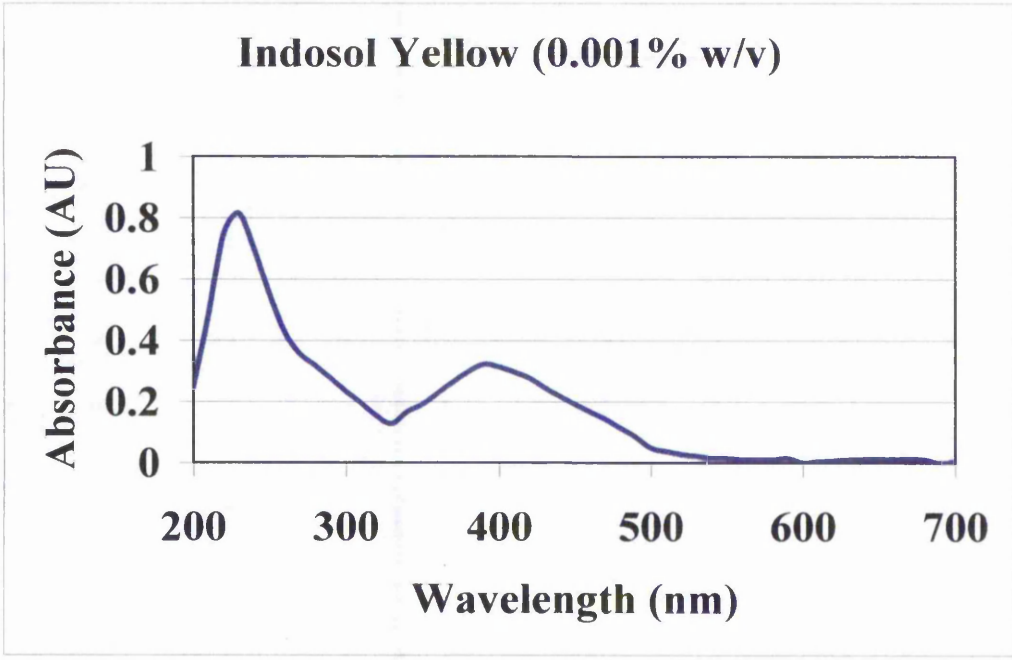

Erionyl Navy Blue (0.005% w/v)
Absorbance (AU)
2
1.5
1
0.5
0
200
300
400
500
600
700
Wavelength (nm)

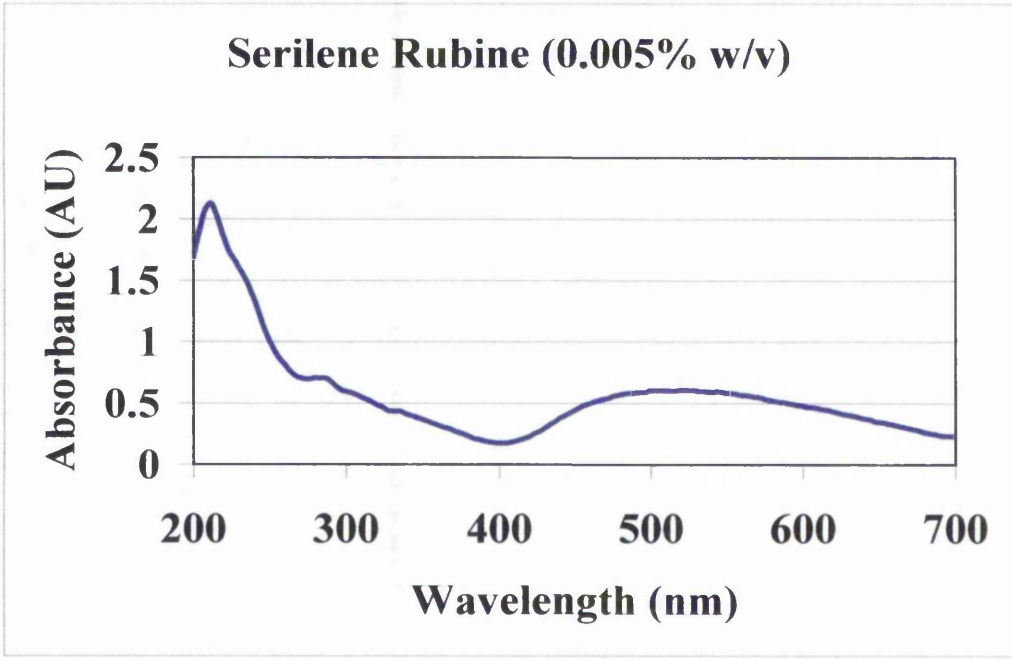

Dispersol Black (0.005% w/v)
Absorbance (AU)
2.5
2
1.5
1
0.5
0
200
300
400
500
600
700
Wavelength (nm)

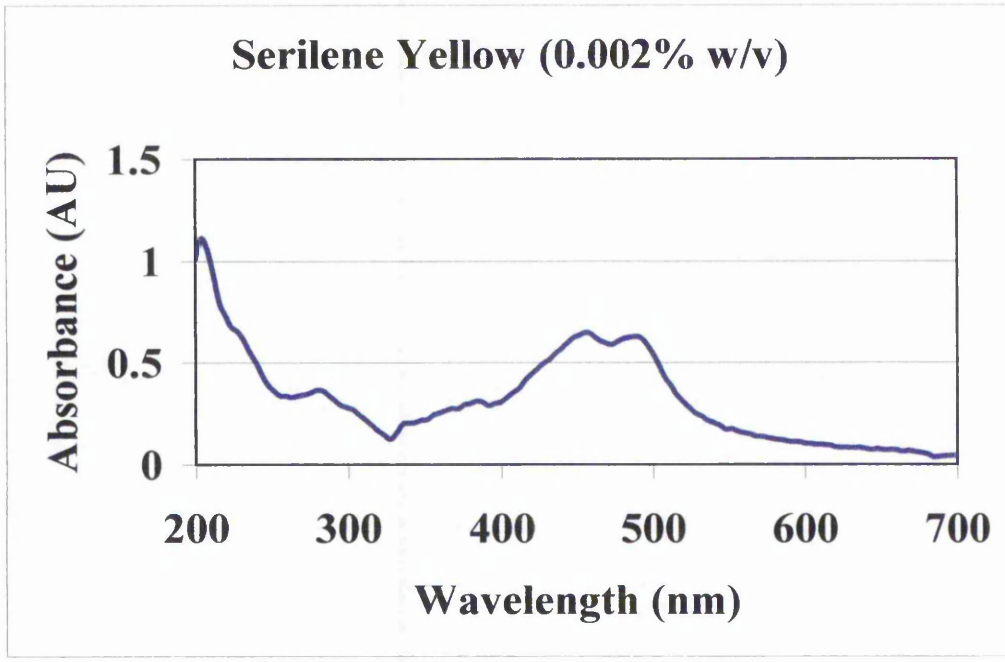

Dispersol Blue (0.005% w/v)
Absorbance (AU)
2.5
2
1.5
1
0.5
0
200
300
400
500
600
700
Wavelength (nm)

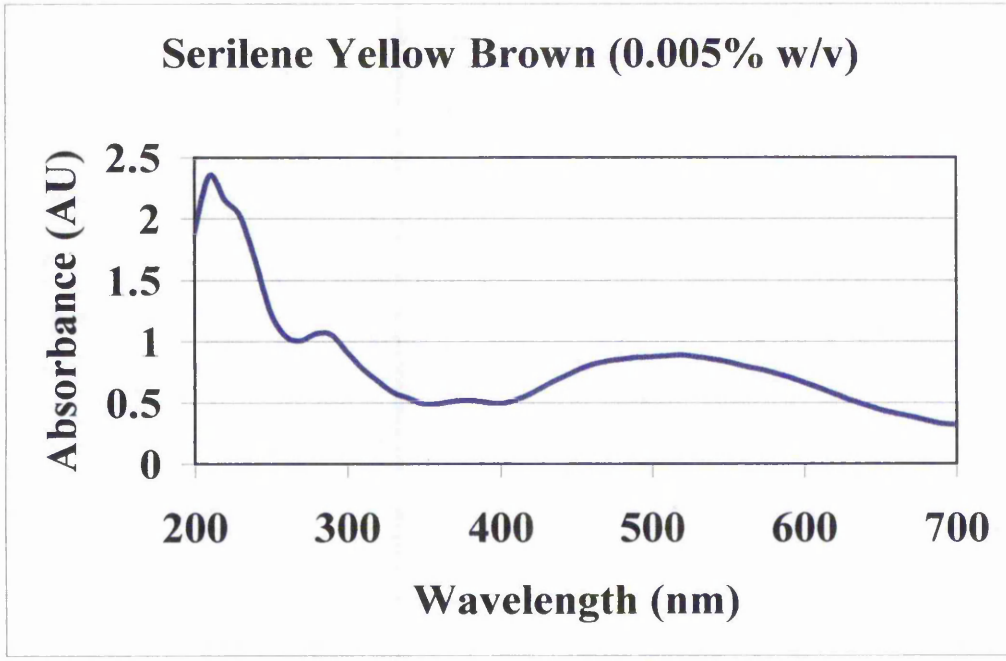

Indosol Blue (0.002% w/v)
Absorbance (AU)
1
0.8
0.6
0.4
0.2
0
200
300
400
500
600
700
Wavelength (nm)

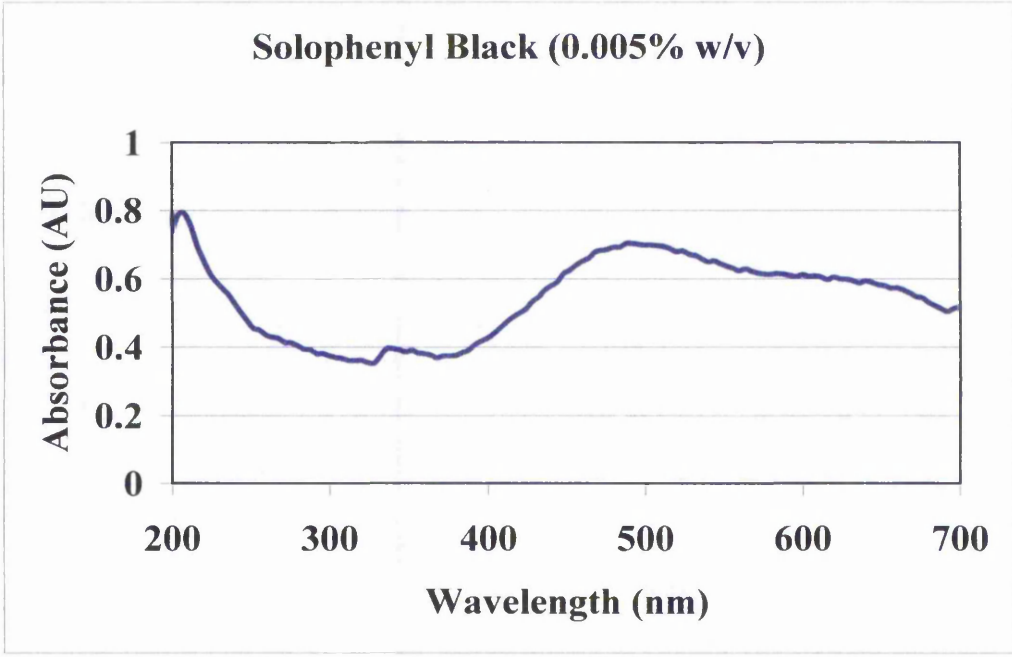

Indosol Rubinole (0.002% w/v)
Absorbance (AU)
1
0.8
0.6
0.4
0.2
0
200
300
400
500
600
700
Wavelength (nm)

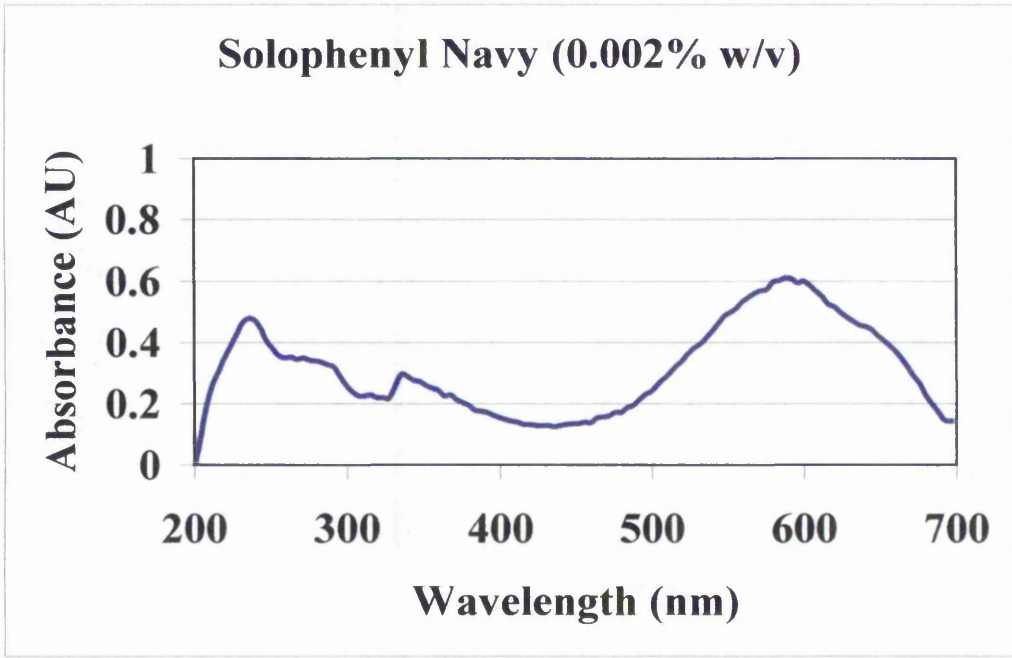

Indosol Yellow (0.001% w/v)
Absorbance (AU)
1
0.8
0.6
0.4
0.2
0
200
300
400
500
600
700
Wavelength (nm)

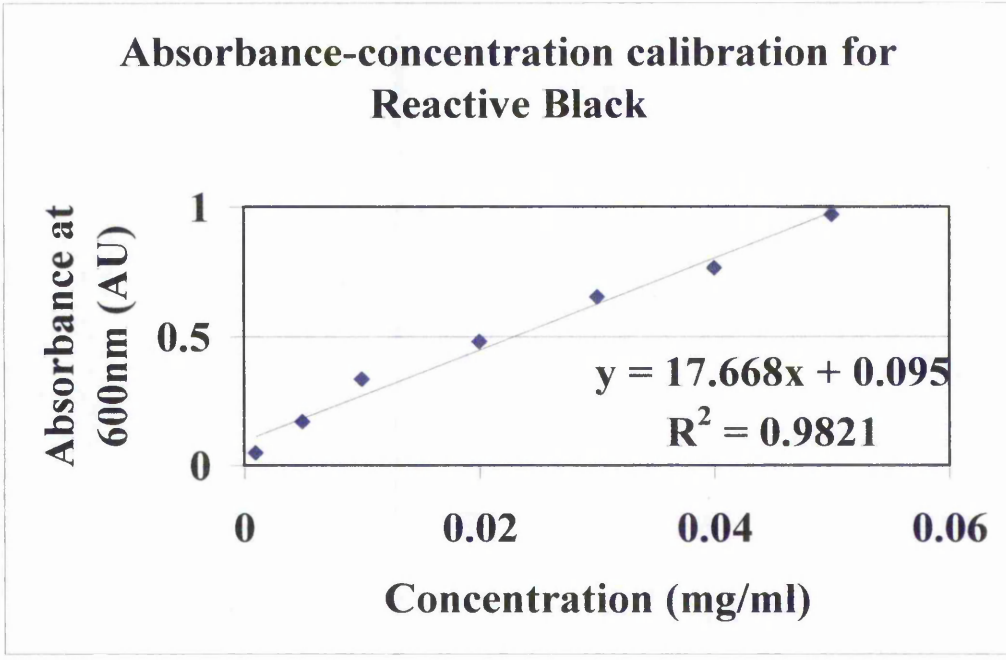

Serilene Rubine (0.005% w/v)
Absorbance (AU)
2.5
2
1.5
1
0.5
0
200
300
400
500
600
700
Wavelength (nm)

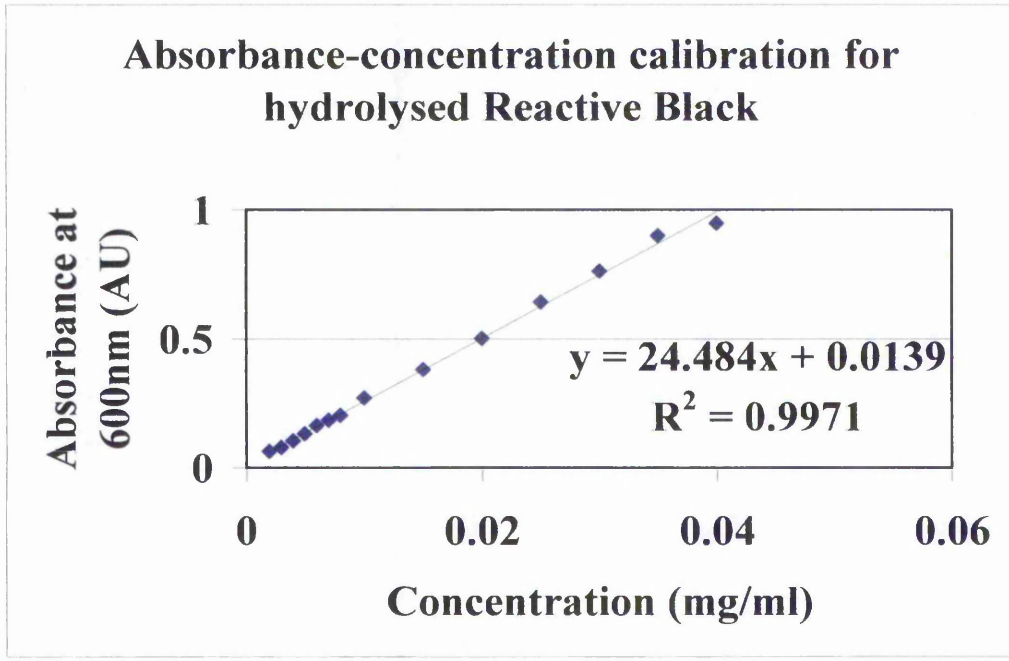

Serilene Yellow (0.002% w/v)
Absorbance (AU)
1.5
1
0.5
0
200
300
400
500
600
700
Wavelength (nm)

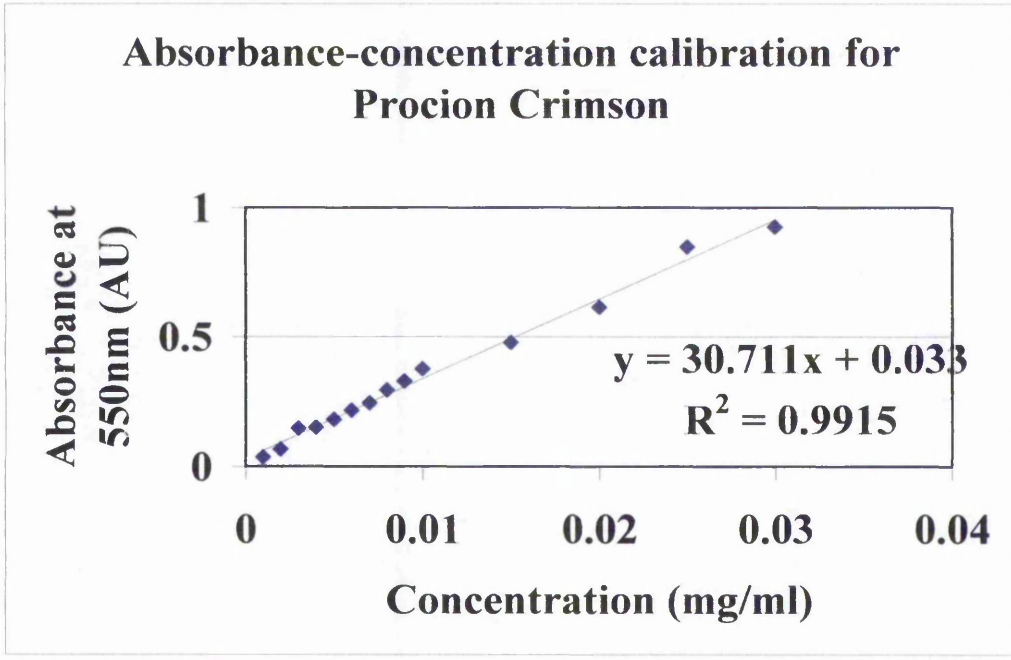

Serilene Yellow Brown (0.005% w/v)
Absorbance (AU)
2.5
2
1.5
1
0.5
0
200
300
400
500
600
700
Wavelength (nm)

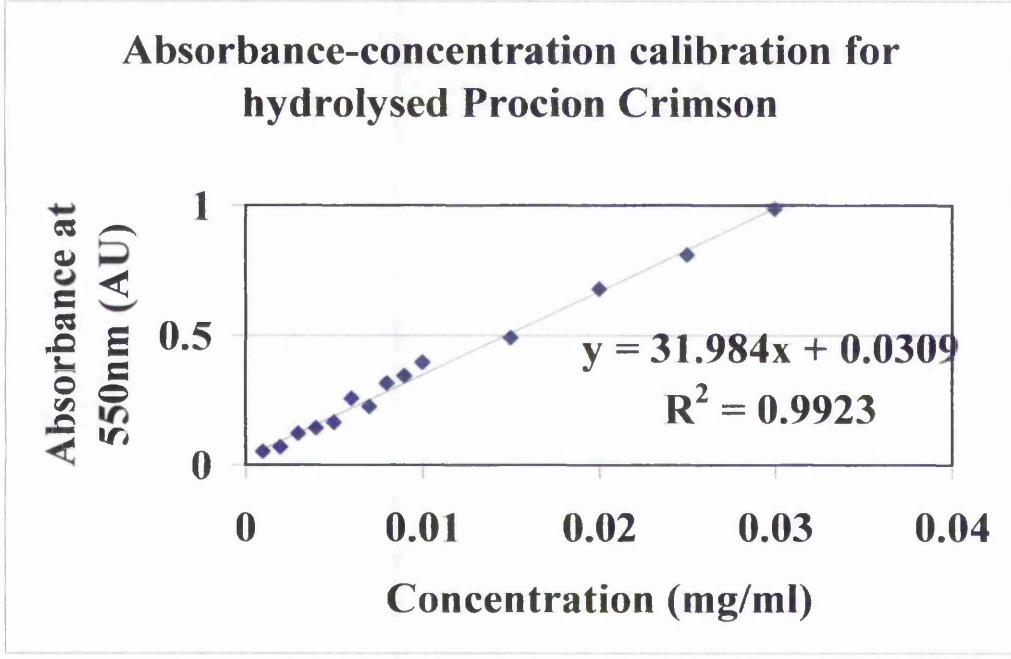

Solophenyl Black (0.005% w/v)
Absorbance (AU)
1
0.8
0.6
0.4
0.2
0
200
300
400
500
600
700
Wavelength (nm)

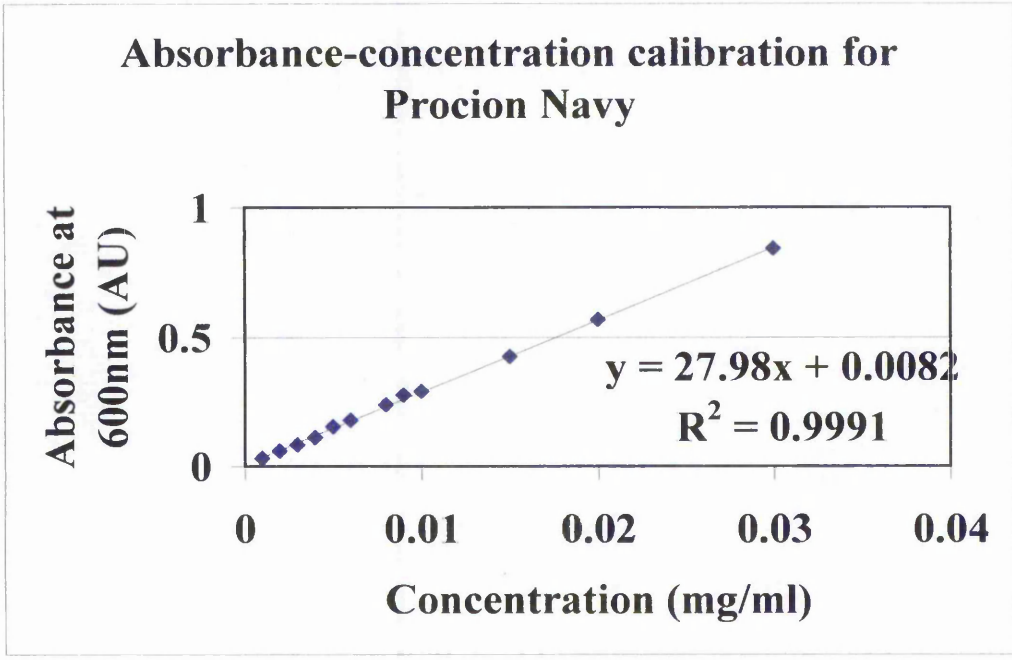

Solophenyl Navy (0.002% w/v)
Absorbance (AU)
1
0.8
0.6
0.4
0.2
0
200
300
400
500
600
700
Wavelength (nm)

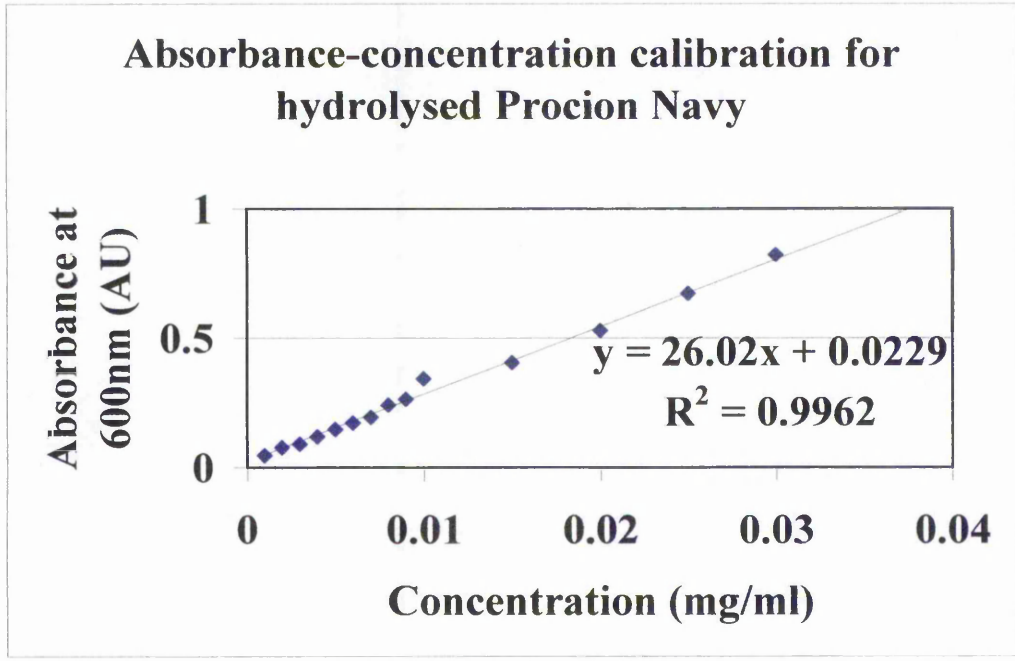

Absorbance-concentration calibration for Reactive Black
Absorbance at 600nm (AU)
1
0.5
0
y = 17.668x + 0.095
R2 = 0.9821
0
0.02
0.04
0.06
Concentration (mg/ml)

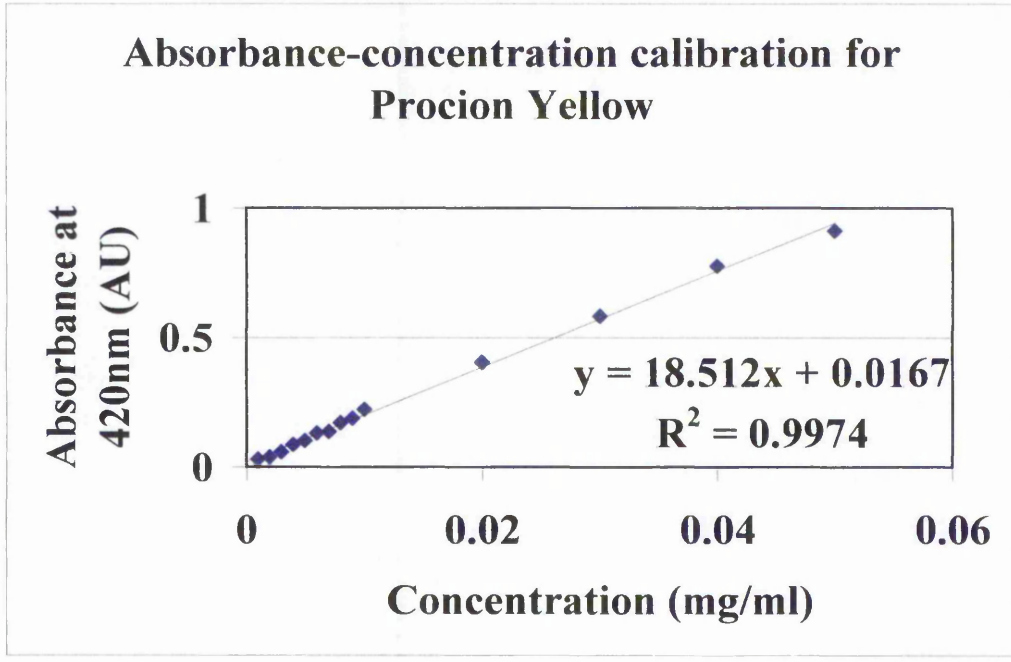

Absorbance-concentration calibration for hydrolysed Reactive Black
Absorbance at 600nm (AU)
1
0.5
0
y = 24.484x + 0.0139
R2 = 0.9971
0
0.02
0.04
0.06
Concentration (mg/ml)

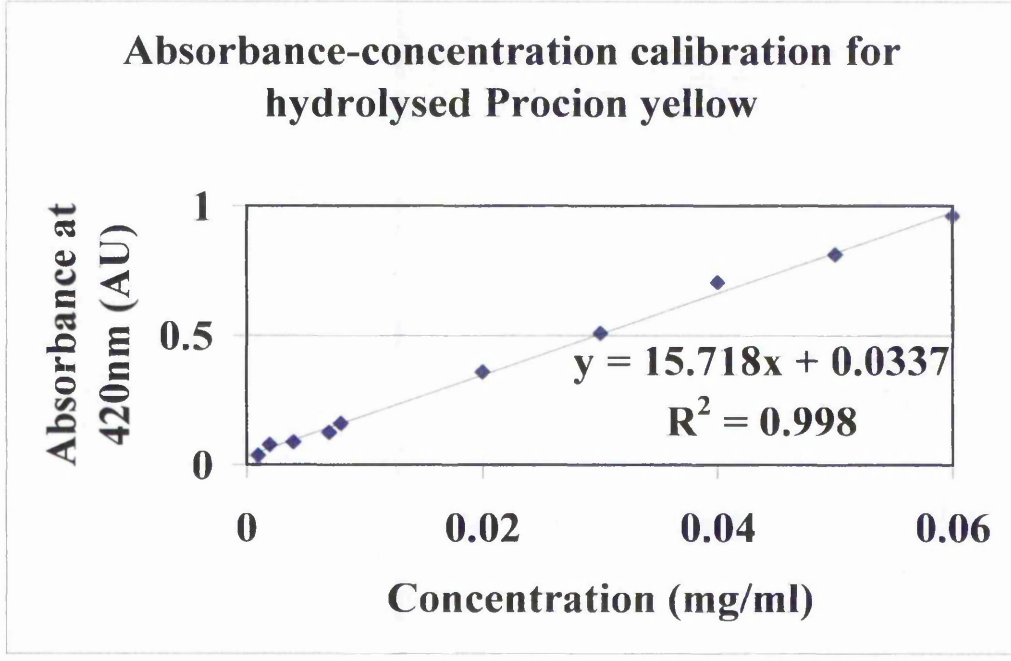

Absorbance-concentration calibration for Procion Crimson
Absorbance at 550nm (AU)
1
0.5
0
y = 30.711x + 0.033
R2 = 0.9915
0
0.01
0.02
0.03
0.04
Concentration (mg/ml)

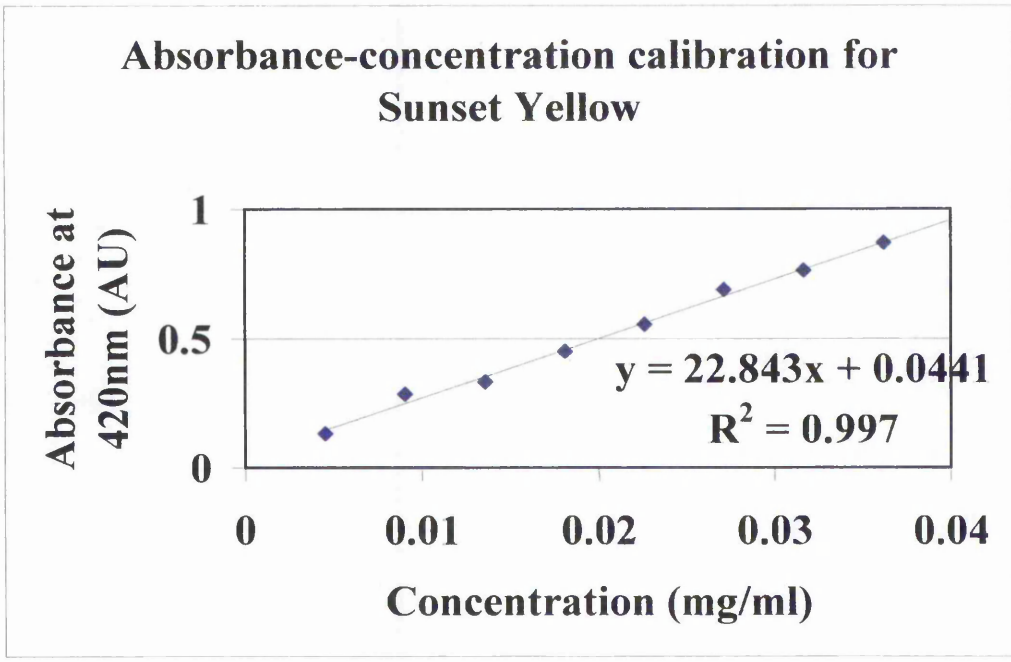

Absorbance-concentration calibration for hydrolysed Procion Crimson
Absorbance at 550nm (AU)
1
0.5
0
y = 31.984x + 0.0309
R2 = 0.9923
0
0.01
0.02
0.03
0.04
Concentration (mg/ml)

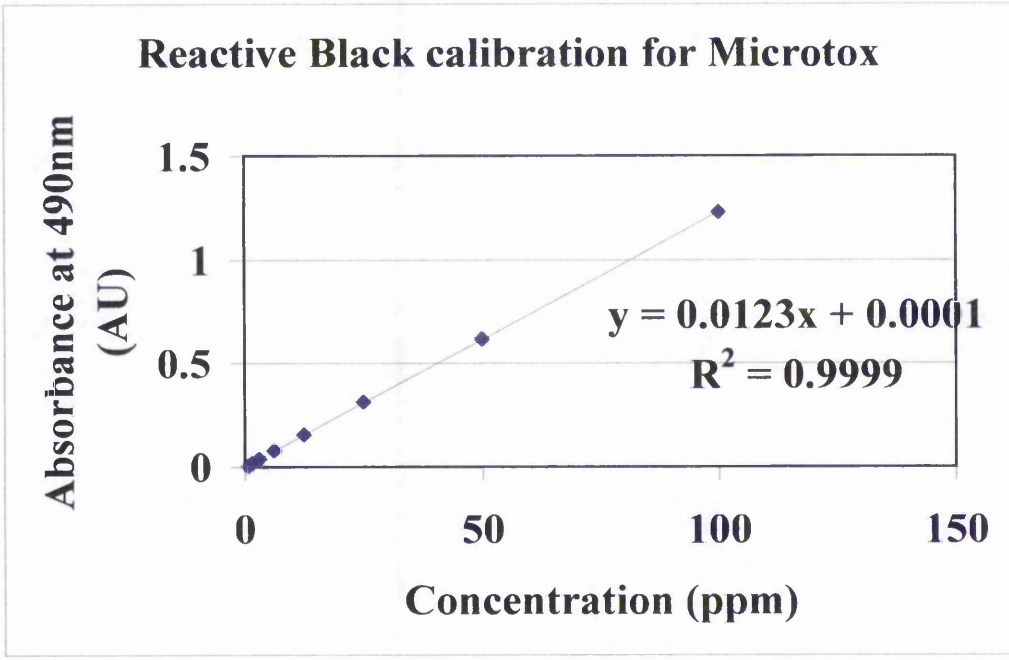

Absorbance-concentration calibration for Procion Navy
Absorbance at 600nm (AU)
1
0.5
0
y = 27.98x + 0.0082
R2 = 0.9991
0
0.01
0.02
0.03
0.04
Concentration (mg/ml)

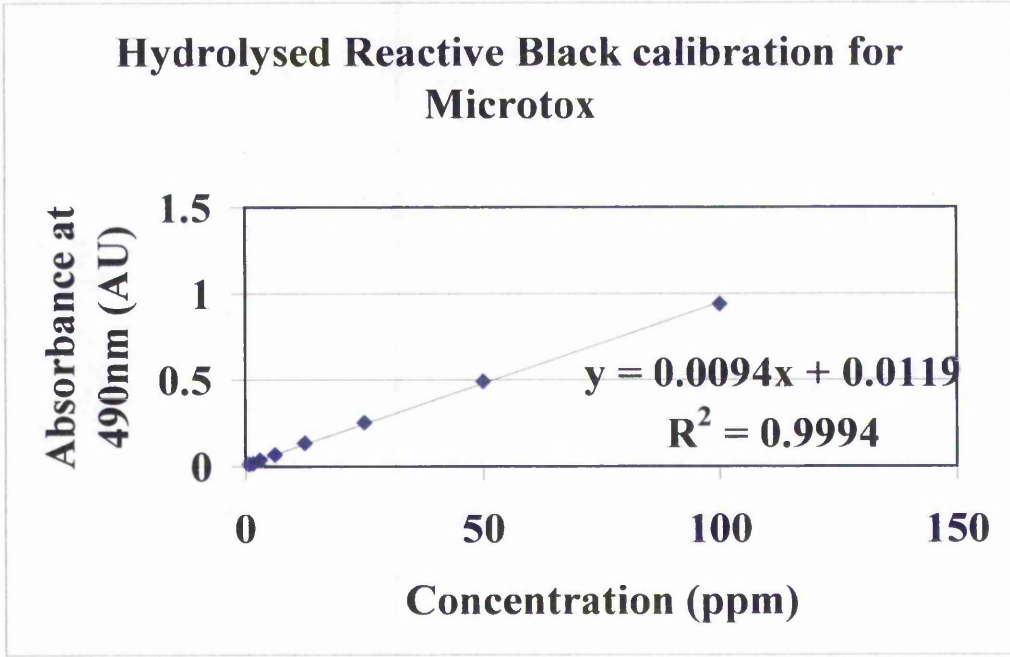

Absorbance-concentration calibration for hydrolysed Procion Navy
Absorbance at 600nm (AU)
1
0.5
0
y = 26.02x + 0.0229
R2 = 0.9962
0
0.01
0.02
0.03
0.04
Concentration (mg/ml)


Absorbance-concentration calibration for Procion Yellow
Absorbance at 420nm (AU)
1
0.5
0
0
0.02
0.04
0.06
Concentration (mg/ml)
y = 18.512x + 0.0167
R2 = 0.9974


Absorbance-concentration calibration for hydrolysed Procion yellow
Absorbance at 420nm (AU)
1
0.5
0
0
0.02
0.04
0.06
Concentration (mg/ml)
y = 15.718x + 0.0337
R2 = 0.998


Absorbance-concentration calibration for Sunset Yellow
Absorbance at 420nm (AU)
1
0.5
0
y = 22.843x + 0.0441
R2 = 0.997
0
0.01
0.02
0.03
0.04
Concentration (mg/ml)


Reactive Black calibration for Microtox
Absorbance at 490nm (AU)
1.5
1
0.5
0
0
50
100
150
Concentration (ppm)
y = 0.0123x + 0.0001
R2 = 0.9999


Hydrolysed Reactive Black calibration for Microtox
Absorbance at 490nm (AU)
1.5
1
0.5
0
0
50
100
150
Concentration (ppm)
y = 0.0094x + 0.0119
R2 = 0.9994

Appendix 3.

(a) Example for the calculation of cell numbers from optical density for *E. faecalis* as discussed in Section 2.8.4.

	1st	2nd	3rd
Wt Eppendorf	0.4664g	0.4736g	0.4585g
Wt Eppendorf + dried cells	0.4685g	0.4757g	0.4599g
Wt dried cells	0.0021g	0.0021g	0.0014g
Wt cells for 1 ml culture	0.021g	0.021g	0.014g
OD_{650nm} (x20dil)	0.76 AU	0.74 AU	0.71 AU
Effective OD_{650nm} for 1 ml	15.2 AU	14.8 AU	14.2 AU
Wt cells equal to OD_{650nm} 1AU	0.0014g	0.0014g	0.0010g

(b) Statistical calculations for decolourisation rate of Reactive Black with different glucose concentrations as discussed in Section 3.5.2.

Calculated decolourisation rates:

Assay with 1% glucose 22.95 mg dye/h/g dry cell wt.

Assay with 0.1% glucose 21.35 mg dye/h/g dry cell wt.

Comparison of variances using the F-test:

	Variable 1	Variable 2
Mean	22.95	21.35
Variance	2.83	1.46
Observations	3	3
Degrees of freedom	2	2
Calculated F	1.95	
Critical F 2 sided test	39.00	

Calculated F < Critical F therefore there is no significant difference between the two sample variances.

So use a T-test assuming equal variances:

	Variable 1	Variable 2
Mean	22.95	21.35
Variance	2.83	1.46
Observations	3	3
Pooled variance	2.14	
Degrees of freedom	4	
Calculated t	1.34	
Critical t	2.13	

Calculated t < critical t therefore there is no significant difference between the two means.

(c) Analysis of variance data for decolourisation rates with different batches of cells as discussed in Section 3.5.4.

Null Hypothesis: There is no significant difference in decolourisation rate between batches of cells.

Calculation for *E. faecalis* and Reactive Black:

	Culture 1	Culture 2	Culture 3
Rate 1	26.35	20.84	21.78
Rate 2	22.78	19.10	21.02
Rate 3	21.81	18.13	19.42

Summary

Groups	Count	Sum	Average	Variance
Culture 1	3	70.95	23.65	5.72
Culture 2	3	58.07	19.36	1.89
Culture 3	3	62.23	20.74	1.46

ANOVA (single factor)

Source of variation	Sum of Squares	Degrees of freedom	Mean Square	F calc.	P-value	F crit.
Between	28.82	2	14.41	4.7702	0.0575	5.1432
Within	18.12	6	3.02			
Total	46.94	8				

Calculated F 4.7701< critical F 5.1432. Therefore the null hypothesis is accepted.

Calculation for *E. faecalis* and hydrolysed Reactive Black:

	Culture 1	Culture 2	Culture 3
Rate 1	53.77	40.72	48.16
Rate 2	53.54	45.18	57.56
Rate 3	55.76	56.43	54.22

Summary

Groups	Count	Sum	Average	Variance
Culture 1	3	163.08	54.36	1.49
Culture 2	3	142.33	47.44	65.48
Culture 3	3	159.94	53.3	22.74

ANOVA (single factor)

Source of variation	Sum of Squares	Degrees of freedom	Mean Square	F calc.	P-value	F crit.
Between	83.38	2	41.69	1.3942	0.318	5.1432
Within	179.42	6	29.90			
Total	262.81	8				

Calculated F 1.3942< critical F 5.1432. Therefore the null hypothesis is accepted.

Calculation for *C. butyricum* (F5) and Reactive Black:

	Culture 1	Culture 2	Culture 3
Rate 1	55.05	61.56	54.91
Rate 2	56.29	70.58	65.80
Rate 3	50.07	67.20	50.18

Summary

Groups	Count	Sum	Average	Variance
Culture 1	3	161.42	53.81	10.84
Culture 2	3	199.29	66.43	20.54
Culture 3	3	170.89	56.96	64.15

ANOVA (single factor)

Source of variation	Sum of Squares	Degrees of freedom	Mean Square	F calc.	P-value	F crit.
Between	258.95	2	129.47	4.0660	0.076	5.1432
Within	191.06	6	31.84			
Total	450.00	8				

Calculated F 4.0660 < critical F 5.1432. Therefore the null hypothesis is accepted.

Calculation for *C. butyricum* (F5) and hydrolysed Reactive Black:

	Culture 1	Culture 2	Culture 3
Rate 1	102.70	100.68	92.53
Rate 2	109.98	123.81	104.13
Rate 3	102.66	116.71	70.78

Summary

Groups	Count	Sum	Average	Variance
Culture 1	3	315.34	105.11	17.74
Culture 2	3	341.20	113.73	140.46
Culture 3	3	267.44	89.15	286.50

ANOVA (single factor)

Source of variation	Sum of Squares	Degrees of freedom	Mean Square	F calc.	P-value	F crit.
Between	933.77	2	466.88	3.1497	0.116	5.1432
Within	889.40	6	148.23			
Total		8				

Calculated F 3.1497 < critical F 5.1432. Therefore the null hypothesis is accepted.

(d) Analysis of variance data for the Sunset Yellow check assays for cell free extract preparations as discussed in Section 3.7.

Null Hypothesis: There is no significant difference in decolourisation rate between batches of cell free extract preparations.

Calculation for *E. faecalis* and Sunset Yellow:

	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5	Extract 6
Rate 1	597.90	531.98	665.35	458.83	542.09	511.07
Rate 2	540.86	542.39	680.35	356.51	542.25	590.88
Rate 3	534.14	517.74	666.74	620.51	641.10	516.83

Summary

Groups	Count	Sum	Average	Variance
Extract 1	3	1654.90	551.63	610.71
Extract 2	3	1592.10	530.70	153.14
Extract 3	3	2012.43	670.81	68.69
Extract 4	3	1435.86	478.62	17718.06
Extract 5	3	1725.44	575.15	3262.84
Extract 6	3	1618.79	539.60	1981.19

ANOVA (single factor)

Source of variation	Sum of Squares	Degrees of freedom	Mean Square	F calc.	P-value	F crit.
Between	61338.01	5	12267.6	3.0933	0.0506	3.1059
Within	47589.25	12	3965.77			
Total	108927.3	17				

Calculated F 3.0933 < critical F 3.1059. Therefore the null hypothesis is accepted.

Calculation for *C. butyricum* (F5) and Sunset Yellow:

	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5	Extract 6	Extract 7	Extract 8
Rate 1	9.30	7.29	6.99	9.70	10.71	17.97	12.12	13.44
Rate 2	13.81	12.20	9.89	10.23	13.21	17.71	17.63	14.25

Summary

Groups	Count	Sum	Average	Variance
Extract 1	2	23.11	11.55	10.20
Extract 2	2	19.49	9.75	12.04
Extract 3	2	16.88	8.44	4.23
Extract 4	2	19.94	9.97	0.14
Extract 5	2	23.92	11.96	3.13
Extract 6	2	35.67	17.84	0.03
Extract 7	2	29.75	14.88	15.18
Extract 8	2	27.69	13.84	0.34

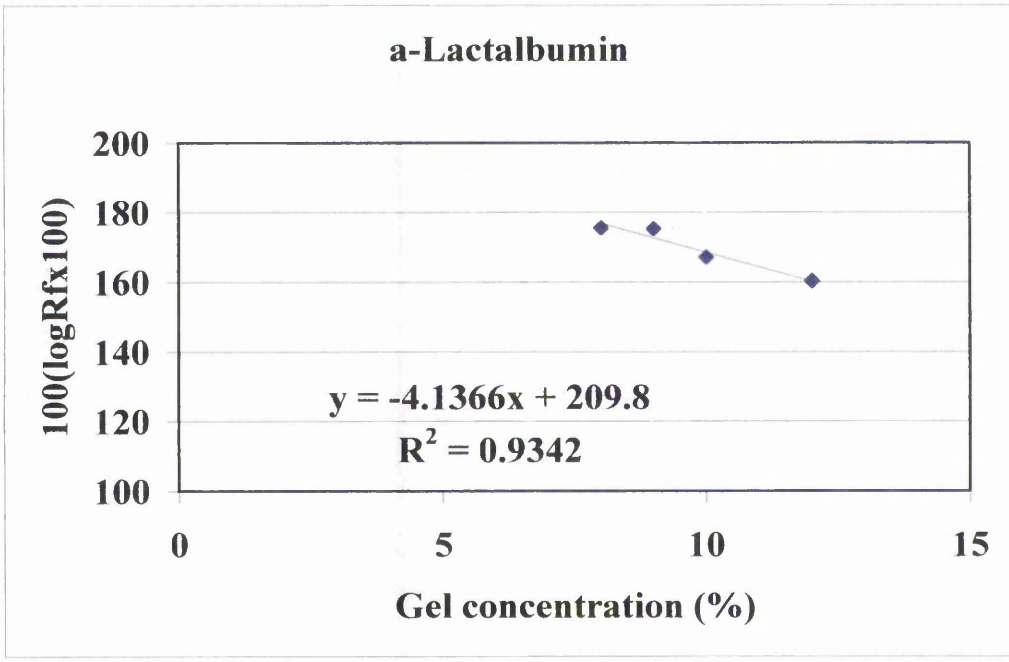
ANOVA (single factor)

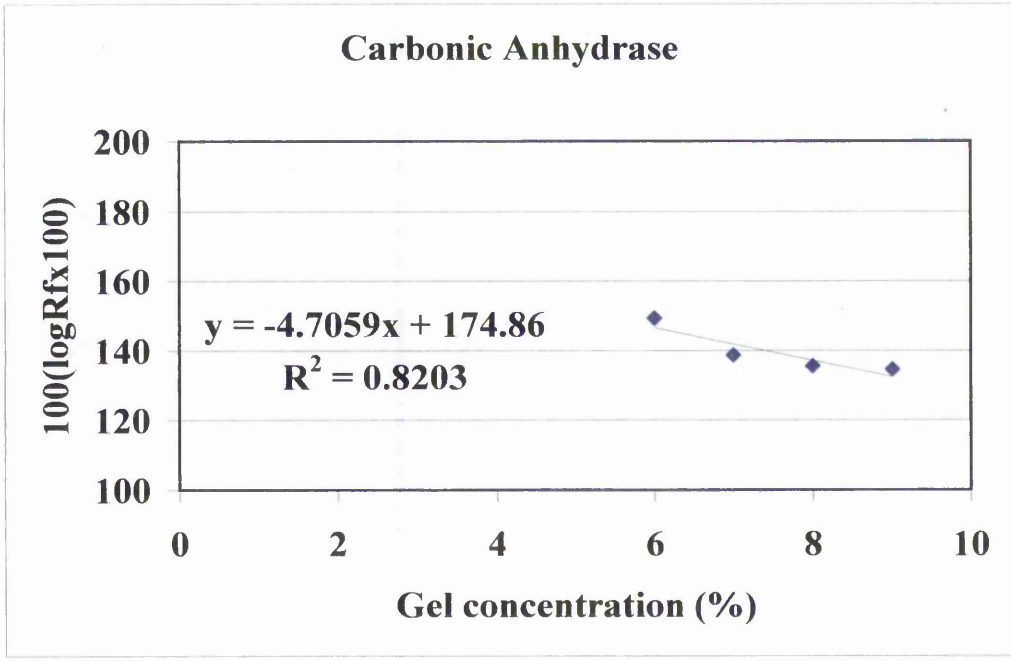
Source of variation	Sum of Squares	Degrees of freedom	Mean Square	F calc.	P-value	F crit.
Between	134.41	7	19.20	3.3908	0.0542	3.5005
Within	45.30	8	5.66			
Total	179.72	15				

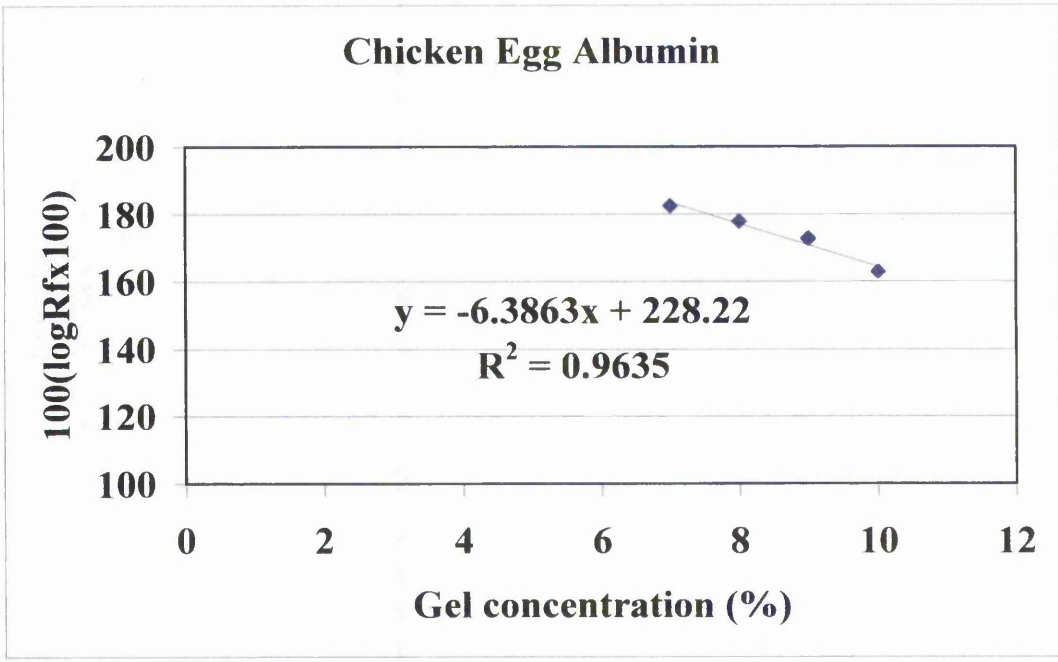
Calculated F 3.3908 < critical F 3.5005. Therefore the null hypothesis is accepted.

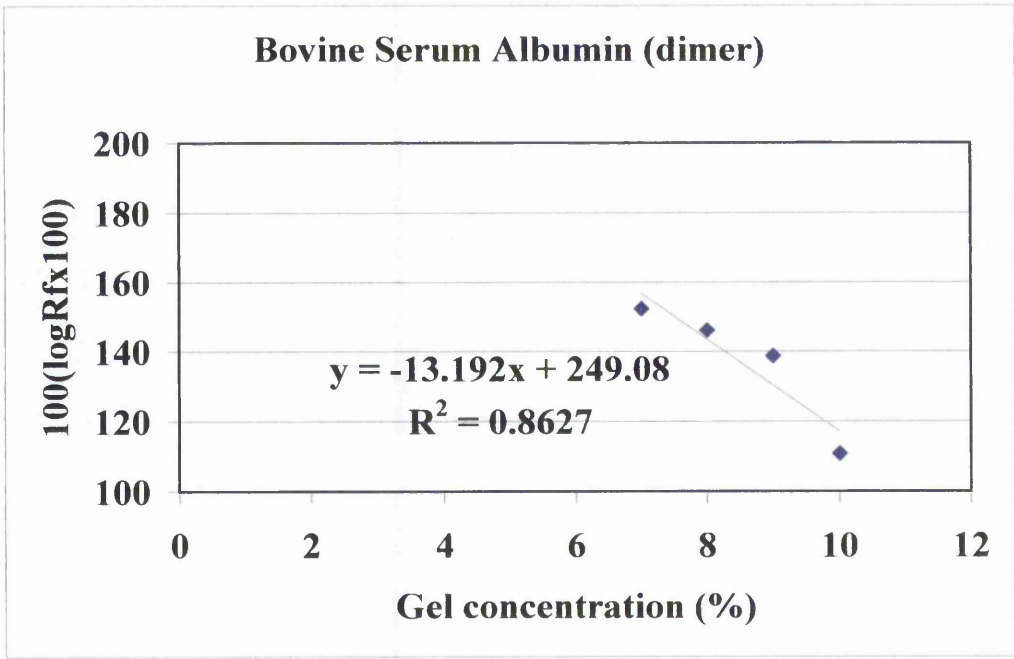
Appendix 4.

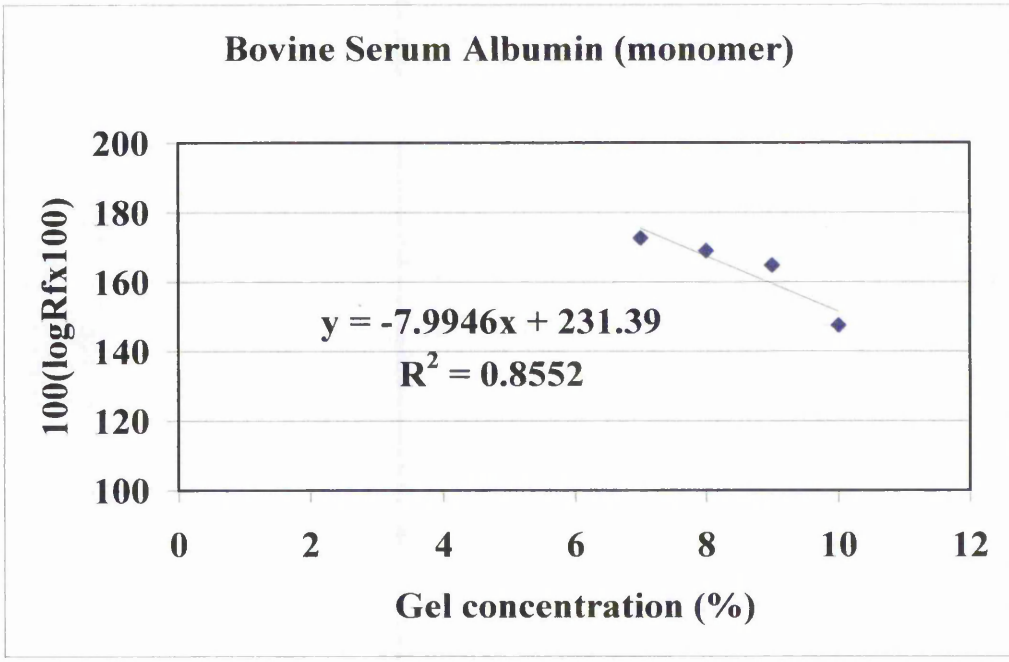
Retardation coefficients for the molecular weight standards used in the determination of the molecular weight of *E. faecalis* azoreductase as discussed in Section 3.9 (Figure 26).

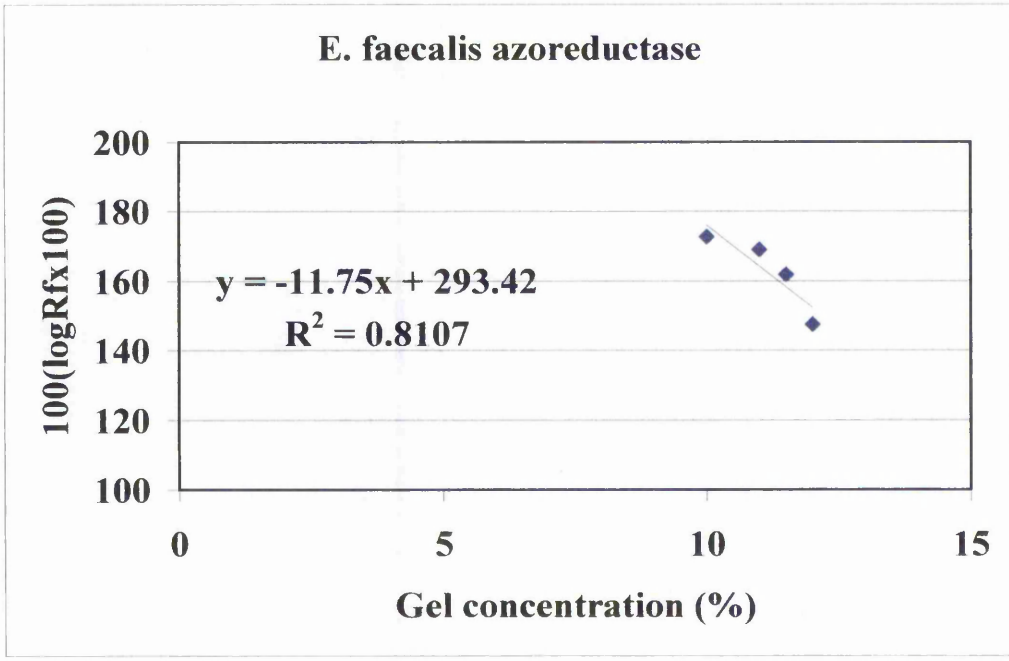

a-Lactalbumin
200
180
160
140
120
100
100(logRfx100)
y = -4.1366x + 209.8
R2 = 0.9342
0
5
10
15
Gel concentration (%)


Carbonic Anhydrase
200
180
160
140
120
100
100(logRfx100)
y = -4.7059x + 174.86
R2 = 0.8203
0
2
4
6
8
10
Gel concentration (%)


Chicken Egg Albumin
100(logRfx100)
200
180
160
140
120
100
0
2
4
6
8
10
12
y = -6.3863x + 228.22
R2 = 0.9635
Gel concentration (%)


Bovine Serum Albumin (dimer)
100(logRfx100)
200
180
160
140
120
100
0
2
4
6
8
10
12
y = -13.192x + 249.08
R2 = 0.8627
Gel concentration (%)


Bovine Serum Albumin (monomer)
200
180
160
140
120
100
100(logRfx100)
y = -7.9946x + 231.39
R2 = 0.8552
0
2
4
6
8
10
12
Gel concentration (%)


E. faecalis azoreductase
200
180
160
140
120
100
100(logRfx100)
y = -11.75x + 293.42
R2 = 0.8107
0
5
10
15
Gel concentration (%)

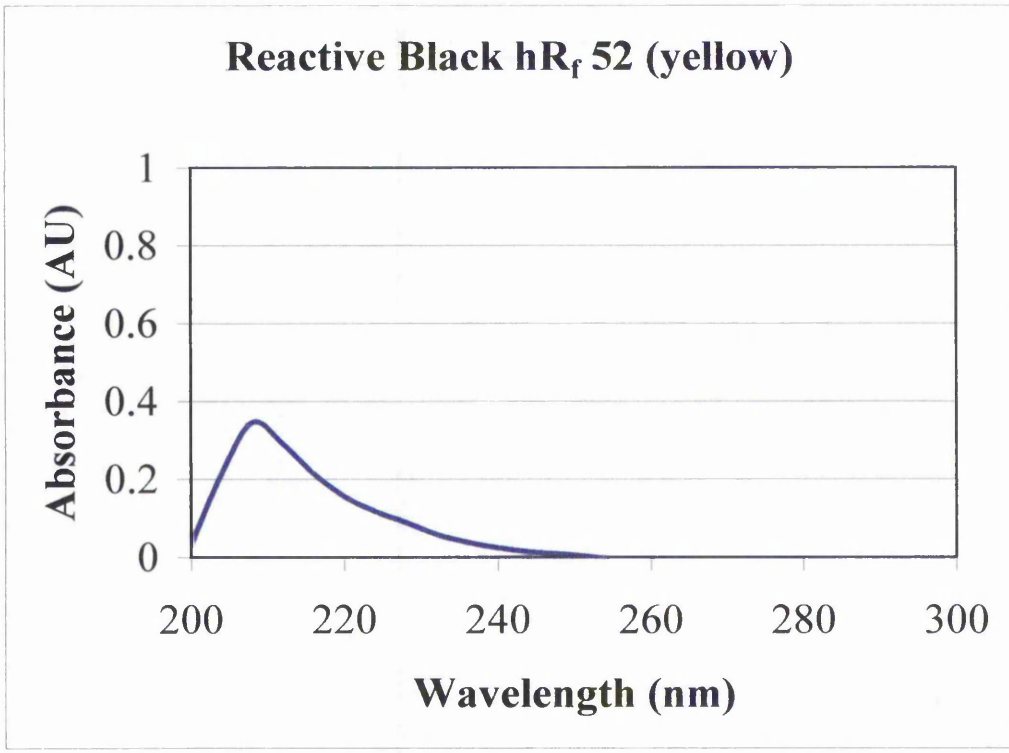
Appendix 5.

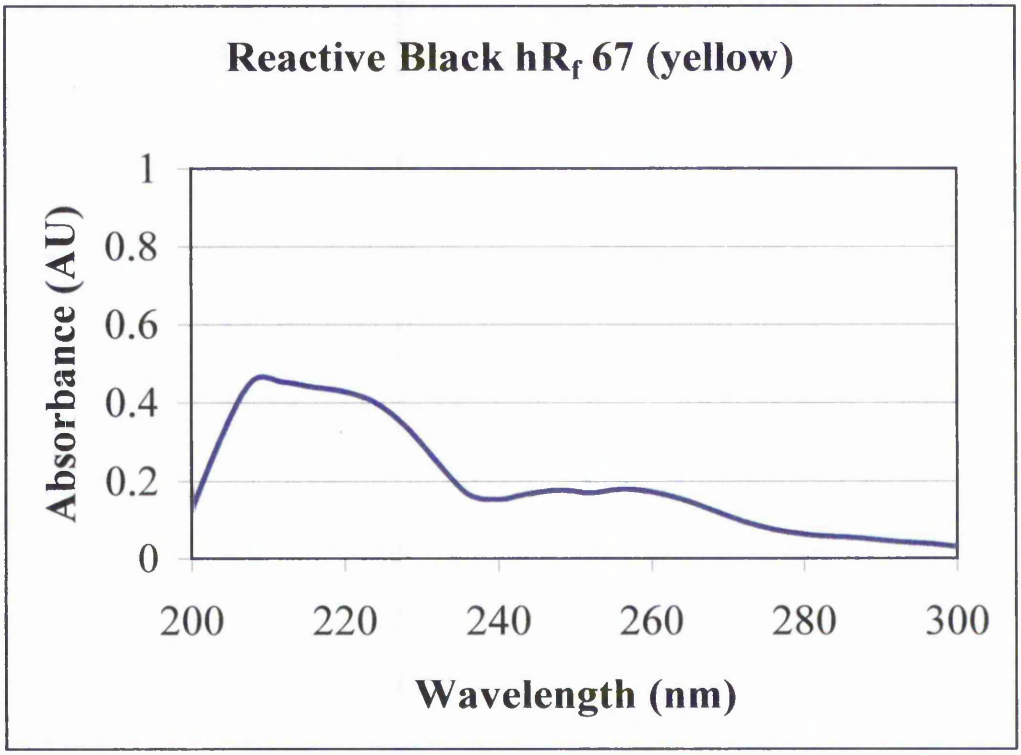
UV/VIS profiles of TLC bands as discussed in Section 3.12.

UV/VIS profiles of TLC bands for Reactive Black and hydrolysed Reactive Black decolourised by *E. faecalis* and for a control assay without dye as detailed in Table 24.

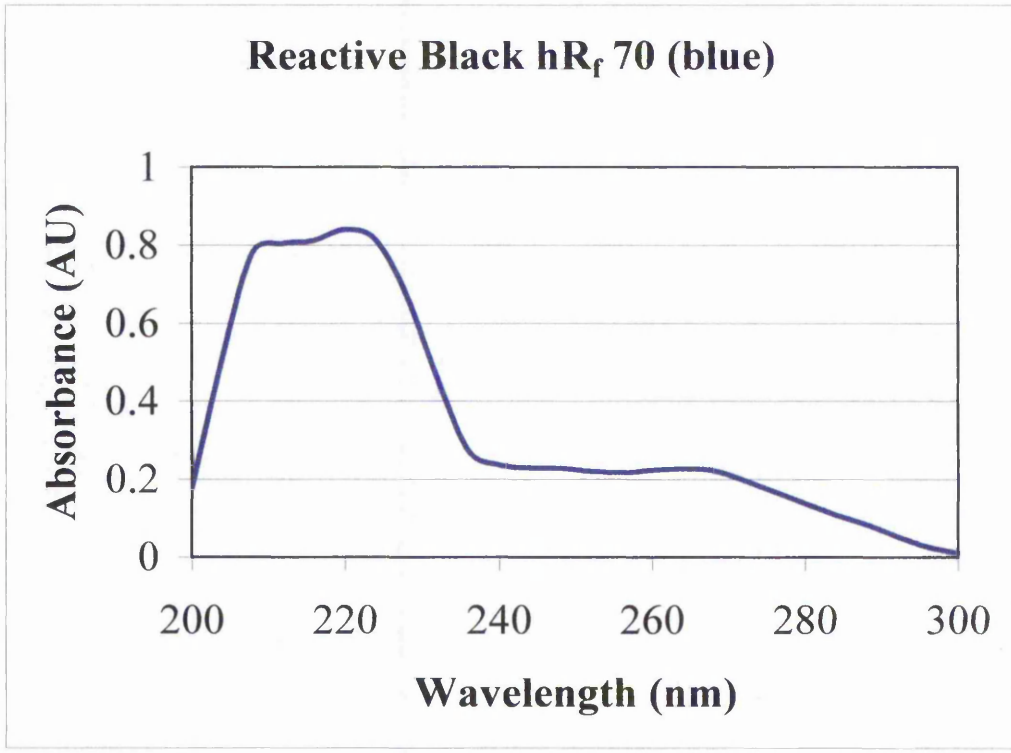
UV/VIS profiles of TLC bands for Reactive Black and hydrolysed Reactive Black decolourised using sodium dithionite as detailed in Table 25.

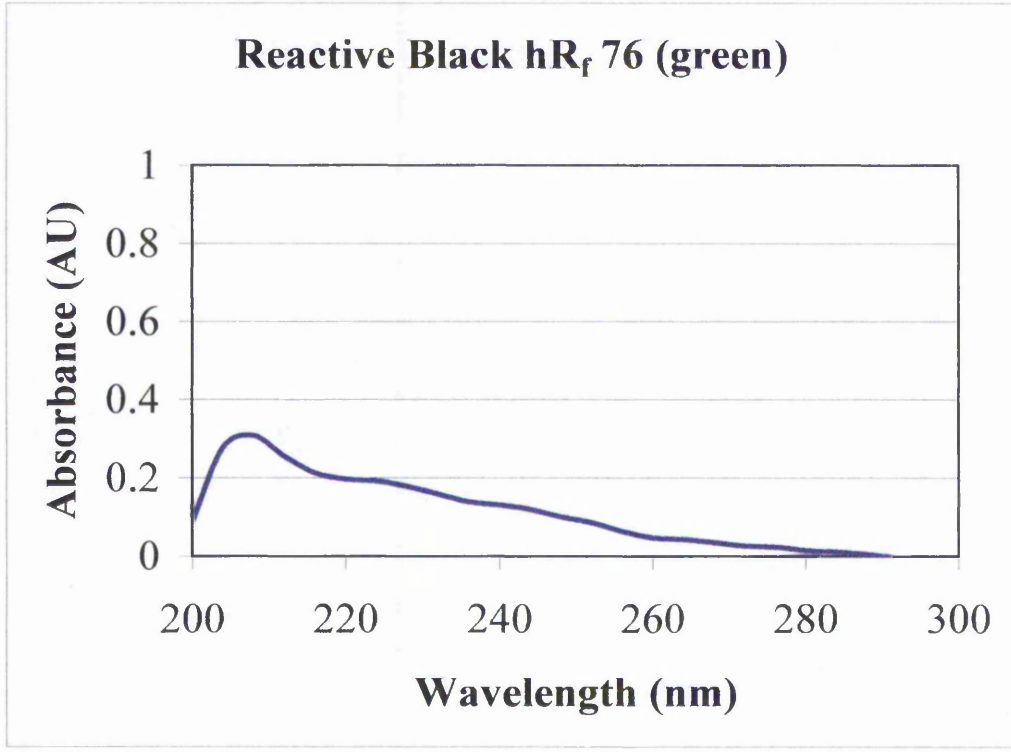
E. faecalis reduced.



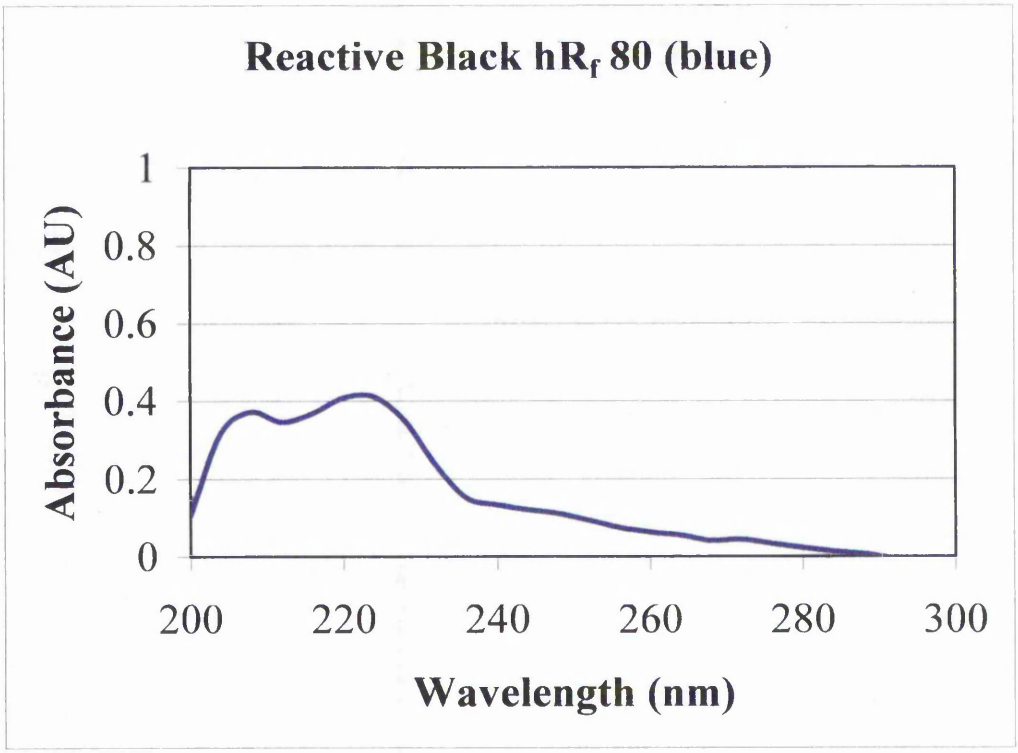


E. faecalis reduced.

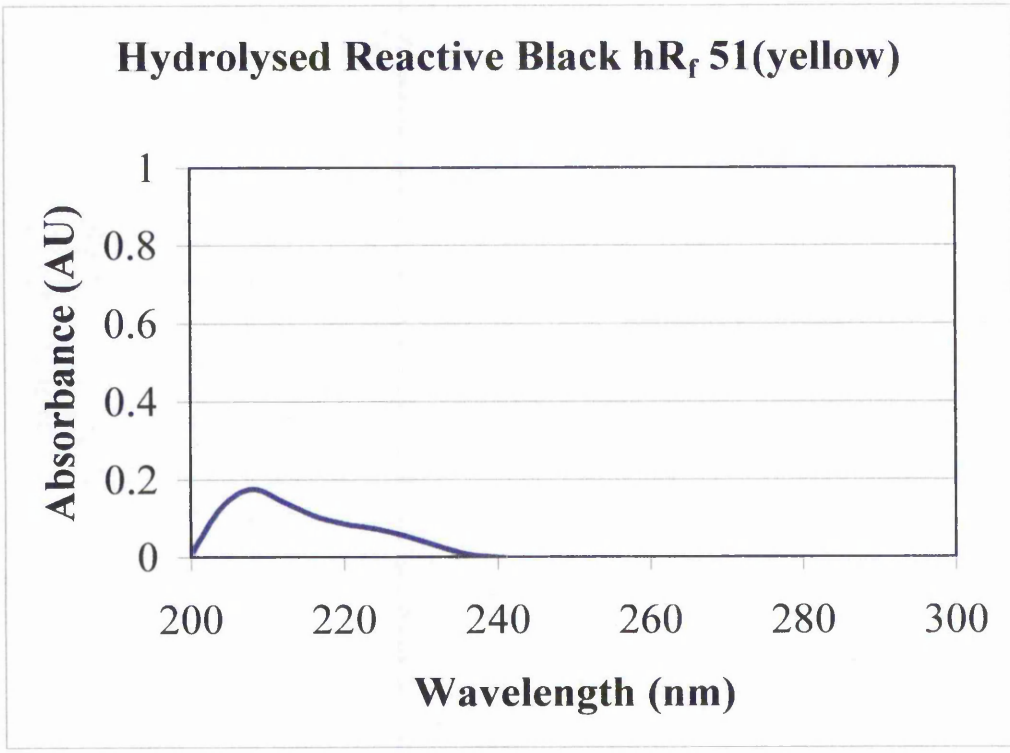


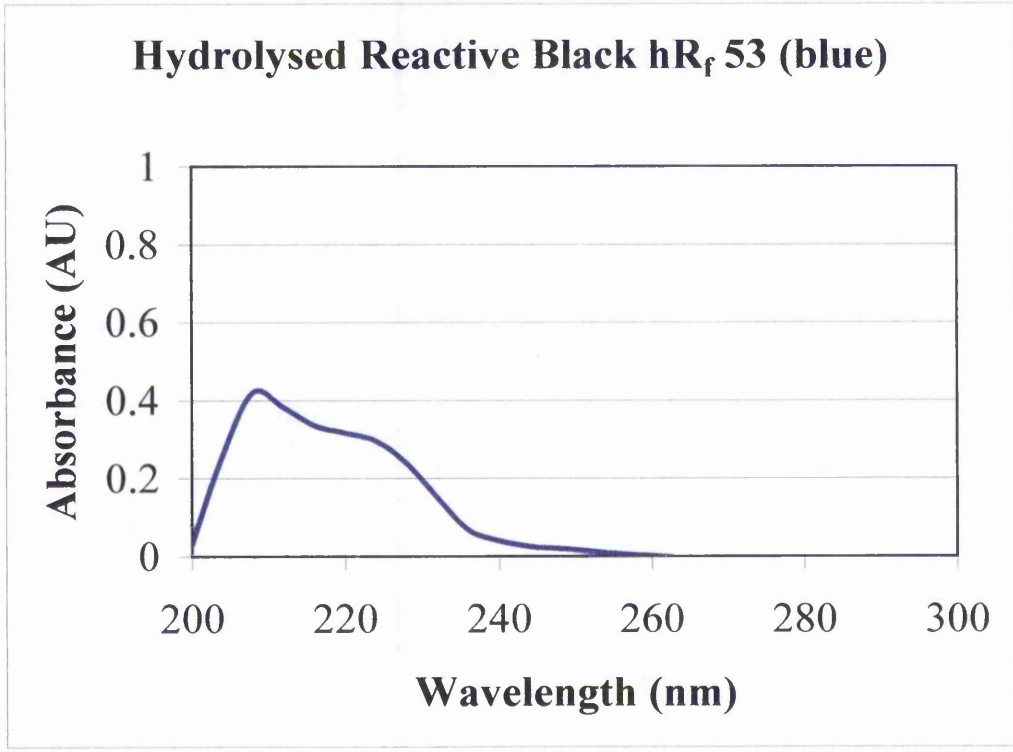


E. faecalis reduced.

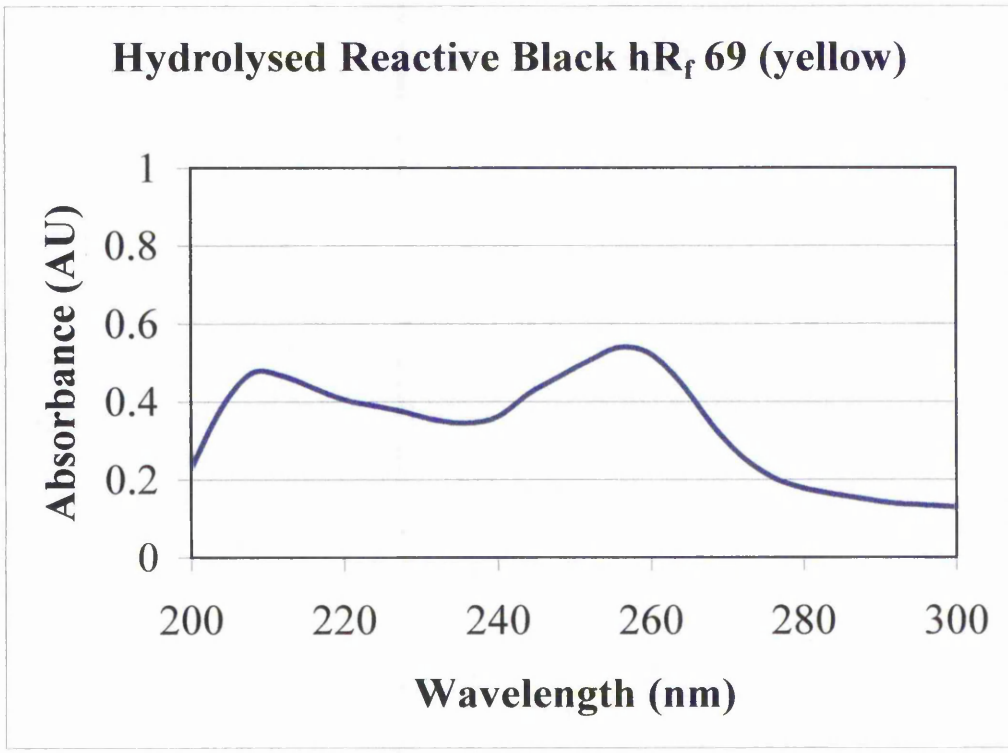


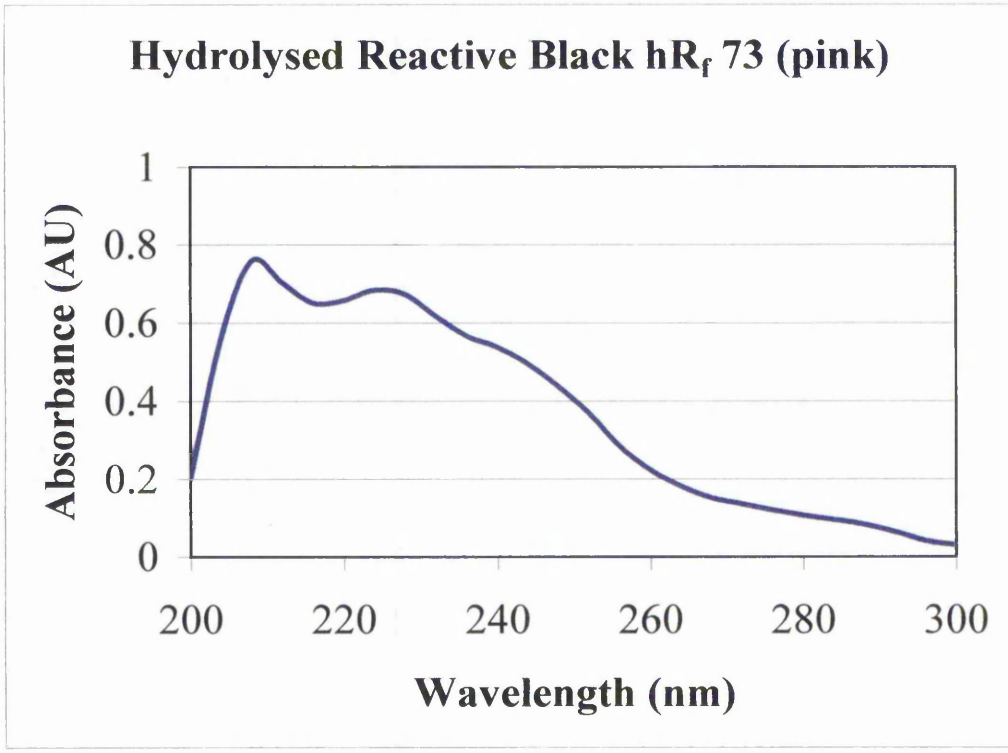
E. faecalis reduced.



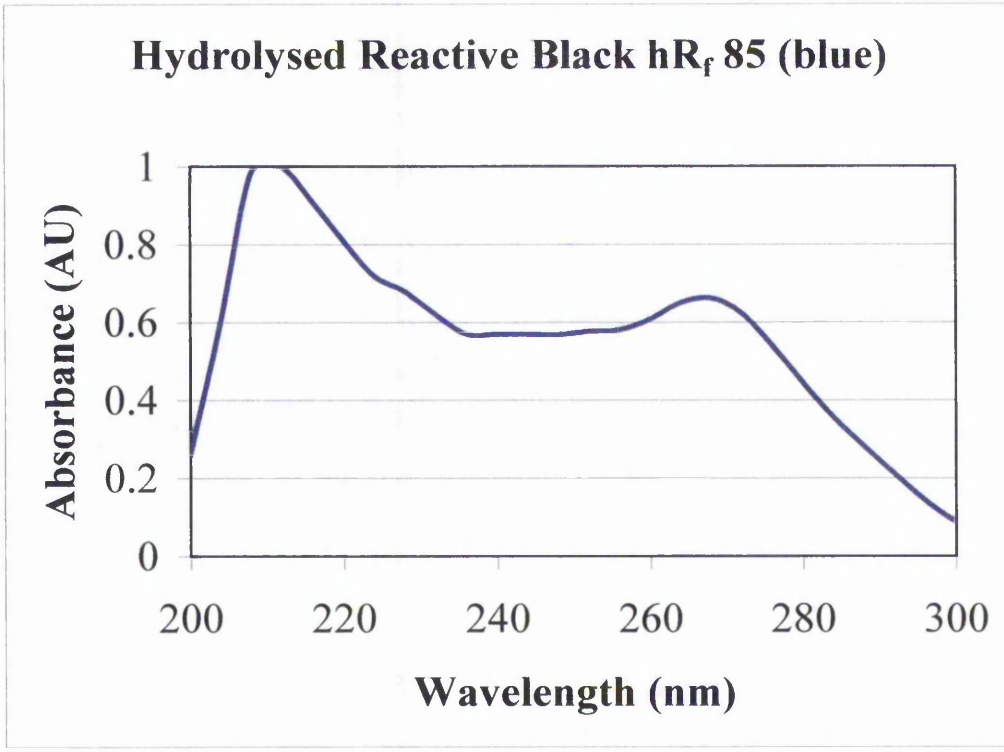


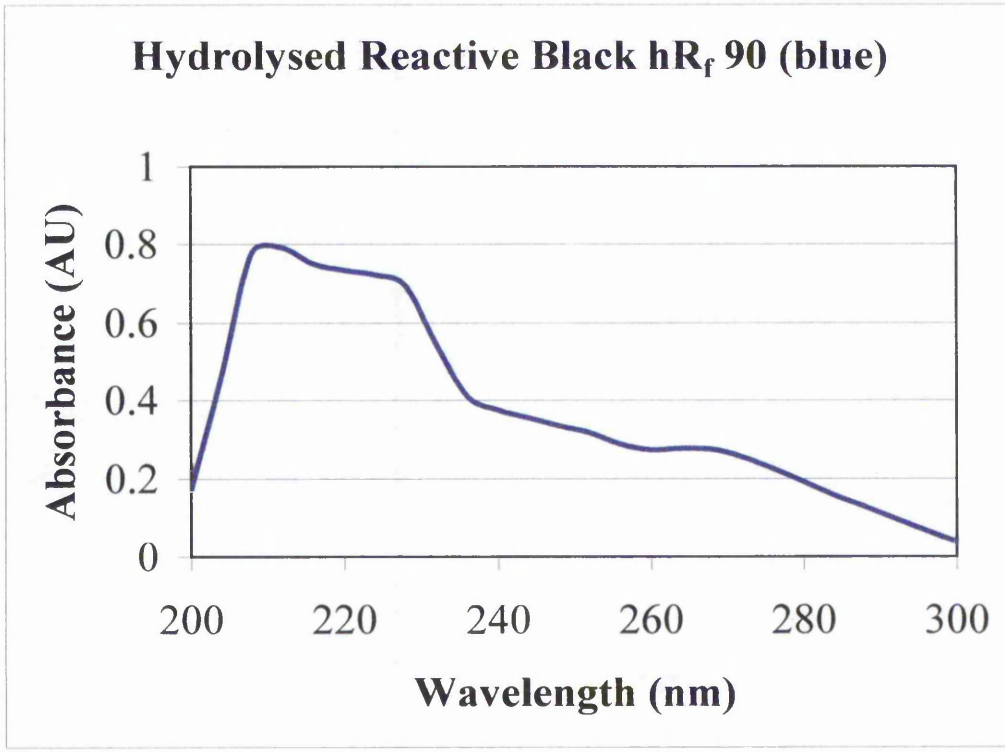
E. faecalis reduced.



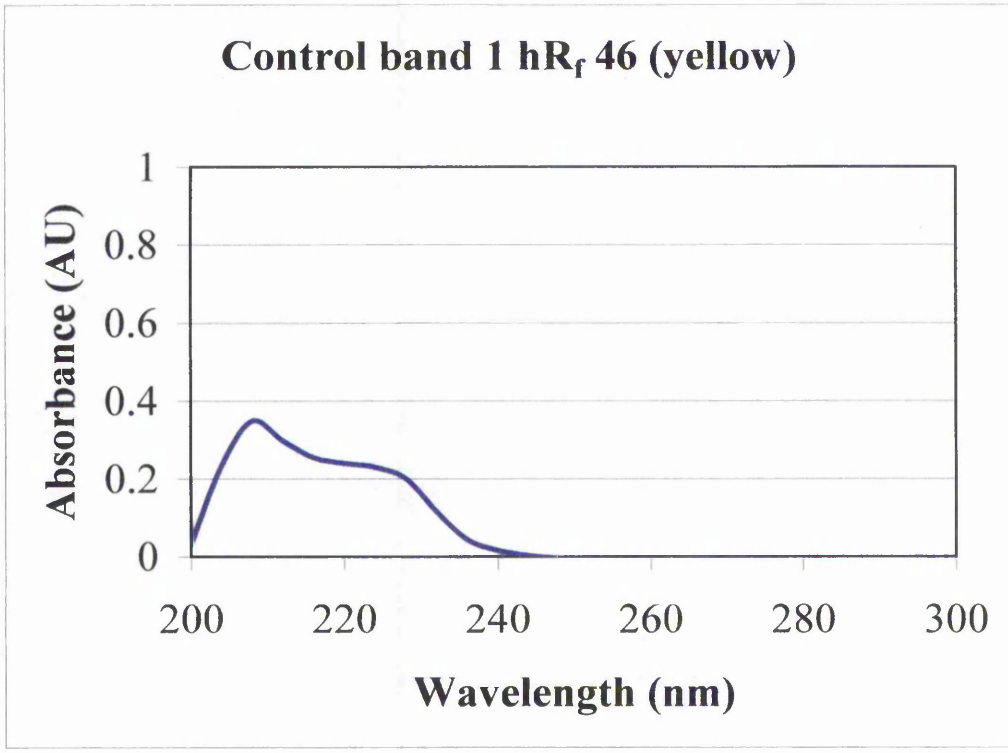


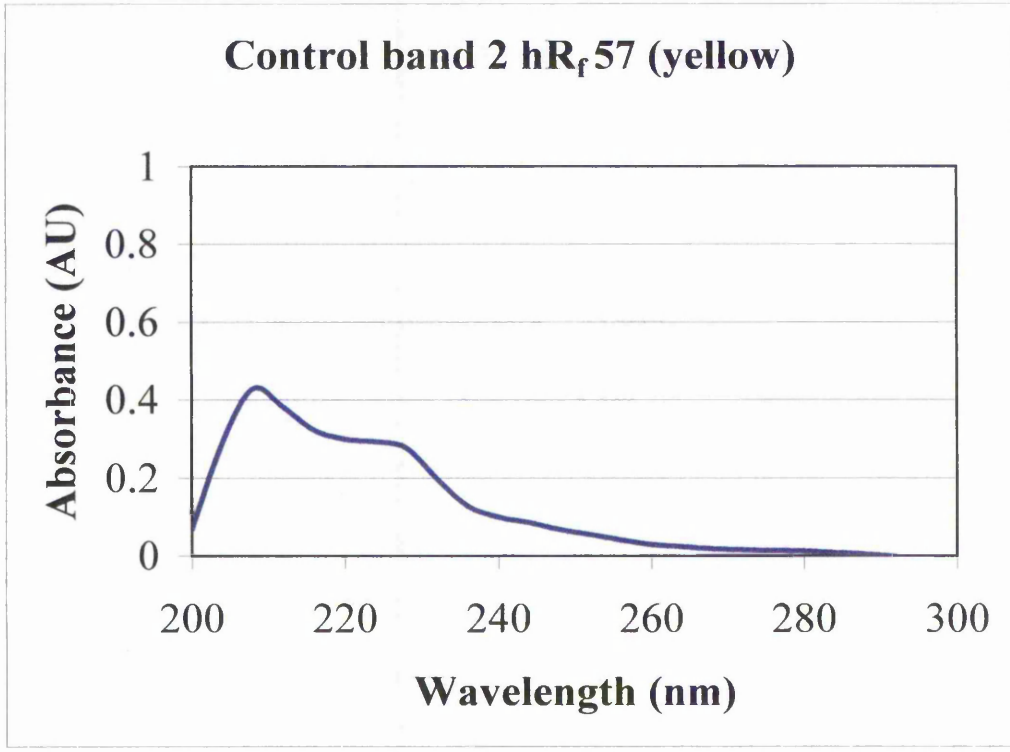
E. faecalis reduced.



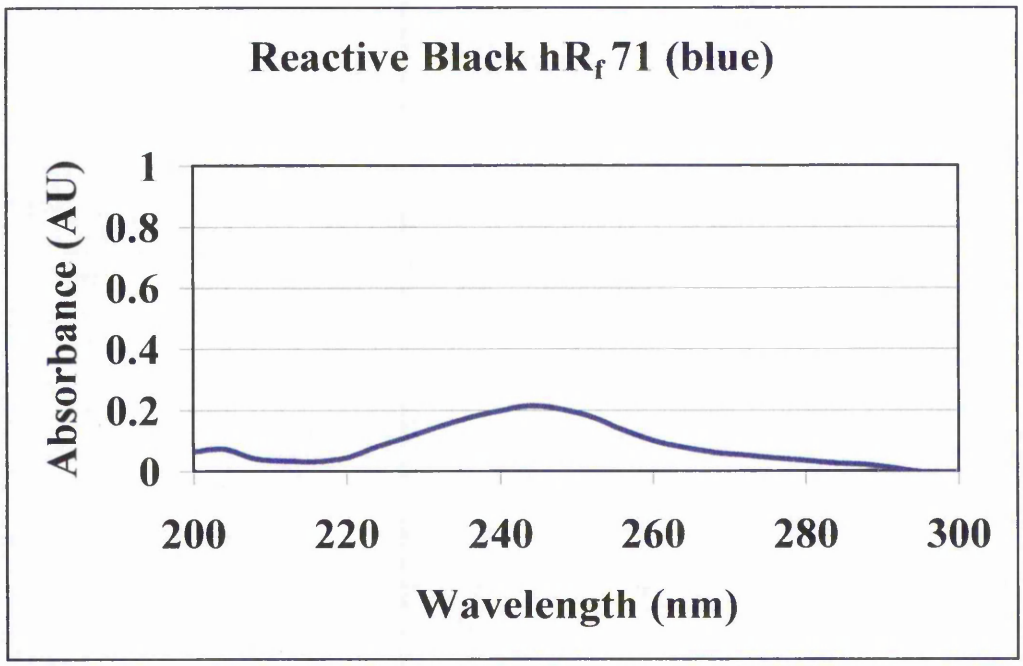


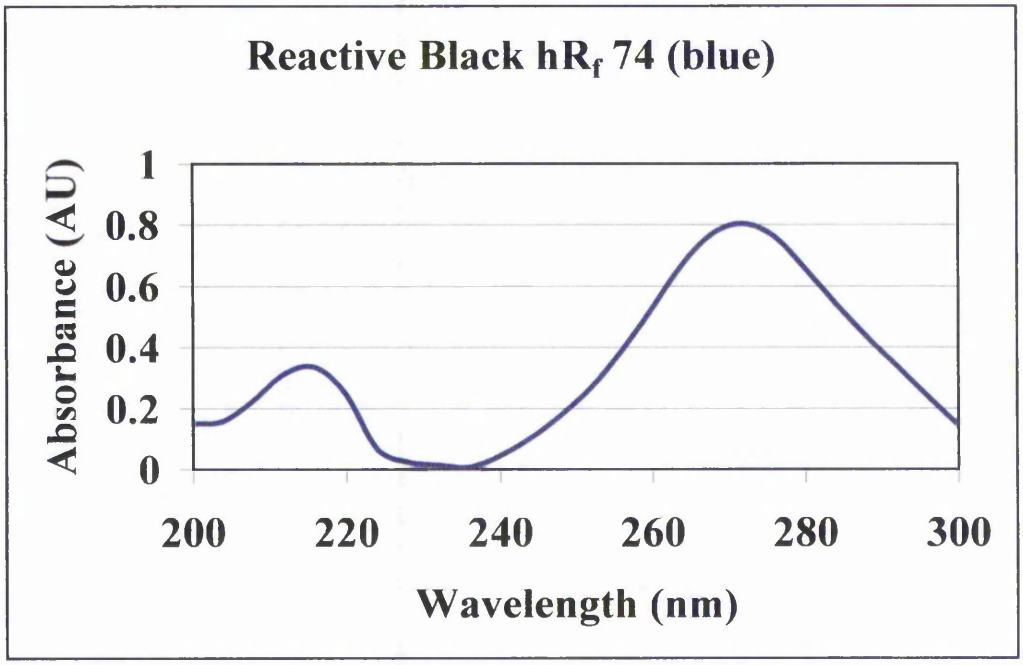
E. faecalis control assay.



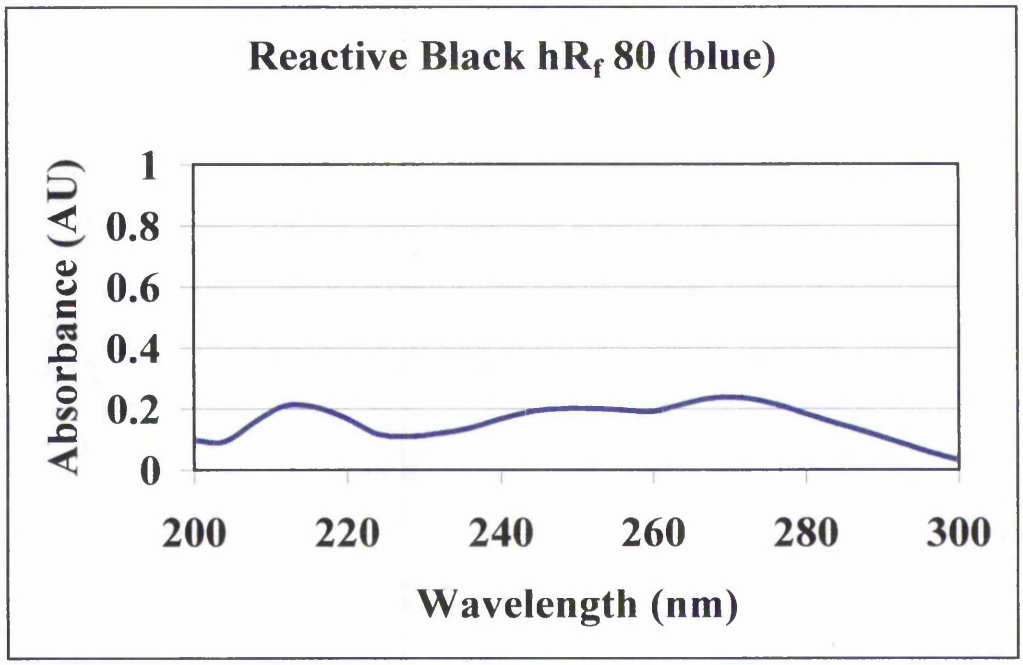


Sodium dithionite reduced.

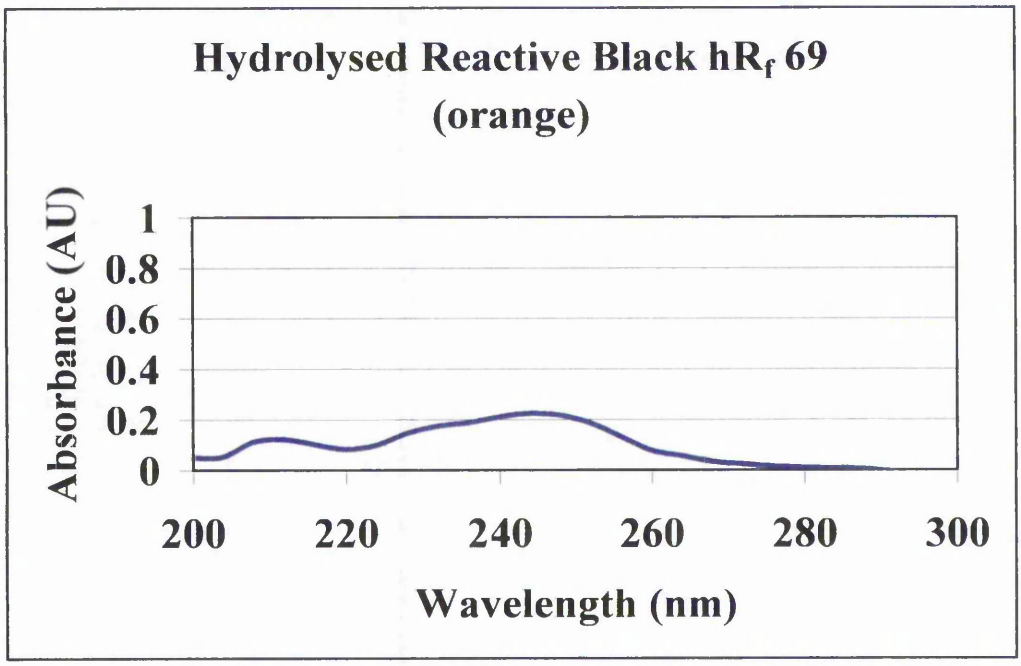


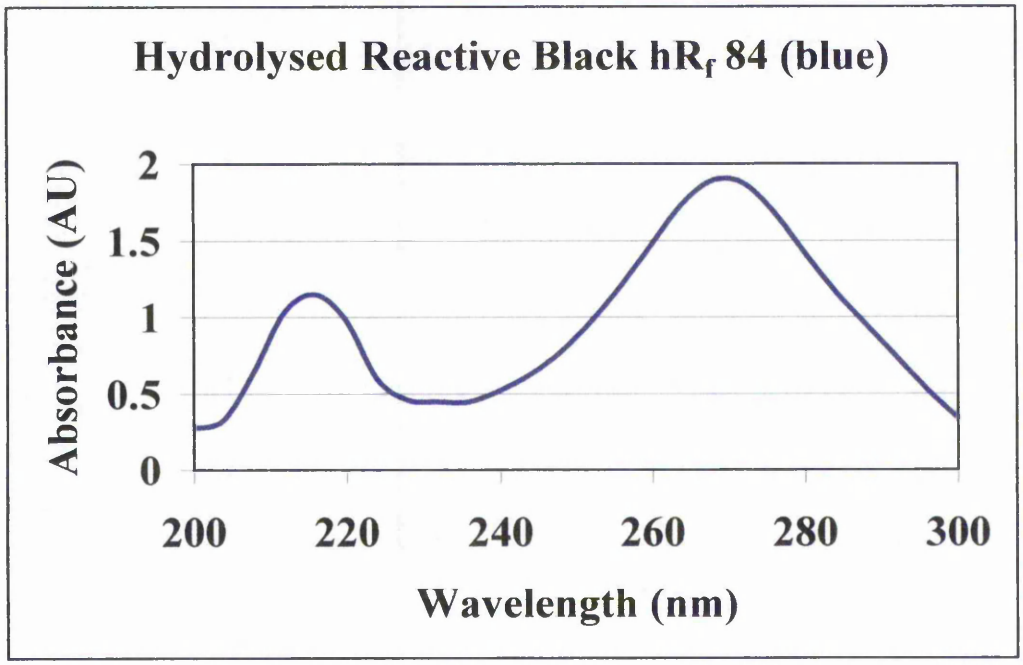


Sodium dithionite reduced.



Sodium dithionite reduced.





Sodium dithionite reduced.