Removal of colour from solutions of azo dyes using bacterial cells (*Shewanella* strain J18 143)

By

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others

Sign: Date:

To my beloved ones:

my mother and father, my brother and my husband, Chao.

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Abstract

Water pollution control is presently one of the major areas of scientific activity. While coloured organic compounds generally impart only a minor fraction of the organic load to wastewater, their colour renders them aesthetically unacceptable. Effluent discharges from the textile industries to neighbouring water bodies and wastewater treatment systems have been given of much concern especially in recent years. Colour removal, in particular, has recently become of major scientific interest, as indicated by the large number of related research reports.

The bacterium, *Shewanella* strain J18 143, has been reported to be capable of directly inducing the reduction of azo bonds in reactive azo dyes and, indirectly being involved in the reduction of azo/ketohydrazone chromophores in pigment molecules. Two approaches have been used to extend the applications of *Shewanella* strain J18 143 in the treatment of textile wastewaters. The first approach concerned the use of this bacterium, in an immobilised form, in removing the colour from reactive azo dye solutions. The second approach was the use of this bacterium, in a free form, in reducing colour from selected metal-complex azo dye solutions.

A grafted cellulose copolymer was chosen as the immobilisation substrate. The cotton fabric was firstly pre-treated and then underwent copolymerisation with the use of acrylic acid as the monomer and potassium persulphate as an initiator. Soxhlet extraction as used for after-treatment of the modified cellulose. The grafting yield of the graft copolymer was controlled at 5.5%.

The bacterial cells were immobilised by three methods. The first method, "growing-in", was carried out by growing the bacterial starter culture with the presence of graft cellulosic copolymer. The cells were therefore bound to the substrate. The immobilisation method of adsorption was carried out by allowing the pre-grown, resting cells to physically adsorb onto the copolymeric substrate. The third method, chemical coupling, was carried out by coupling the bacterial cells onto

the substrate using a coupling agent, carbodi-imide (CMC). Protein assay of immobilised cells was studied. The method derived from the use of the BCA kit was applied for a qualitative confidence of the presence of the immobilised cells.

Decoloration of Remazol Black B solution was carried out using the immobilised cells. In all of the three methods the immobilised cells were able to decolorise the dye. The immobilisation methods of "growing-in" and chemical coupling were found more effective in decoloration. Complete decoloration of the dye solutions was observed. The potential use of the graft copolymer substrate was confirmed.

Four Irgalan series metal-complex azo dyes, Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL, were decolorised using planktonic cells of *Shewanella* strain J18 143.

The bacterial cells completely decolorised the solution of Irgalan Grey GLN, apart from that some coloured by-products were observed in the system. Irgalan Grey GLN is a mixture of Irgalan Black RBLN and Irgalan Blue 3GL. Colour reductions of these two metal-complex azo dyes were achieved using planktonic bacterial cells. The results obtained were similar to that of the Irgalan Grey GLN. The colour of the solutions was reduced. Some coloured materials were produced at the bottom and at the top of the aqueous treating system.

Colour reduction of Irgalan Yellow 3RL KWL was carried out using the planktonic biological cells *Shewanella* strain J18 143. Not too much colour reduction can be found from the visual results and from the UV-visible spectra. However, some changes were made by the biological cells. Some precipitation was observed at the bottom the evaluating system which contained Irgalan Yellow 3RL KWL and the cells. This observation was further proved by the particle sizing analysis. The particle cells analysis has shown the standard dye solution has 35% of particles with a size no more than 1.1 nm. The biological treated the aqueous system contained particles, up to 20%, with a size range of 500 nm to 7000nm.

The effect of the dye concentration, the incubation temperature and the pH on the colour reduction of the selected metal-complex azo dyes was studied. The evaluations were carried out by evaluating the colour reduction rate against the varied factors.

For the colour reduction of Irgalan Grey GLN, the colour reduction rate was increased as the dye concentration increased, the optimum temperature of for the colour reduction was around 50°C, and the reduction rate has shown to be higher at pH range 6 to 9. Same results have shown to the colour reduction of the Irgalan Black RBLN and Irgalan Blue 3GL, except that the optimum operating temperature for the Irgalan Blue 3GL was at 40°C. Although the colour reduction of the Irgalan Yellow 3RL KWL was not significant, the effects of these factors were investigated. The maximum colour reduction was achieved at a dye concentration of 0.11 g dm^{-3}, at a temperature of 40°C and pH range from 6 to 8.

List of abbreviations

A	Absorbance of colorant
Ad	Adsorption
AOP	Advanced oxidation process
AOX	Adsorbed organic halides
AQDS	Anthraquinone-2,6-disulphonic acid
BCA	Bicinchoninic acid
BOD	Biological oxygen demand
B.S.A.	Bovine serum albumin
CC	Chemical coupling
C.I.	Colour Index
CMC	Carboxymethyl cellulose
CMC	1-cyclohexyl-3-[2-morphilinyl-4-ethyl]carbodi-imide metho-p-toluene sulphonate
COD	Chemical oxygen demand
c	Concentration (mol dm^{-3})
DS	Dissolved Solids
EF	Electro-Fenton
EHS	Northern Ireland Environment and Heritage Service
ε	Absorption coefficient
ETAD	Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers
GC	Grafted cellulose
GI	Growing-in
I_0	Intensity of incident light
I_T	Intensity of transmitted light
IPPC	Integrated Pollution Prevention and Control
l	Path length of the light travels through colorant solution

MAA	Methacrylic acid
NRA	National Rivers Authority
P.B.S.	Phosphate buffered saline solution
PMAA	Poly(methacrylic acid)
PPC	Pollution Prevention and Control
ppm	Parts per million
PTFE	Poly(tetrafluoroethylene)
PVA	Poly(vinyl alcohol)
RQO	River quality objectives
SEM	Scanning electron microscopy
SEPA	Scottish Environment Protection Agency
SS	Suspended Solids
SS304	Stainless Steel 304
STW	Sewage treatment works
T	Transmission
TOC	Total organic carbon
TS	Total solids
T.S.A.	Tryptone Soy Agar
T.S.B.	Tryptone Soy Broth
TSS	Total Suspended Solids
t	Time
U.S.P.	United States Pharmacopeia
UV	Ultraviolet
WAO	Wet-air oxidation

Contents

Title Page i

Acknowledgements iii

Abstract iv

List of Abbreviations vii

Table of Contents ix

List of Figures xviii

List of Tables xxiv

1 Introduction 1

1.1 Textile coloured wastewater 2

1.1.1 Source of textile wastewater 2

1.1.2 Environmental concerns regarding textile wastewaters 4

1.1.3 Colour in textile effluent 11

1.2 Reactive dye effluents 13

1.2.1 The development and use of reactive dyes 14

1.2.2 The structure and chemistry of reactive dyes 15

1.2.3 Reactive dyes in textile effluents 20

1.2.4 Reactive azo dyes 22

1.3 Metal-complex azo dye effluents 23

1.3.1 General information concerning metal-complex azo dyes 23

1.3.2 Aspects of the chemistry of metal-complex azo dyes 25

1.3.3 Metal-complex azo dyes in textile effluents 27

1.4 Methods of colour removal from textile effluents 28

1.4.1 Physico-chemical processes for the treatment of coloured wastewater. 32

1.4.1.1 Advanced oxidative processes (AOPs) 32

1.4.1.2 Reductive processes 40

1.4.1.3 Electrochemical processes 40

1.4.1.4 Coagulation/ flocculation processes 41

1.4.1.5 Adsorption treatments 43

1.4.1.6 Ion exchange treatments 44

1.4.1.7 Membrane processes 45

1.4.2 Biological processes for the treatment of coloured wastewater 47

1.4.2.1 Colour removal of textile wastewaters involving fungi 48

1.4.2.2 Colour removal of textile wastewaters involving bacteria 50

1.4.3 Metabolic diversity of *Shewanella* species 56

1.4.3.1 *Shewanella* strain J18 143 59

1.5 Immobilisation of microbial cells and applications in the decoloration of textile wastewaters 60

1.5.1 Methods for immobilisation of microbial cells and enzymes 61

1.5.2 Applications of immobilised microbial cells and enzymes in the decoloration of textile wastewaters 62

1.5.3 The options of support materials for immobilising microbial cells and enzymes in the decoloration of textile wastewaters 64

2 Materials and Methods 67

2.1 Materials and methods for the preparation of the immobilisation substrate and for the immobilisation of biological cells 68

2.1.1 Materials and methods for the preparation of the cellulosic copolymer-based immobilisation substrate 68

2.1.1.1 Materials used in preparation of the cellulosic graft copolymer 68

2.1.1.2 Grafting copolymerisation 68

2.1.2 Materials and methods for the preparation of biological cells – *Shewanella* strain J18 143 70

2.1.2.1 Preparation of growth medium 71

2.1.2.2 Streak-plate technique 71

2.1.2.3 Culture storage 73

2.1.2.4 Aseptic techniques 74

2.1.2.5 Preparation of planktonic *Shewanella* strain J18 143 cells 75

2.1.3 Immobilisation of *Shewanella* strain J18 143 cells 76

2.1.3.1 Immobilisation of biological cells onto the substrate using the method of "growing-in" 77

2.1.3.2 Immobilisation of biological cells onto substrates using the method of physical adsorption 77

2.1.3.3 Immobilisation of biological cells onto the substrate using the method of chemical coupling 77

2.1.4 Protein assay of immobilised biological cells 78

2.2 Decoloration of Remazol Black B dye solution using immobilised *Shewanella* strain J18 143 cells 78

2.2.1 Materials 78

2.2.2 Calibration curve of Remazol Black B dye solutions 79

2.2.3 Establishment of standards 80

2.2.3.1 Immobilised cells standard 81

2.2.3.2 Dye solution standard 81

2.2.3.3 Cell growth medium solution standards 82

2.2.3.4 Graft cellulosic copolymer standard 82

2.2.3.5 Free cells standard 82

2.2.4 Colour removal from Remazol Black B dye solutions using immobilised *Shewanella* strain J18 143 cells 83

2.3 Colour reduction of metal-complex azo dye solutions using planktonic cells of *Shewanella* strain J18 143 84

2.3.1 Materials 84

2.3.2 Preparation of the *Shewanella* strain J18 143 cells 87

2.3.3 Colour reduction of solutions of the Irgalan Grey GLN metal-complex azo dye using the planktonic cells of *Shewanella* strain J18 143 88

2.3.3.1 Calibration curve of the solutions of Irgalan Grey GLN 88

2.3.3.2 Reduction of solutions of the Irgalan Grey GLN metal-complex dye using the planktonic cells of *Shewanella* strain J18 143 89

2.3.3.3 Establishment of dye solution standards 89

2.3.3.4 Establishment of planktonic cells standard 90

2.3.3.5 Particle size analysis 90

2.3.4 Colour reduction of metal-complex azo dye solutions (Irgalan Black RBLN and Irgalan Blue 3GL) using the planktonic cells of *Shewanella* strain J18 143 91

2.3.4.1 Calibration curve of the solutions of Irgalan Black RBLN and Irgalan Blue 3GL 91

2.3.4.2 Colour Reduction from solutions of Irgalan Black RBLN and Irgalan Blue 3GL using the planktonic cells of *Shewanella* strain J18 143 .. 92

2.3.4.3 Establishment of dye solution standards 93

2.3.4.4 Establishment of planktonic cells standard 94

2.3.5 Colour reduction from solutions of Irgalan Yellow 3RL KWL dye using the planktonic cells of *Shewanella* strain J18 143 94

2.3.5.1 Calibration curve for solutions of Irgalan Yellow 3RL KWL 94

2.3.5.2 Reduction of solutions of Irgalan Yellow 3RL KWL using the planktonic cells of *Shewanella* strain J18 143 95

2.3.5.3 Establishment of dye solution standards 95

2.3.5.4 Establishment of planktonic cells standard 96

2.3.5.5 Particle size analysis 96

2.4 Factors affecting the colour reduction of the metal-complex dye solutions using the planktonic cells of *Shewanella* strain J18 143 97

2.4.1 Materials 97

2.4.2 Protein assay of planktonic cells of *Shewanella* strain J18 143 98

2.4.3 Effect of dye concentration on the colour reduction of the metal-complex azo dyes 99

2.4.4 Effect of temperature on the colour reduction of the metal-complex azo dyes 100

2.4.5 Effect of pH on the colour reduction of metal-complex azo dyes ... 101

2.5 Analysis and characterisation methods 102

2.5.1 Gravimetric analysis 102

2.5.2 Analysis using scanning electron microscopy (SEM) 102

2.5.3 Analysis using UV-visible spectrophotometry 103

2.5.4 Particle sizing analysis 105

2.5.5 Protein assay 105

2.5.5.1 Protein assay of planktonic cells 105

2.5.5.2 Protein assay of immobilised biological cells 106

3 Results and Discussion 108

3.1 Preparation of the immobilisation substrate and the immobilisation of biological cells 109

3.1.1 Preparation of cellulosic copolymer-based immobilisation substrate.... 109

3.1.2 Preparation of biological cells – *Shewanella* strain J18 143..... 113

3.1.2.1 Culture storage 114

3.1.3 Immobilisation of *Shewanella* strain J18 143 116

3.1.3.1 Attempts at protein assay of immobilised *Shewanella* strain J18 143 116

3.2 Decoloration of Remazol Black B solutions using immobilised *Shewanella* strain J18 143 cells 119

3.2.1 Calibration curve of the solutions of Remazol Black B..... 119

3.2.2 Visual appraisal arising from the decoloration of the Remazol Black B solutions by the immobilised cells of *Shewanella* strain J18 143..... 121

3.2.3 Results from the established standard controls 124

3.2.3.1 Standard of the cell growth medium solution 124

3.2.3.2 Standard of graft cellulosic copolymer 125

3.2.3.3 Decoloration of solutions of Remazol Black B using free resting *Shewnaella* strain J18 143 cells 126

3.2.4 Measuring results from the decoloration of Remazol Black B solutions using immobilised cells 127

3.2.4.1 Results from the decoloration of the of Remazol Black B solution using the "growing-in" immobilised cells 128

3.2.4.2 Results from the decoloration of the Remazol Black B solution using the chemically coupled immobilised cells..... 130

3.2.4.3 Results from the decoloration of the Remazol Black B solution using the physically adsorbed immobilised cells..... 132

3.2.4.4 Comparison of decoloration effect of the immobilised cells between the three immobilisation methods..... 134

3.3 Colour reduction of metal-complex azo dye solutions using free cells of *Shewanella* strain J18 143 136

3.3.1 Colour reduction of solutions of Irgalan Grey GLN using the free cells of *Shewanella* strain J18 143 136

3.3.1.1 Calibration curve for solutions of Irgalan Grey GLN metal-complex dye..... 136

3.3.1.2 Visual results from the reduction of solutions of Irgalan Grey GLN metal-complex dye using the free cells of *Shewanella* strain J18 143..... 137

3.3.1.3 Particle size investigation..... 138

3.3.1.4 Measured results from the reduction of solutions of Irgalan Grey GLN using the free cells of *Shewanella* strain J18 143 141

3.3.2 Colour reduction of solutions of Irgalan Black RBLN using the free cells of *Shewanella* strain J18 143 144

3.3.2.1 Calibration plot of the solutions of Irgalan Black RBLN 144

3.3.2.2 Visual results from the colour reduction of Irgalan Black RBLN dye solutions using the free cells of *Shewanella* strain J18 143 145

3.3.2.3 Measured results from the reduction of solutions of Irgalan Black RBLN using the free cells of *Shewanella* strain J18 143..... 147

3.3.3 Colour reduction of solutions of Irgalan Blue 3GL using the free cells of *Shewanella* strain J18 143 149

3.3.3.1 Calibration plot of the solutions of Irgalan Blue 3GL 149

3.3.3.2 Visual results from the reduction of solutions of Irgalan Blue 3GL using the free cells of *Shewanella* strain J18 143 150

3.3.3.3 Measured results from the reduction of solutions of Irgalan Blue 3GL using the free cells of *Shewanella* strain J18 143 152

3.3.4 Colour reduction of solutions of Irgalan Yellow 3RL KWL using the free cells of *Shewanella* strain J18 143 154

3.3.4.1 Calibration plot of the solutions of Irgalan Yellow 3RL KWL ... 155

3.3.4.2 Visual results from the reduction of solutions of Irgalan Yellow 3RL KWL using the free cells of *Shewanella* strain J18 143 155

3.3.4.3 Measured results from the reduction of solutions of Irgalan Yellow 3RL KWL using the free cells of *Shewanella* strain J18 143 157

3.3.4.4 Particle size investigation..... 159

3.4 Factors affecting the reduction of solutions of metal-complex azo dyes using free resting cells of *Shewanella* strain J18 143 162

3.4.1 Protein assay 162

3.4.2 Factors affecting the reduction of Irgalan Grey GLN solutions using free resting cells of *Shewanella* strain J18 143 163

3.4.2.1 Effect of dye concentration on the dye reduction 163

3.4.2.2 Effect of temperature on dye reduction..... 165

3.4.2.3 Effect of pH on dye reduction..... 166

3.4.3 Factors affecting the reduction of Irgalan Black RBLN solutions using free resting cells of *Shewanella* strain J18 143 168

3.4.3.1 Effect of dye concentration on dye reduction 168

3.4.3.2 Effect of temperature on dye reduction..... 169

3.4.3.3 Effect of pH on dye reduction..... 171

3.4.4 Factors affecting the reduction of Irgalan Blue 3GL solutions using free resting cells of *Shewanella* strain J18 143 172

3.4.4.1 Effect of dye concentration on dye reduction 172

3.4.4.2 Effect of temperature on dye reduction..... 174

3.4.4.3 Effect of pH on dye reduction..... 175

3.4.5 Factors affecting the reduction of Irgalan Yellow 3RL KWL solutions using free resting cells of *Shewanella* strain J18 143 177

3.4.5.1 Effect of dye concentration on dye reduction 177

3.4.5.2 Effect of temperature on dye reduction 179
3.4.5.3 Effect of pH on dye reduction 180
4 General discussion 182
4.1 Studies involving colour removal from Remazol Black B solutions using immobilised cells 182
4.2 Colour reduction of solutions of metal-complex azo dyes using the planktonic cells 185
4.3 Investigation of the factors affecting the biological colour reduction of the metal-complex azo dye solutions 188
5 Conclusions 190
6 Suggestions for further work 191
References 192

List of Figures

Figure 1.3.1 The Most Important Synthesised Types of Metal-Complex Dyes 26

Figure 1.3.2 Molecular Structure of the More Common 1:2 Metal-Complex Azo Dyes 27

Figure 2.1.1 Scheme of Process of Grafting Copolymerisation.....69

Figure 2.1.2 Scheme of Methods of Biological Cells Immobilisation.....76

Figure 2.3.1 The Chemical Structure of the Chromophore in Irgalan Black RBLN.....85

Figure 2.3.2 The Chemical Structure of the Chromophore in Irgalan Blue 3GL 86

Figure 2.3.3 The Chemical Structure of the Chromophore in Irgalan Yellow 3RL KWL.....86

Figure 3.1.1 Scanning Electron Micrograph (SEM) of Pre-treated Cellulose 110

Figure 3.1.2 Scanning Electron Micrograph (SEM) of Grafted Cellulose 113

Figure 3.1.3 Bacterial Culture Stored on Agar Slope 114

Figure 3.1.4 Bacterial Culture Streaked on a Agar Plate.....115

Figure 3.1.5 Scanning Electron Micrograph of Prepared Cell Suspension 115

Figure 3.1.6 Calibration Plot of Standard Protein Solution Assay 117

Figure 3.2.1 Calibration Curve of Dye Solutions of Remazol Black B 120

Figure 3.2.2 Visual Results from the Decoloration of Dye Solutions of Remazol Black B.....122

Figure 3.2.3 Established Standard of Immobilisation Substrate-Contains Solutions of Tryptone Soy Broth (Sample 6) 125

Figure 3.2.4 Established Standard of Immobilisation Substrate (Sample 7).....126

Figure 3.2.5 Decoloration Flow Profile for the Decoloration with Free *Shewanella* Cells (Sample 8) 127

Figure 3.2.6 Results from the Decoloration of the Remazol Black B Dye Solution using Immobilised Biological Cells 128

Figure 3.2.7 UV-Visible Spectra for Dye Solutions of Remazol Black B after 24 Hours Incubation without (Black dye std) and with (Black dye with "growing-in" immobilised cells) "Growing-in" Immobilised Cells of *Shewanella* Strain J18 143 at 30°C.....129

Figure 3.2.8 Results from the Decoloration of Remazol Black B Dye Solutions by Immobilised Biological Cells.....130

Figure 3.2.9 UV-Visible Spectra for Dye Solutions of Remazol Black B after 24 Hours Incubation without (Black dye std) and with (Black dye with coupling immobilised cells after 24 hours of incubation and after 48 hours of incubation) "Growing-in" Immobilised Cells of *Shewanella* Strain J18 143 at 30°C 131

Figure 3.2.10 Results from the Decoloration of Remazol Black B Dye Solutions using the Immobilised Biological Cells 132

Figure 3.2.11 UV-Visible Spectra for Dye Solutions of Remazol Black B after 24 Hours Incubation without (Black dye std) and with (Black dye with adsorbed cells after 24 hours of incubation and after 48 hours of incubation) Physically Adsorbed Cells of *Shewanella* Strain J18 143 at 30°C 133

Figure 3.2.12 Comparison of the Effect of Colour Removal from Remazol Black B using Immobilised Cells based on the Three Different Immobilisation Methods 135

Figure 3.3.1 Calibration Plot for Solutions of Irgalan Grey GLN 137

Figure 3.3.2 Flow File of Reduction of Dye Solution of Irgalan Grey GLN 138

Figure 3.3.3 Particle Size Distribution of Standard Irgalan Grey GLN Dye "Solution" after 18 Hours of Incubation at 30°C 139

Figure 3.3.4 Particle Size Distribution of Irgalan Grey GLN Dye "Solution" after 18 Hours of Incubation with an Anaerobic Cell Suspension of *Shewanella* Strain J18 143 at 30°C 140

Figure 3.3.5 Particle Size Distribution of Standard Cell Suspension of *Shewanella* Strain J18 143 after 18 Hours of Incubation at 30°C 140

Figure 3.3.6 Decoloration of Solutions of Irgalan Grey GLN using Free Resting Biological Cells 142

Figure 3.3.7 Corrected Results from the Decoloration of Solutions of Irgalan Grey GLN using Free Resting Biological Cells 142

Figure 3.3.8 UV-Visible Spectra for Solutions of Irgalan Grey GLN after 18 Hours of Incubation, without and with Anaerobic Cell Suspension of *Shewanella* Strain J18 143, at 30°C 143

Figure 3.3.9 Calibration Plot for Solutions of Irgalan Black RBLN 145

Figure 3.3.10 Flow File of Reduction of Solutions of Irgalan Black RBLN 146

Figure 3.3.11 Photographs of Standard Dye Solutions of Irgalan Black RBLN and Reduction of the Dye Solutions using *Shewanella* Strain J18 143 after 18 Hours of Incubation, at 30°C 147

Figure 3.3.12 Measured Results from the Colour Reduction of Solutions of Irgalan Black RBLN using Free Resting Biological Cells 148

Figure 3.3.13 UV-Visible Spectra for Solutions of Irgalan Black RBLN after 24 Hours Incubation without and with Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 at 30°C 149

Figure 3.3.14 Calibration Curve for Solutions of Irgalan Blue 3GL 150

Figure 3.3.15 Sequence in the Reduction of Solutions of Irgalan Blue 3GL 151

Figure 3.3.16 Photographs of Standard Solutions of Irgalan Blue 3GL and Reduction of the Dye Solutions using *Shewanella* Strain J18 143 after 18 Hours' Incubation, at 30°C pH 7 152

Figure 3.3.17 Results from the Colour Reduction of Solutions of Irgalan Blue 3GL using Free Resting Biological Cells 153

Figure 3.3.18 UV-Visible Spectra for Solutions of Irgalan Blue 3GL after 24 Hours Incubation without (Blue dye std) and with Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 (Blue dye with cells) at 30°C 154

Figure 3.3.19 Calibration Plot for Solutions of Irgalan Yellow 3RL KWL 155

Figure 3.3.20 Flow File of Reduction of Solutions of Irgalan Yellow 3RL KWL 156

Figure 3.3.21 Results from the Colour Reduction of Solutions of Irgalan Yellow 3RL KWL using Free Resting Biological Cells 157

Figure 3.3.22 "Corrected" Results from the Decoloration of Solutions of Irgalan Yellow 3RL KWL using Free Resting Biological Cells 158

Figure 3.3.23 UV-Visible Spectra for the Solution of Irgalan Yellow 3RL KWL and for the Dye Solution that was Treated with Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 after 18 Hours Incubation, at 30°C 159

Figure 3.3.24 Particle Size Distribution of Standard Cell Suspension of *Shewanella* Strain J18 143 after 18 Hours of Incubation at 30°C 160

Figure 3.3.25 Particle Size Distribution of Standard Irgalan Yellow 3RL KWL Dye "Solution" after 18 Hours of Incubation at 30°C.....160

Figure 3.3.26 Particle Size Distribution of Irgalan Yellow 3RL KWL "Solution" after 18 Hours of Incubation with Anaerobic Cell Suspension of *Shewanella* Strain J18 143 at 30°C.....161

Figure 3.4.1 Calibration Curve of Standard Bovine Serum Albumin Protein Assay 163

Figure 3.4.2 Reduction of Irgalan Grey GLN Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C 164

Figure 3.4.3 Effect of Concentration on the Rate of the Irgalan Grey GLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 164

Figure 3.4.4 Reduction of Irgalan Grey GLN Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8)....165

Figure 3.4.5 Effect of Temperature on the Rate of Irgalan Grey GLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 166

Figure 3.4.6 Reduction of Irgalan Grey GLN Solutions under Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C).....167

Figure 3.4.7 Effect of pH Conditions on the Rate of Irgalan Grey GLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 167

Figure 3.4.8 Reduction of Irgalan Black RBLN Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C 168

Figure 3.4.9 Effect of Concentration on the Rate of the Irgalan Black RBLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 169

Figure 3.4.10 Reduction of Irgalan Black RBLN Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8)....170

Figure 3.4.11 Effect of Temperature on the Rate of Irgalan Black RBLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 170

Figure 3.4.12 Reduction of Irgalan Black RBLN Solutions under Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C) 171

Figure 3.4.13 Effect of pH Conditions on the Rate of Irgalan Black RBLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 172

Figure 3.4.14 Reduction of Irgalan Blue 3GL Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C 173

Figure 3.4.15 Effect of Concentration on the Rate of the Irgalan Blue 3GL Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 173

Figure 3.4.16 Reduction of Irgalan Blue 3GL Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8)....174

Figure 3.4.17 Effect of Temperature on the Rate of Irgalan Blue 3GL Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 175

Figure 3.4.18 Reduction of Irgalan Blue 3GL Solutions under Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C).....176

Figure 3.4.19 Effect of pH Conditions on the Rate of Irgalan Blue 3GL Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143 176

Figure 3.4.20 Reduction of Irgalan Yellow 3RL KWL Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C.....177

Figure 3.4.21 Effect of Concentration on the Rate of Irgalan Yellow 3RL KWL Dye Reduction in Solution using Resting Cell Suspension of *Shewanella* Strain J18 143.....178

Figure 3.4.22 Reduction of Irgalan Yellow 3RL KWL Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) 179

Figure 3.4.23 Effect of Temperature on the Rate of Irgalan Yellow 3RL KWL Dye Reduction in Solutions using Resting Cell Suspension of *Shewanella* Strain J18 143.....179

Figure 3.4.24 Reduction of Irgalan Yellow 3RL KWL Solutions at Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C) 180

Figure 3.4.25 Effect of pH Conditions on the Rate of Irgalan Yellow 3RL KWL Dye Reduction in Solutions using Resting Cell Suspension of *Shewanella* Strain J18 143 181

Figure 4.2.1 Suggested Biological Mechanism for the Reduction of Remazol Black B by Bacterial Cells of *Shewanella* strain J18 143 (Pearce, 2004) 189

List of Tables

Table 1.1.1 Inputs and Outputs from Various Textile-Processing Stages.....3

Table 1.1.2 Average Water Consumption for Dyeing of Different Materials.....4

Table 1.1.3 List of Contaminants Found in Textile Finishing Effluents 4

Table 1.1.4 Effluent Characteristics from the Textile Industry.....5

Table 1.1.5 Pollution Loads of Textile Wet Operations.....7

Table 1.1.6 Consents and Standards for discharge of Aqueous Effluents.....8

Table 1.1.7 Colour of 'Average' Effluent Leaving a Dyehouse and of RQO Consents 12

Table 1.1.8 Estimated Degree of Fixation for Different Dye/Fibre Combinations.....13

Table 1.2.1 Chemical Classes of Reactive Dyes 16

Table 1.2.2 Typical Reactive Groups in Commercial Reactive Dyes.....18

Table 1.3.1 Summary of Major Events in the Development of Metal-complex Dyes.....24

Table 1.3.2 Distribution of Metal-complex Azo Dyes between Application Ranges.....25

Table 1.4.1 Conventional Wastewater Treatment Processes.....29

Table 1.4.2 Advantages and Disadvantages of Physico-chemical Treatment Processes for Colour Removal from Textile Wastewaters.....31

Table 1.4.3 Comparisons of Aerobic and Anaerobic Biolgical Treatments 51

Table 1.4.4 Reduction of a Range of Electron Acceptors by Various *Shewanella* Strains: Review of Studies Reported in the Literature 57

Table 2.1.1 Composition of Tryptone Soy Broth and Tryptone Soy Agar.....71

Table 2.4.1 Buffer Solutions 98

Table 2.5.1 Standard Bovine Serum Albumin (B.S.A.) Solutions of Known Protein Concentration 106

Table 2.5.2 Preparation of Protein Assay Samples in 24-well Plate 107

Table 3.1.1 Results from Protein Analysis of Immobilised Bacterial Cells.....118

Table 3.2.1 Comparison of Dye Reduction Rate of Decoloration of Remazol Black B for the Different Immobilisation Methods.....134

1 Introduction

In Section 1.1, the introductory material and literature reviews are focused on the occurrence and treatment of coloured textile wastewaters including the source of textile wastewaters, the environmental impact of textile wastewaters and the problem of colour in textile wastewaters.

In Section 1.2, matters relating to the issues of reactive dye effluents are considered. This section begins with an introduction to basic aspects, including the development and the chemistry of reactive dyes. The problems that have been brought by the reactive dyes in textile wastewaters, especially those related to reactive azo dyes are addressed in this section.

Matters relating to the use of metal-complex azo dyes in the textile wastewaters are included in Section 1.3. These include general information of the development of metal-complex dyes, aspects of the chemistry of metal-complex azo dyes and the environmental impact of metal-complex azo dyes in textile wastewaters.

The colour removal techniques, in the treatment of textile wastewaters are introduced in Section 1.4. These techniques are considered in three categories, the physical methods, the chemical methods and the biological methods. These three categories are treated in two sub-sections in Section 1.4. These two sub-sections are physio-chemical methods and biological methods. The applications of *Shewanella* strains are introduced at the end of the section.

Matters relating to the use of immobilised biological cells in colour removal are given in Section 1.5. The immobilisation methods and aspects of the immobilisation substrates are considered in this section. The use of cellulose and of modified celluloses as immobilisation substrates are introduced at the end of this section.

1.1 Textile coloured wastewater

Textile industry is very energy-, water-, and chemical- use intensive. Worldwide, the industry is one of the major water consumers. In localised situations, textile coloration can be considered to be a major source of waterborne pollutions.

1.1.1 Source of textile wastewater

Within the textile industry, approximately 60 percent of the total energy that is consumed is within the wet-processing stages. In wet processing, almost all of the chemicals, dyes etc., are discharged into effluents. It is not surprising that wet processing operations in the textile industry are among the major causes for waterborne pollution (Mishra and Tripathy, 1993). The chemical treatments that are used during the wet processing of textiles include desizing, scouring, bleaching, dyeing, printing and finishing. Table 1.1.1 gives the various stages involved and also the inputs and outputs from these stages (Source: Integrated Pollution Prevention and Control, Sector guidance note 6.05). Water is mainly used as a solvent for the processing of chemicals and as a washing and rinsing medium. Most wet processing involves treatments with chemicals-containing baths, which often require washing, rinsing, and drying stages between key treatment steps. Consequently, large volumes of wastewater are generated, with a very diverse range of contaminants that must be treated prior to disposal. Much energy is consumed in heating and cooling chemical-treatment baths, to heat the wash waters and to dry the fabrics or yarns (Snowden-Swan, 1995). Wet processing is associated with dyeing and finishing that takes place in the dyehouse or finishing mill. Finishing is a final stage in the manufacture of a textile. The textile finishing industry has a high specific water demand that may be as much as 500 dm^3 kg^{-1} in a process as shown in Table 1.1.2 (Karcher *et al.*, 2002; Menezes, 2003). It has been established that the UK textile dyeing and finishing industry uses over 60,000 million litres of water every year

(Source: Environmental Agency, 2006). A large amount of wastewater is generated from the dyeing processes and subsequent rinsing steps, forming one of the larger contributions to wastewater generation in the textile industry. Some of the common pollutants that exist in textile finishing effluents are shown in Table 1.1.3 (Broadbent, 2001). No doubt, the industry faces increasing pressure regarding environmental and waste-related concerns as a result of the quantity and toxicity of generated wastewaters.

Table 1.1.1 Inputs and Outputs from Various Textile-Processing Stages (Source: Integrated Pollution Prevention and Control Sector guidance note 6.05)

Inputs (raw materials/utilities)	**Process**	**Outputs (Product/wastes)**
Hot water Detergent	Desize	Hot water Dilute detergent Dissolved size
Alkali Water	Scour	Dilute spent chemical Hot water Trace contaminants
Bleaching agent Water	Bleach	Bleach Water
Dyes Water	Dye	Unfixed dye Water
Inks Water wash	Print	Unfixed inks Water
Heat Resin Retardants	Finish	Unfixed chemicals Waste heat

Table 1.1.2 Average Water Consumption for Dyeing of Different Materials (Source: Menezes, 2003)

Material	Consumption (dm^3 kg^{-1})
Wool garments	265–465
Nylon socks	125–150
Nylon hose	100-240
Acrylic garments	100–230
Synthetic polymer fabrics	65–190
Wool hanks	40-65

Table 1.1.3 List of Contaminants Found in Textile Finishing Effluents (Source: Broadbent, 2001)

Water pollutants	Examples
Heavy metals	Chromium, copper, zinc (as ions)
Organochlorine compounds	Moth-proofing agents
Insecticides	Aldrin
Sulphides	Sodium sulphide from dyeing
Colour	Dyes
Surfactants	Detergents, dispersing agents
Oils	Emulsions form scouring
Carbohydrates	Starch sizing
Solvents	Degreasing solvents from scouring
Acids and alkalis	Dyeing assistants

1.1.2 Environmental concerns regarding textile wastewaters

Textile wastewaters cause serious problems to the environment. Such problems are of both national and international concern. The effluent from textile processing is often either discharged into a municipal sewage treatment plant or directly into

waterways. Textile wastewaters may include many types of dyes, detergents, insecticides, pesticides, greases and oils, sulphide compounds, solvents, heavy metal salts, inorganic salts and fibres (Šostar-Turk *et al.*, 2005), as listed in Table 1.1.3. It is not surprising that the wastewater discharged from the textile industries is characterised by strong colour, a high organic load (COD/BOD, COD refers to the chemical oxygen demand and BOD is the biochemical oxygen demand), a large amount of suspended solids, a highly fluctuating pH, high temperatures and low biodegradability (Joo *et al.*, 2007; Karim *et al.*, 2006; Şayan, 2006). Table 1.1.4 shows the effluent characteristics from different operations in textile processes.

Table 1.1.4 Effluent Characteristics from the Textile Industry (Source: Wynne *et al.*, 2001)

Process	Effluent composition	Nature
Sizing	Starch, waxes, carboxymethyl cellulose (CMC), poly(vinyl alcohol) (PVA), wetting agents	High in BOD, COD
Desizing	Starch, CMC, PVA, fats, waxes, pectin	High in BOD, COD, Suspended Solids (SS) and Dissolved Solids (DS)
Bleaching	Sodium hypochlorite, chlorine, sodium hydroxide, hydrogen peroxide, acids, surfactants, sodium silicate, sodium phosphate, short cotton fibre	High alkalinity, high SS
Mercerising	Sodium hydroxide, cotton, waxes	High pH, low BOD, high DS
Dyeing	Dyestuffs, urea, reducing agents, oxidising agent, acetic acid, detergents, wetting agents	Strongly coloured, high in BOD and DS, low in SS and heavy metals
Printing	Pastes, urea, starches, gums, oils binders, acids, thickeners, cross-linkers, reducing agents, alkaline solutions	Highly coloured, high in BOD, oily appearance, and SS
Finishing	Inorganic salts, formaldehyde	Slightly alkaline, low BOD

The biological oxygen demand (BOD) is an important measure of the impact of industrial wastewaters on the environment. It is the amount of oxygen consumed in the biological processes that break down organic matter in water, often during a five-day test. It is expressed in milligrams of oxygen per litre of wastewater. Although the BOD is not a specific parameter, it is used as an indirect measure of the concentration of biologically degradable materials that are present in the wastes. BOD can be used as an indicator of pollutant level where the greater the BOD, the more pronounced is the degree of pollution (Willmott, 1997).

Table 1.1.5 summarises the expected or most probable pollution loads of BOD, along with pH, total solids (TS) and water consumption, resulting from each processing operation of the various raw materials.

The chemical oxygen demand (COD) is also a very important factor concerning the impact of industrial wastewaters on the environment. It is amount of the oxygen consumed in the complete chemical oxidation of soluble organic compounds to produce CO_2 and H_2O. It is also expressed in mg dm^{-3}, indicating the mass of oxygen that is consumed per litre of wastewater. The COD is commonly monitored by quantifying the amount of the oxygen that is used when the contaminants are oxidised in a boiling aqueous acidic dichromate ion solution (Willmott, 1997).

The COD level can be determined more readily than the BOD. However, this measurement does not indicate how much of the waste can be decomposed by biological oxidation. The COD is often used as a measure of the organic matter that is in industrial and municipal wastes that contain compounds that are toxic to bacteria. The COD represents the level of pollution of water caused by the compounds. Organic matter is the most common source of reducible compounds that are found in the water. Therefore, the COD can be used to indicate the level of the organic pollution in the wastewater; the greater the COD, the greater the degree and impact of the pollution.

Table 1.1.5 Pollution Loads of Textile Wet Operations (Source: Correia *et al.*, 1994)

Fibre	Process	BOD ($mg\ dm^{-3}$)	pH	TS ($mg\ dm^{-3}$)	Water usage ($dm^3\ kg^{-1}$)
Cotton	Desizing	1700-5200	—	16000-32000	3-9
	Scouring	50-2900	10-13	7600-17400	26-43
	Bleaching	90-1700	8.5-9.6	2300-14400	3-124
	Mercerising	45-65	5.5-9.5	600-1900	232-308
	Dyeing	11-1800	5-10	500-14100	8-300
Wool	Scouring	30000-40000	9-14	1129-64448	46-100
	Dyeing	380-2200	4.8-8	3855-8315	16-22
	Washing	4000-11455	7.3-10.3	4830-19267	334-835
	Neutralisation	28	1.9-9	1241-4830	104-131
	Bleaching	390	6	908	3-22
Nylon	Scouring	1360	10.4	1882	50-67
	Dyeing	368	8.4	641	17-33
Acrylic/ modacrylic	Scouring	2190	9.7	1874	50-67
	Dyeing	175-2000	1.5-3.7	833-1968	17-33
	Final Scour	668	7.1	1191	67-83
Polyester	Scouring	500-800	—	—	25-42
	Dyeing	480-27000	—	—	17-33
	Final Scour	650	—	—	17-33
Viscose	Scouring and dyeing	2832	8.5	3334	17-33
	Salt bath	58	6.8	4890	4-13
Acetate	Scouring and dyeing	2000	9.3	1778	33-50

As mentioned above, a larger value of COD indicates that the content of the reducible compounds (organic matter) is greater. The reducible compounds can lower the amount of dissolved oxygen in water. As a result, the position of fish and

aquatic life can become untenable. In addition, theses species can become sensitised to other toxic chemicals, noxious odours and toxic organics that may be produced subsequently.

Dyeing auxiliaries or organic substances that are non-recyclable are more likely to be responsible for the high COD/BOD of the effluents (Allègre *et al.*, 2006). The maximum acceptable concentrations for effluent discharge into the public sewer are 1200–2500 mg of COD dm^{-3} of wastewater and 600 mg of BOD dm^{-3} of wastewater (Skelly, 2003). The discharges to surface water in the UK are limited to 50 mg of COD dm^{-3} of wastewater and 30 mg of BOD dm^{-3} of wastewater, as listed in Table 1.1.6 (Kamilaki, 2000).

Table 1.1.6 Consents and Standards for discharge of Aqueous Effluents (Source: Kamilaki, 2000)

Parameter	**Standard/Consent**
Temperature	Below 42 °C at point of discharge
pH	Between 6 and 9 at point of discharge
BOD	30 mg dm^{-3} to surface waters
COD	50 mg dm^{-3} to surface waters
	Consented to sewer
Suspended solids	20 mg dm^{-3} to surface waters
	Consented to sewer
Colour	Below 1ppm consented
Toxic substances	Restricted by legislation
Volume and flow	Basis for charging consented

The pH of textile wastewater may vary. This is because acids and alkalis are used in the dyeing process, depending on the type of the dye involved. Also, large quantities of alkali are used in bleaching, desizing, scouring and mercerising (Delée *et al.*, 1998). According to Fox and Pickle (1996), the pH of textile effluents ranges from 1

to 13 in standard pH units, as shown in Table 1.1.5. The temperature of the textile effluent also varies, depending on the type of the dye and on the dyeing process. In the UK, the pH of the discharged wastewater is limited between 6 and 9. The temperature is limited to below 42°C at the point of discharge, as shown in Table 1.1.6.

The Total Suspended Solids, generally referred to as TSS, is a measure of the settleable solids and the non-settleable solids in the wastewater. Suspended solids in water reduce light penetration in the water and are often associated with toxic contaminants because organics and metal ions tend to bind to particles. TSS, like BOD, is an indicator of the relative strength of the liquid. Accordingly, the higher the TSS concentration, the greater is the pollution caused by the wastewater. The maximum acceptable concentrations of TSS and settleable SS (Suspended Solids) for effluent discharge into the public sewer are respectively 650-1200 mg dm^{-3} and 15-40 mg dm^{-3} (Skelly, 2003). The amount of all of the solids in the water, including dissolved, suspended, and settleable components is defined as Total Solids (TS). Details of the TS in each textile wet operation are listed in Table 1.1.5.

Textile industries generate large volumes of wastewaters that are polluted with dyes. Due to their high molecular weights, their complex structures and especially their high solubilities in water, they persist once discharged into a natural environment (Cañizares *et al.*, 2006). The dyes are difficult to biodegrade and some, particularly hydrolysed reactive dyes and certain acid dyes, are not readily absorbed by active sludge and, therefore pass through the treatment works into rivers and streams. Some dyes and auxiliaries contain heavy metal ions such as copper ions, zinc ions, and chromium ions that are not totally exhausted onto the fibre. These can be discharged and, clearly, have an environmental impact (Cooper, 1989). More information regarding the impact of coloured wastewater is given in Section 1.1.3.

The consents and standards of the wastewater discharging limits vary across the different countries or the different regions. Hewson (1999) presents numerical

consents and standards of the major parameters for the discharge of aqueous discharges in the UK (Kamilaki, 2000). These are listed in Table 1.1.6.

Concentrations of dye as low as 1 mg dm^{-3} can give rise to public complaint (Easton, 1995). Textile processing wastewaters, typically with a dye content in the range 10–200 mg dm^{-3}, are therefore highly coloured and not acceptable (van der Zee, 2002). There is increasing public pressure for improvements in river water quality and regulations governing contaminant release in water streams from industry are becoming increasingly stringent. ETAD (Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers) was set up in 1974, as an international association, to minimise any possible negative impacts of colorants on human health and on the environment. ETAD involves currently 41 member companies, based in 15 countries worldwide, including China, Japan and some European countries.

A UK government agency was launched in 1989 as the National Rivers Authority (NRA). It had responsibility for managing water resources, investigating and regulating pollution and taking over flood controls and land drainage from the former ten regional water authorities of England and Wales. In 1990, the National Rivers Authority (now the Environment Agency) set colour standards for discharges from sewage treatment works. These are covered under the Water Resources Act of 1991, as amended by the Environment Act 1995, Protection of water environment. In April 1996, the NRA was replaced by the Environment Agency, having begun to establish a reputation for being supportive to wildlife projects and being tough on polluters. Following a judicial review of the authority in 1991, for allegedly failing to carry out its statutory duty to protect rivers and seas from pollution, river quality improved by 26% in the period 1993–1996 (www.environmentagency.com).

In 1999, legislation was introduced in the form of an Integrated Pollution Prevention and Control (IPPC) order for the UK and Europe. IPPC is a regulatory system that employs an integrated approach to control the environmental impact of certain industrial activities under the Pollution Prevention and Control (PPC) Act of 1999. Textile sector guidance, IPPC S6.05, has been produced by the Environment Agency

for England and Wales in collaboration with the Scottish Environment Protection Agency (SEPA) and the Northern Ireland Environment and Heritage Service (EHS). It covers the textile treatment processes based on the IPPC Reference Document on Best Available Techniques in the European textile commission.

1.1.3 Colour in textile effluent

Colour is the most problematic feature of the textile wastewaters. Colour has to be removed from the waterborne effluent before such water can be discharged into water bodies or on land. The primary concern of effluent, colour is not only important because of the toxicity aspects but also because of the undesirable aesthetic impact it has on receiving waters. Historically, the discharge of coloured waste waters from textile dyehouses has prompted more complaints to the water companies than any other form of pollution (Willmott *et al.*, 1998). Coloured wastewater is highly visible in very small concentrations, Pierce (1994) has pointed out that the human eye can detect concentrations of 0.005 mg dm^{-3} of reactive dye in water. Therefore, the presence of dye exceeding this limit would not be permitted on aesthetic grounds. The coloured compounds are not only aesthetically displeasing, also inhibit sunlight from penetrating into the stream and reduce photosynthetic reactions, representing serious problems to the environment. In some cases, dyes in low concentration are harmful to aquatic life (Eren and Acar, 2006; Karim *et al.*, 2006; Kwok *et al.*, 2003). The absorbance values of an average textile discharging effluent and river quality objectives (RQO) colour consent limits have been summarised, according to different wavelengths, as shown in Table 1.1.7.

The problem of coloured wastewaters comes from the release of the dyes to the environment. Dyes are discharged into environment via wastewaters from batch processes in both the dye-manufacturing and the dye-consuming industries. It has been stated that, from over 700,000 tons of dye produced annually worldwide, about 2% were discharged in aqueous effluents from manufacturing operations and 9%

from the coloration sector (Al-Ghouti *et al.*, 2003; Easton. 1995; Khehra *et al.*, 2006; Robinson *et al.*, 2001). That is, about 10-15% of the used dyes are discharged as wastewater (Tsui *et al.*, 2003). The U.S. Department of Commerce predicted a 3.5 fold increase in textile manufacturing between 1975 and 2020 (Wallace. 2001). This implies that the consumption of the dyes and the development of the new dyes would increase correspondingly.

Table 1.1.7 Colour of 'Average' Effluent Leaving a Dyehouse and of RQO Consents (Source: Southern, 1995; Waters, 1995)

Wavelength (nm)	Discharging effluent (Absorbance)	Consent limits (Absorbance)
Colour at 400 nm	0.96	0.025
Colour at 450 nm	1.11	0.015
Colour at 500 nm	1.37	0.012
Colour at 550 nm	1.49	0.010
Colour at 600 nm	1.56	0.008
Colour at 650 nm	0.87	0.005
Colour at 700 nm	0.13	0.003

The vast variety of dyes brings up complicated problems that have their origin in the coloured wastewaters. The Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists published the Colour Index, 3rd Edition, in 1971 and the Colour Index International (Electronic resource), 4th Edition, in 2002. The Colour Index lists about 28,000 commercial dye names, representing ~10,500 different dyes (van der Zee, 2002). Based on the application characteristics, the dyes used in the textile industries has been assigned as acid dyes, basic dyes, direct dyes, disperse dyes, mordant dyes and reactive dyes. The degree of the dye fixation on the substrate is one of the major factors in determining the discharge of a dye to the receiving waters. Although the dye manufacturers have made considerable

attempts at improving the levels of fixation, significant amounts of dyes are being released in the effluent, as shown in Table 1.1.8. It can be seen from Table 1.1.8, that up to 50% of reactive dye applied may be lost to the wastewaters.

Table 1.1.8 Estimated Degree of Fixation for Different Dye/Fibre Combinations (Source: Easton, 1995)

Dye Class	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

The problems brought about by the degradation of the dyes in solution are significant. Dyes are very difficult to treat due to their synthetic origin and their complex aromatic molecular structures. The structures are often constructed to resist fading on exposure to sweat, soap, water, light or oxidizing agents. These render the dyes towards being more resistant to the colour removal treatments (Banat *et al.*, 1996). Brightly coloured, water-soluble reactive dyes and acid dyes are particularly problematic, passing unaffected through conventional treatment systems at the sewage works and entering watercourses (Willmott *et al.*, 1998).

1.2 Reactive dye effluents

According to Colour Terms and Definitions (1988), a reactive dye is "a dye that, under suitable conditions, is capable of reacting chemically with a substrate to form a

covalent dye-substrate linkage". This unique characteristic of this dye class causes reactive dyes to have good properties in their wide range of brilliant shades. There is great versatility in the methods of their application and in their high wet fastness. There is also the increasing problem of "colour" in the textile discharging wastewaters.

1.2.1 The development and use of reactive dyes

It seems that all reviews on dyes begin with mention of mauveine, the first synthetic dye that was discovered in 1856 by William Henry Perkin (Venkataraman, 1952). This discovery stimulated chemists towards much more interest in synthetic dyes. Since then, over 100,000 synthetic dyes have been developed (Meyer, 1981; Willmott, 1997). The first reactive dyes for cellulosic fibres appeared on the market in 1956, introduced by ICI under the trade name Procion (Siegel, 1972), although many attempts had been made previously. Cross and Bevan (in 1895) recognised the fixation of a dye to a textile substrate via a covalent bond (Rys and Zollinger, 1989). In 1929, German workers introduced chloroacylamino groups into wool dyes. This work was developed by ICI. This led to the production of the first commercially available reactive dye, Supramine Orange R, brought out by I. G. Farben in the 1930s for the dyeing of wool (Bahrini, 1996; Shore, 2002). In 1953, Stephen and Rattee worked on sulphonated monoazo compounds containing dichlorotriazinyl-amino groups, for wool dyeing, leading to the possibility of applying these dyes to cellulosic fibres, in the presence of alkali. This led to the marketing of Procion reactive dyestuffs in 1956 (Bahrini, 1996; Holme, 2006). In 1957, the Cibacron monochlorotriazinyl (Ciba) and Remazol vinyl sulphonyl (Hoechst) ranges were introduced. Since that time, many important reactive-dye ranges have been developed for cellulosic fibres (Holme, 2006). Nowadays, reactive dyes form the second largest dye class in the Colour Index with respect to the amount of active

entries. About 600 of the ~1050 different reactive dyes listed are in current production (van der Zee, 2002).

Reactive dyes have a big market share and their use is still expanding due to their many advantages, such as availability in bright colours covering the complete colour gamut, good fastness properties, especially washfastness – due to the ease of covalent bonding, there being a reasonably wide range of application methods and the ease of dyeing by selection of suitable ranges (Tapley, 2002). It has been established that 33% of the worldwide production of colorants for cellulosic fibres by weight are reactive dyes. This figure rises to 43% if the reactive dyes are considered in terms of the cost of consumption (Yang and Yang, 2001). Reactive dyes have some disadvantages. These include incomplete fixation, the need for salt during application to promote dye uptake and extended washing-off times at the end of dye application to remove unfixed dye from the fabric. There is also a need to ensure that high wash fastness properties are actually achieved and that colour pollution in the discharging water is as low as possible.

1.2.2 The structure and chemistry of reactive dyes

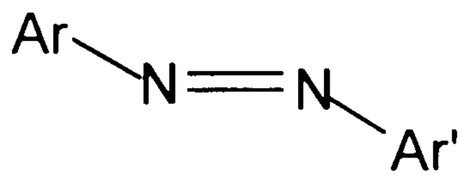
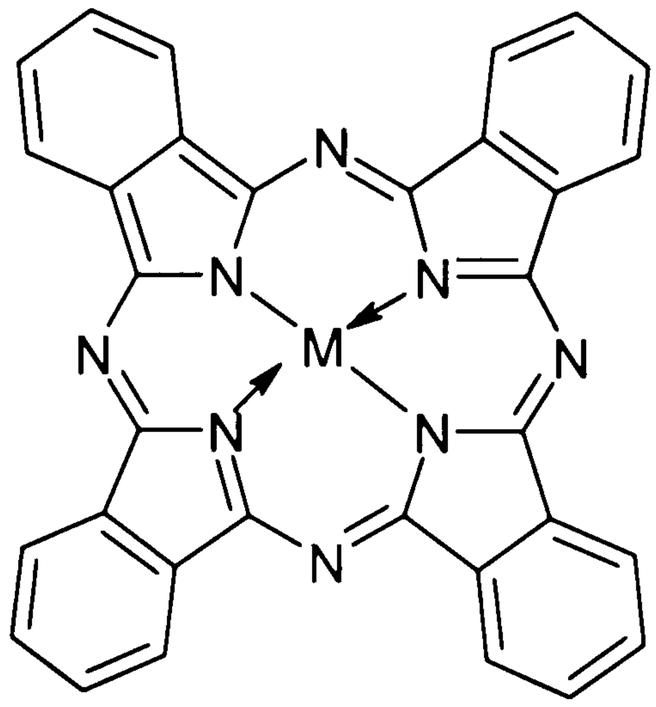
Reactive dyes are a unique class of dyes in that they covalently react with the substrate. Other dyes are retained through physical forces of attraction or containment. Dye molecules often contain specific functional groups that can undergo addition or substitution reactions with the –OH, –SH and $–NH_2$ groups that are present in textile fibres.

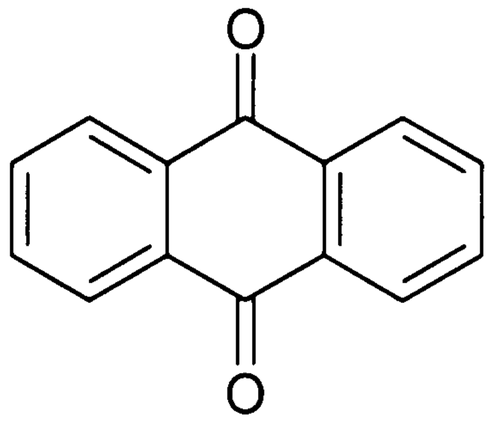
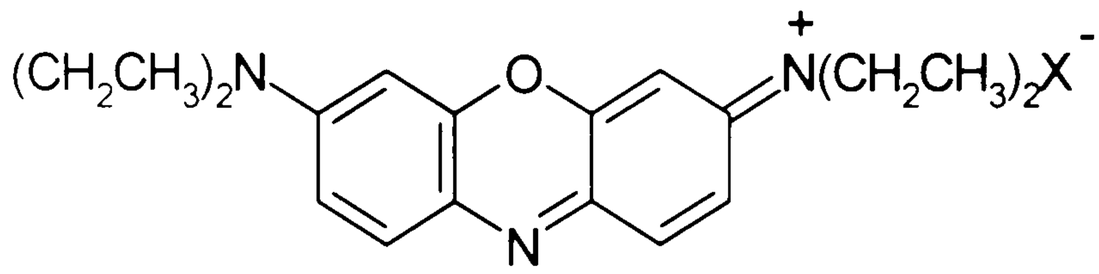
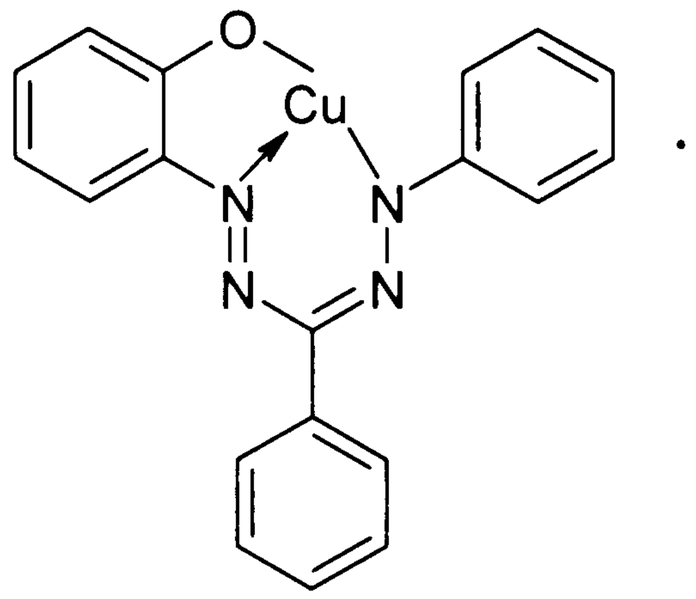
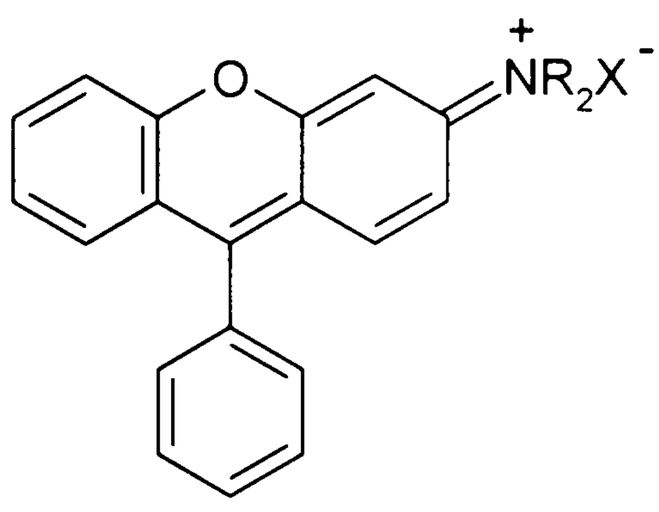
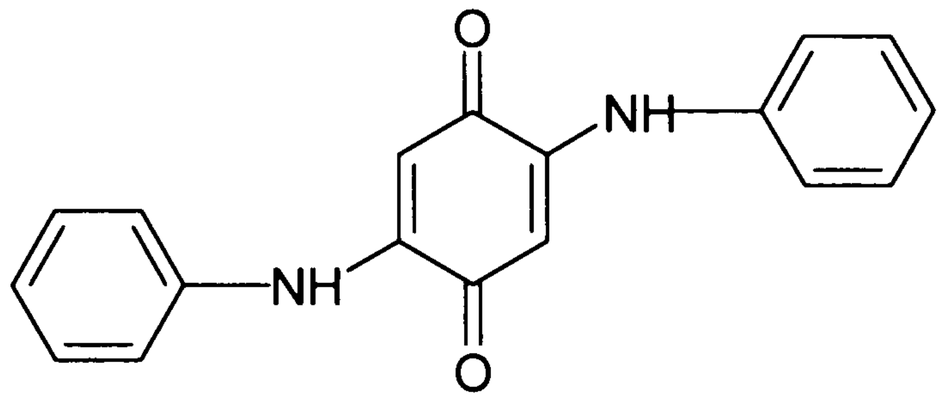
The characteristic structure of a reactive dye generally consists of water solubilising group(s), the actual chromogen, the bridging linker, electrophilic reactive group(s) and a nucleofugic leaving group (Zollinger, 2003).

The solubilising groups within reactive dyes are usually sulphonic acid groups, ranging in number between one and four. Generally, the substantivity of the dye decreases as a result of the increasing number of solubilising groups (Clark, 2002).

The chromogen in reactive dyes contributes to the colour and to much of the substantivity for the fibre. The major chromogens are azo, anthraquinone, phthalocyanine, formazan, oxazine, xanthene, and aminoketone (Shore, 2002: Colour Index, 3rd Ed.). About 81% of reactive dyes are azo chromogens, which include 15% of the metallised azo species (Shore, 2002). Generally, reactive yellows, reactive oranges and reactive reds are unmetallised azo dyes. Anthraquinones and phthalocyanines and their derivatives are particularly important for the greens and blues. Most of violet, dark blue, brown and greyish black shades are metal-complex monoazo dyes. Reactive dyes with a chromogen of formazan are mainly blue and green metal complexes (Wang, 1987). Reactive dyes can be classified according to the chromophore or to the reactive system. Table 1.2.1 lists the chromogens of reactive dyes, the percentage of reactive dyes in each chemical class and the chemical structure of each chromogen.

Table 1.2.1 Chemical Classes of Reactive Dyes (Source: Shore, 2002; Colour Index, 3rd Ed.)

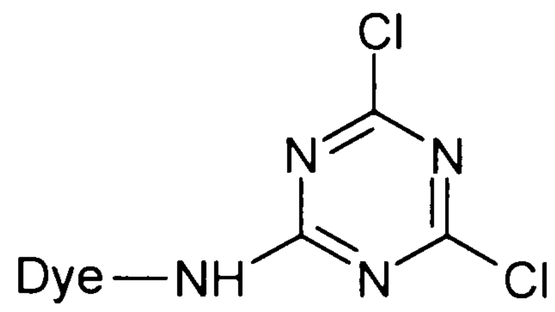
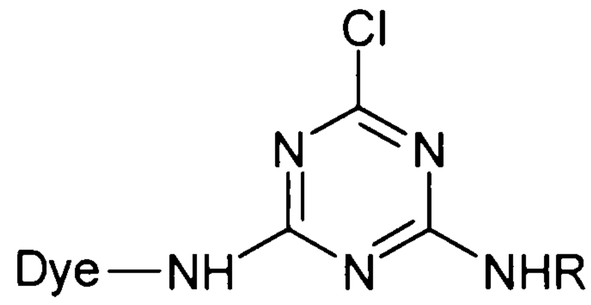
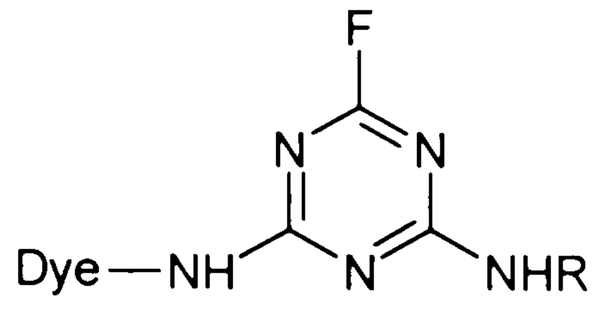
Chemical Class	**Chemical Structure of the Chromophore and Percentage of Reactive Dyes in Each Application Class (%)**	
Azo (including metallised azo)		(23%)
Phthalocyanine		(43%)

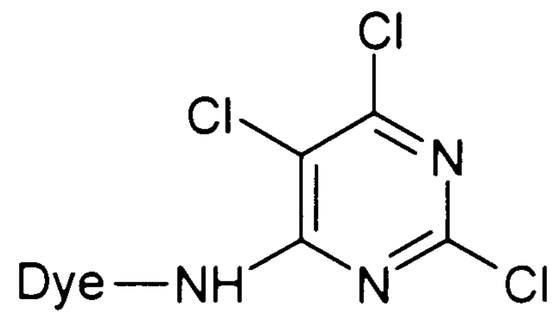
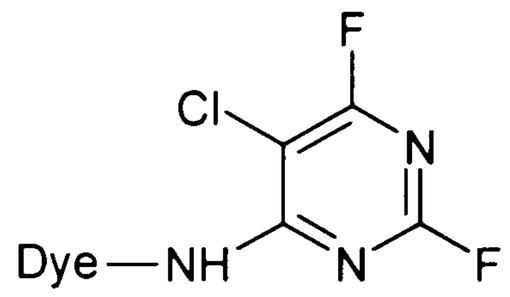
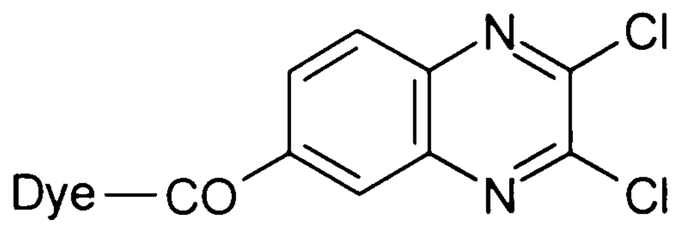
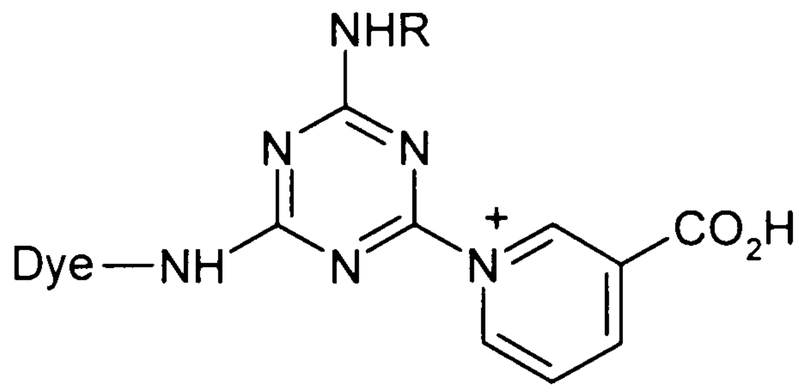
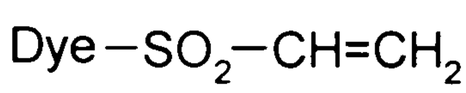
Anthraquinone		(6%)
Oxazine		(10%)
Formazan		(30%)
Xanthene		(2%)
Aminoketone		(3%)

The chromogen and reactive systems of the reactive dye are joined together through a bridging group, which is usually an amide (–NH–) group, although –NHCO– and –NH–SO_2– groups are also used (Willmott, 1997). Bridging groups can influence the reactivity of the reactive system, the consistency of the reactive dyeing process and the degree of fixation of the dye (Bahrini, 1996; Rys and Zollinger, 1989).

The reactive system contains a reactive group and a leaving group, which enables the dye to form a covalent bond with the fibre and often also contributes some substantivity. There are several reactive groups in commercial reactive dyes: dichlorotriazinyl, monochlorotriazinyl, monofluorotriazinyl, trichloropyrimidyl, difluorochloropyrimidyl, dichloroquinoxalinyl, nicotinyltriazinyl, and vinyl-sulphonyl, as shown in Table 1.2.2 (Broadbent, 2001). According to the reaction mechanisms, the reactive groups can be classified as halogenoheterocyclic reactive dyes (nucleophilic substitution), vinyl sulphone reactive dyes (nucleophilic addition) and other types of reactive dyes, depending on the type of reaction undergone, such as multiple addition/elimination reactions and single addition/elimination processes (Wang, 1987; Zollinger, 2003). Vinylsulphonyl dyes offer intermediate reactivity, lower substantivity but higher stability under acidic conditions than are possessed by other reactive dyes (Kamilaki, 2000).

Table 1.2.2 Typical Reactive Groups in Commercial Reactive Dyes (Source: Broadbent, 2001)

Reactive group	**Structure**
Dichlorotriazinyl	
Monochlorotriazinyl	
Monofluorotriazinyl	

Trichloropyrimidyl	
Difluorochloropyrimidyl	
Dichloroquinoxalinyl	
Nicotinyltriazinyl	
Vinylsulphonyl	

Many of the early reactive dyes had only one reactive group in the dyestuff molecule. To enhance the possibilities of reaction between the dyes and the fibres, many of the newer reactive dyes are bifunctional, containing two or more identical (homobifunctional) or different (heterobifunctional) reactive groups. Those two reactive groups can be linked to a same chromogen or to different chromogens. The first bifunctional reactive dye, Remazol Black B (C.I. Reactive Black 5), was commercially produced by Hoechst in 1957 (Kamilaki, 2000). This dye contains two sulphatoethylsulphone groups and is almost symmetrical in structure. A detailed list of reactive groups in reactive dyes of the major dye manufacturers was presented by Rys and Zollinger in "The Theory of Coloration of Textiles" in 1989. Aspects of

reactive groups of reactive dyes have been well featured in several reviews (Venkataraman, 1972; Wang, 1987; Rys and Zollinger, 1989; Aspland, 1997; Shore, 2002; Hunger, 2003 and Zollinger, 2003).

1.2.3 Reactive dyes in textile effluents

Reactive dyes have been and are considered to be the most environmental problematic compounds in textile dye effluents (García-Montaño *et al.*, 2006). Reactive dyes are relatively poorly substantive to fibres. Thus, a substantial amount of electrolytes such as NaCl, Na_2SO_4 and NaOH must be added in the dyeing process. These electrolytes are additionally polluting to the wastewater. The dyeing of cotton fibres with reactive vinylsulphone dyes is carried out using NaCl, NaOH and urea, added to the dye-bath (Kurbus *et al.*, 2003). Therefore, reactive dyes in dyeing wastewater have been identified as recalcitrant compounds since they provide high alkalinity in their use, contain a high concentration of organic materials and give strong colours in comparison with other dyes (Joo *et al.*, 2007).

In the dyeing of cellulosic fibres with reactive dyes, the addition of alkali to the dyebath not only promotes the formation of a covalent bond between the dye and the cellulosic substitute, it also causes the hydrolysis of the reactive groups in the dye. The reaction of the electrophilic group of the reactive dye with water (hydrolysis) competes with the fixation reaction (formation of a covalent bond between the dye and textile substrate). A large fraction, typically around 30%, of the applied reactive dye is wasted because of dye hydrolysis in the alkaline dyebath (Papić *et al.*, 2004). In the case of cellulose-based substrates, both bond formation reactions (heteroaromatic nucleophilic substitution and addition to an active alkene function) rely largely on the ionisation of the substrate with aqueous alkaline solution that, in turn, leads to competitive hydroxyl group attack and the destruction of the reactive moiety (Renfrew and Taylor, 1989). This process can be summarised as:

$$\text{Dye} + \text{Cellulose} + \text{Water} \xrightarrow{alkali} \text{Fixed Dye on Cellulose} + \text{Hydrolysed Dye in Solution}$$

After the reactive dyeing process is complete, 5 to 1500 mg of hydrolysed dye dm^{-3} effluent remains in the bath (Gottlieb *et al.*, 2003; Santhy and Selvapathy, 2006). Hydrolysed reactive dyes do not react with the substrate, though they may be temporarily adsorbed, only to be washed off in later stages. This residual amount is responsible for the coloration of the effluents and cannot be recycled. Dyeing auxiliaries or organic substances are non-recyclable and are also partly responsible for the high BOD/COD of the effluents, as discussed in Section 1.1.2. In the textile industry, in order to accelerate or to increase the fixation of reactive dyes onto the fibres, it is necessary to use high electrolyte concentrations in the dyebath to decrease the negatively charged barrier between the substrate and anionic dyes (Carneiro *et al.*, 2004). About 60 to 100 g of electrolytes, especially sodium chloride and sodium carbonate, per litre of effluent, are responsible for the very high saline content of the wastewater (Allègre *et al.*, 2006). All of the above factors are the basis of the fundamental problems that are associated with reactive dye effluents.

Conventional processes such as coagulation, flocculation and biological methods that have been adopted for decolorising effluents that contain dissolved reactive dyes are no longer able to achieve adequate colour removal. The mechanism for the removal of soluble dyes by an aerobic biological treatment may be straightforward biodegradation, but it operates mainly by adsorption of the dye on to the biomass and destruction of the dye in subsequent sludge processing. Most classes of water-soluble dyes are removed from effluent streams by bioelimination (Easton, 1995). Reactive dyes form a notable exception. Unlike other water soluble dyestuff classes, reactive dyes, in both their ordinary forms and in their hydrolysed forms are not easily biodegradable, with a maximum of 30% of breakdown being achieved, and only 10% being achieved on average. As a result, ~90% of the reactive dye that enters an

activated sludge sewage treatment works (STW) will pass through unchanged and be discharged to river (Pierce, 1994).

1.2.4 Reactive azo dyes

Azo dyes are characterised by the presence of one or more azo groups (–N=N–) having aromatic rings that are mostly substituted by sulphonate groups. These dyes form the largest chemical class of dyes that are used regularly for textile dyeing and for paper printing (Chen, 2002). As previously mentioned, there about 65% of reactive dyes are unmetallised azo dyes and 15% are metallised azo dyes.

Approaches that involve physical and/or chemical processes to remove the azo dyes, and therefore the colour, from the aqueous wastewater are often costly (Pearce *et al.*, 2006). However, the anaerobic reduction of azo dyes using microbial sludges can be an effective and economic treatment process for removing colour from reactive dye wastewaters (Wallace, 2001; Pearce *et al.*, 2003). Most azo dyes are non-toxic. However, the anaerobic degradation of reactive azo dyes involves not only a reduction of the azo bond but also the production of aromatic amines. The resultant aromatic amines resist further degradation and can be even more toxic than the dyes themselves (Brown and DeVito, 1993; Frijters *et al.*, 2006). Because of the toxic potential of the aromatic amines, further degradation of the dye compound is necessary if toxicity is to be eliminated or reduced (Gottlieb *et al.*, 2003). A high percentage of the intermediates of the azo dyes have been identified as carcinogens (Brown and DeVito, 1993). A proportion of these aromatic amines can be aerobically degraded (Razo-Flores *et al.*, 1997). The remaining proportion of the aromatic amines will autoxidise to products that are expected to be easily removed, e.g. by attachment to flocculants that can then be easily separated (Frijters *et al.*, 2006). A two stage mechanism for azo-reduction has been proposed, as shown below (Sponza and Işik, 2002):

$$2e^- + 2H^+ + (R-N=N-R') \rightarrow (R-NH-NH-R') \qquad (1)$$

$$2e^- + 2H^+ + (R-NH-NH-R') \rightarrow (R-NH_2) + (R'-NH_2) \qquad (2)$$

Here, it is speculated that the intermediate product of reaction (1) is an unstable colourless compound and the azo bond can become reformed upon oxidation, regaining some of the colour.

1.3 Metal-complex azo dye effluents

Two practical methods are used for the classification of the dyes on an international basis. One is based on the application characteristics. Dyes can be assigned as vat dyes, sulphur dyes, reactive dyes, direct dyes, disperse dyes, basic dyes, acid dyes and mordant dyes in the application ranges of textile colorants. Another classification route is according to the chemical structure of the dyes. The dyes then can be classified as azo dyes, anthraquinone dyes, phthalocyanine dyes, etc. Reactive dyes, as one of the textile application ranges were discussed in Section 1.2, with respect to their impact on textile wastewaters. A literature research of metal-complex azo dyes and their presence in textile effluents was undertaken with respect to their chemical classification as textile dyes.

1.3.1 General information concerning metal-complex azo dyes

Metal complexes have figured prominently in dyestuff chemistry developments. Metal-complex dyes are coordination compounds in which a metal ion is linked with one or more electron-donating (ion or molecule) ligands. Hunger (2003) has summarised some important events in the development of metal-complex dyes, as shown in Table 1.3.1.

Table 1.3.1 Summary of Major Events in the Development of Metal-complex Dyes (Source: Hunger, 2003)

Year	Milestones of metal-complex dye developments
1887	Synthesis of the first metallisable azo dye, Alizarin Yellow (R. Nietzk)
1891	Formation of the theory of coordination (A. Werner)
1893	Synthesis of the first metallisable 2,2'-dihydroxyazo dye (E. Bergmann and O. Borgmann)
1908	Elucidation of stereochemistry of metal complexes (A. Werner)
1912	Synthesis of the first pre-metallised 1:1 chromium complex (R. Bohn, BASF)
1920	Marketing of 1:1-chromium complex dyes as Palatin Fast series (BASF) and Neolan series (Ciba)
1927	First synthesis of copper phthalocyanine (H. de Diesbach and E. von der Weid)
1949	First water-soluble 2:1 chromium complexes lacking sulpho groups (G. Schetty, Geigy)
1962	Unsymmetrical 2:1 complex dyes containing only one sulpho group
1970	2:1 chromium complexes with two sulpho groups

The preparation of water-soluble, 1:1 chromium complexes of sulphonated dihyroxyanthraquinone dyes was achieved by Bohn in 1912. Similar complexes of azo mordant dyes in the same year, led to the introduction of the Palatin Fast (BASF) and Neolan (Geigy) ranges of 1:1 premetallised dyes for wool (Burkinshaw, 1990). In 1949, Geigy introduced the first example of a 2:1 chromium complex dyestuff having enhanced solubility, derived from a non-ionic solubilising group. This was the first member of their Irgalan range of dyestuffs. These are chromium complex

and cobalt complex dyestuffs containing methylsulphonyl groups, recommended for the fast dyeing of wool from neutral baths or from weakly acidic baths (Price, 1970).

Metal complexes comprise an important class of chromophores. Metal-complex azo dyes are covered in applications of acid dyes, solvent dyes, reactive dyes and direct dyes. Table 1.3.2 summarises the distribution percentage of metal-complex azo dyes in each application range, the percentage of metal-complex azo dyes in each application and the distribution in the hue sector of metal-complex azo dyes, in each application (Shore, 2002; Colour Index, 3rd Ed.).

Table 1.3.2 Distribution of Metal-complex Azo Dyes between Application Ranges (source: Shore, 2002; Colour Index, 3rd Ed.)

		Acid	Solvent	Reactive	Direct
% of Metal-complex azo		65	13	12	10
% in each application		31	18	15	5
Distribution in hue sector %	Yellow	31	12	2	—
	Orange	42	22	10	—
	Red	29	17	9	3
	Violet	44	11	32	9
	Blue	21	—	17	12
	Green	39	1	5	4
	Brown	13	24	43	1
	Black	46	37	55	8

1.3.2 Aspects of the chemistry of metal-complex azo dyes

Metal-complex dyes are strong complexes of one metal atom (usually chromium, cobalt, copper or nickel) and one or two dye molecules, respectively 1:1 and 1:2 metal complex dyes (van de Zee, 2002). Metal-complex-forming dyes need to have two or more electron-donating ligands, e.g., –OH, –COOH, $–NH_2$, or =N– (azo dyes).

The class may be divided conventionally into metal-complex azo dyes and metal-complex non-azo dyes, the former being the more important group (Price, 1970). The metal-complex azo dyes contain azo group(s) to form a part of the metallisable system, which is most commonly derived from azo dyestuffs having metalllisable substituents in at least one ortho position relative to the azo group (Price, 1970).

The most important metal-complex dyes are synthesised from 2,2'-dihydroxy- (a), 2-carboxy-2'-hydroxy- (b), 2-amino-2'-hydroxy-substituted azo dyes (c) and 2,2'-dihydroxy- or 2-carboxy-2'-hydroxy- azomethine (d) dyes, (Zollinger, 2003), as shown in Figure 1.3.1. The aromatic nuclei are, in most cases, benzyl, naphthyl, or pyrazolonyl derivatives, as well as open-chain keto/enol systems.

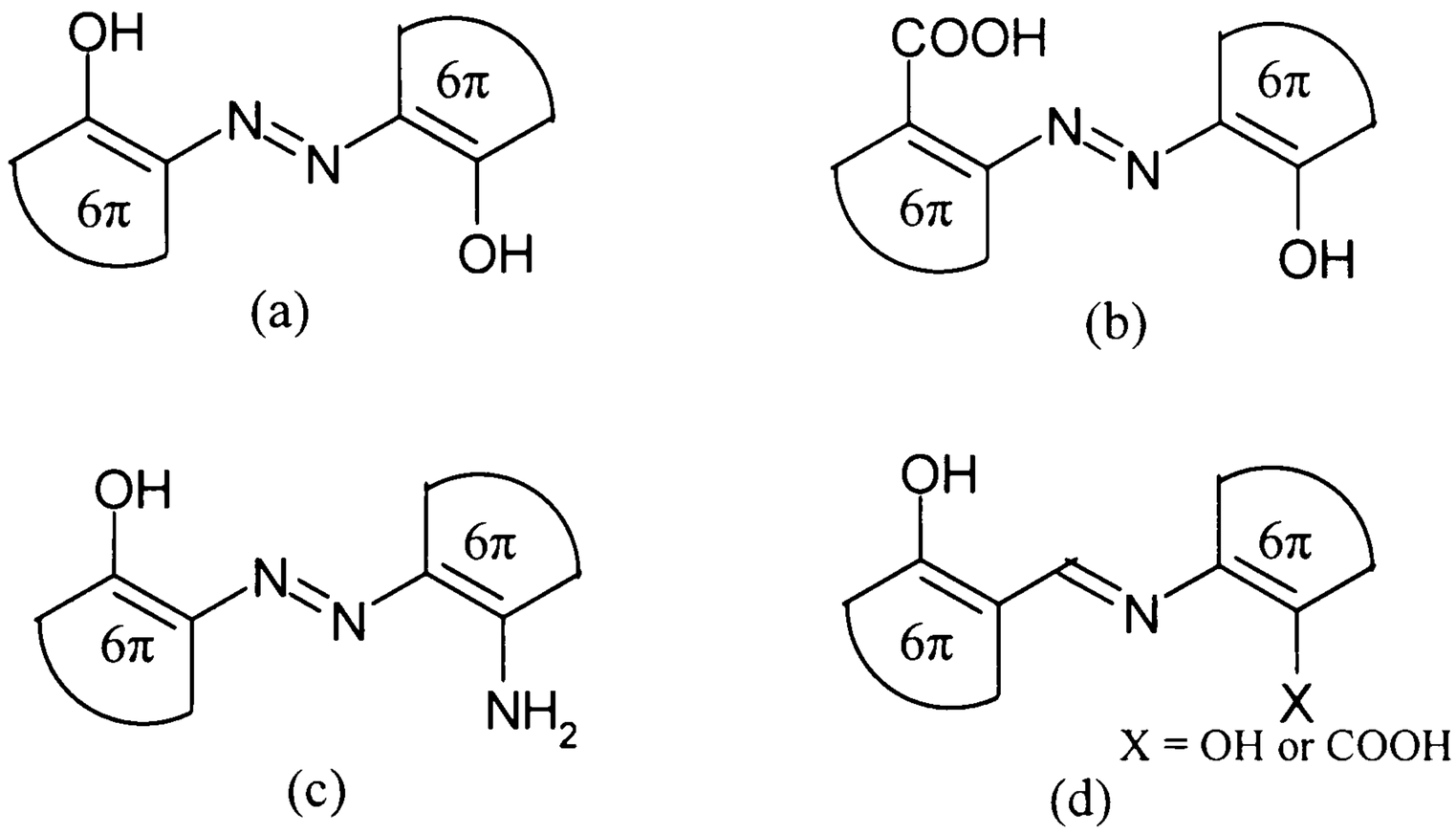


Figure 1.3.1 The Most Important Synthesised Types of Metal-Complex Dyes (Source: Zollinger, 2003)

Coordination of these trifunctional ligands (Figure 1.3.1) with a metal ion, involves the loss of a proton from each of the two substituted hydroxyl, amino or carboxylic groups of the azo or azomethine dye. This results in a structure comprising one five and one six-membered ring in the derived dye structure. The fundamental structure

of 1:2 metal-complex azo dyes are shown as (a) and (b) in Figure 1.3.2 (Burkinshaw, 1992).

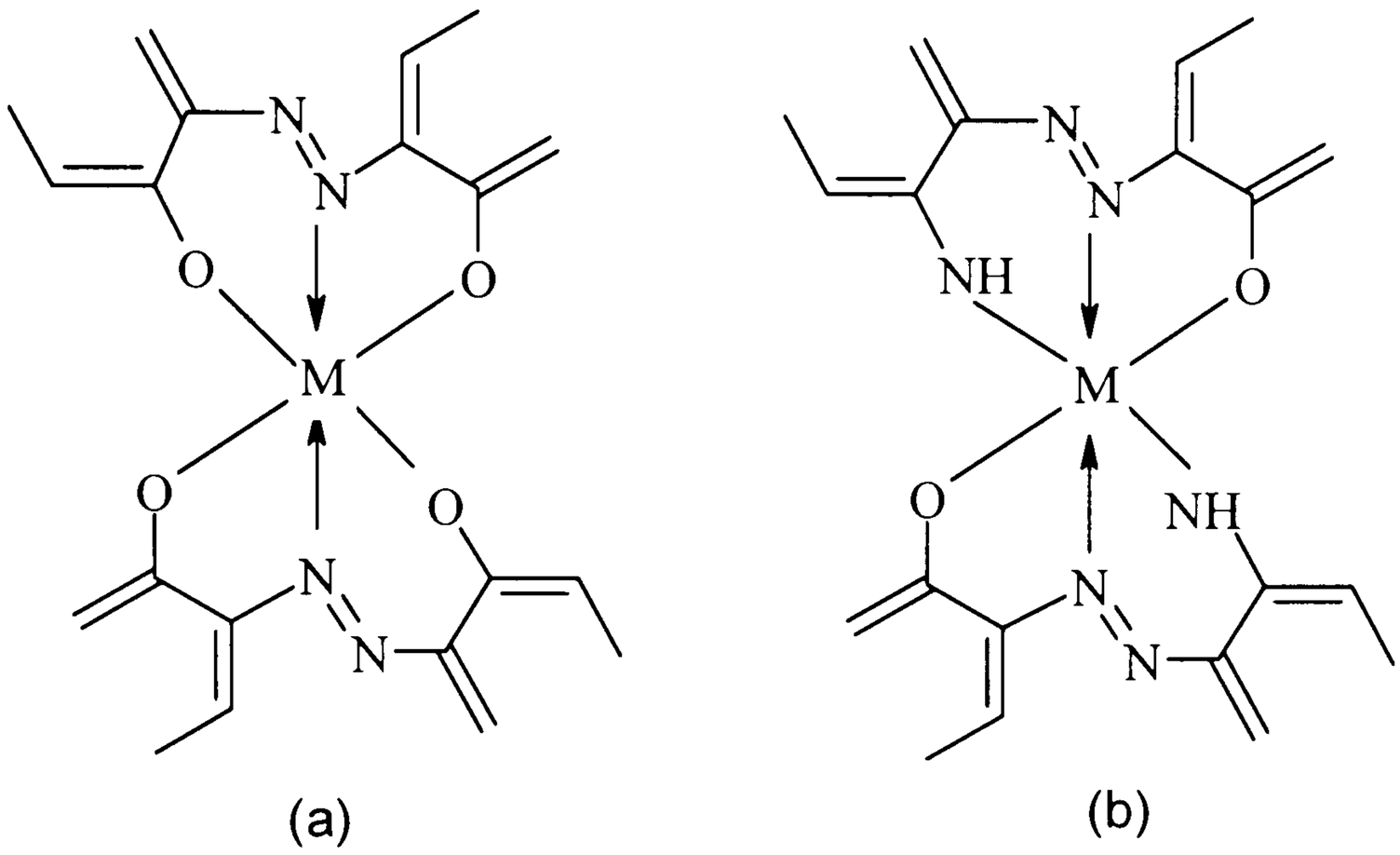


Figure 1.3.2 Molecular Structure of the More Common 1:2 Metal-Complex Azo Dyes

The metal ion used is most commonly trivalent and hexa-coordinate. There are three metal ions that are of importance in metal-complex azo dyes, copper ions, chromium ions and cobalt ions (Price, 1970; Hunger, 2003). The copper ion, Cu^{2+}, has a coordination number of 4, and can form complexes with two bidentate ligands, or with one tri-or tetradentate ligand. Metal ions with a coordination number of 6 can form 1:1 and 1:2 complexes with tridentate ligands, such as Cr^{3+} and Co^{3+}.

1.3.3 Metal-complex azo dyes in textile effluents

The metal-complex azo dyes in textile effluents contribute to two problems. One concerns addition to the heavy metals that exist in the environment and one concerns the presence of the residual metal-complex dyes with a "visible" problem. Therefore, two tasks need to be met in the treatment of textile wastewaters, removing heavy metals ions and decoloration.

Heavy metal ions can affect the growth, the extracellular enzyme production and the dye-decolorizing ability of the white rot fungi (Hatvani and Mécs, 2003). This is unfortunate, because metal-complex dyes are invaluable for their ability to produce photostable colours on polyamide and protein substrates (Edwards and Freeman, 2005). The metal complex dyes are mostly based on chromium ions and cobalt ions. Even a small concentration of chromium ions in the water supply is not acceptable (Mishra and Tripathy, 1993). Chromium ions that are present in the dye effluents sometimes cause myelotoxicological, carcinogenic, mutagenic, teratological, and other severe effects on animals (Gupta, 1990). Much research has been carried out on reducing/removing the metal ions from polluted water and wastewaters (Hatvani and Mécs, 2003; Myers *et al.*, 2000; Yu *et al.*, 2003). However, only a few studies of the decoloration of metal-complex azo dyes have been established (Gupta *et al.*, 1990; Van der Bruggen *et al.*, 2001; Vijaykumar *et al.*, 2006).

1.4 Methods of colour removal from textile effluents

The process of textile wastewater treatment is divided into a number of stages to deal with the waste effluents that contain a wide range of organic components and inorganic components that may or may not be soluble (Pearce, 2004). The unit processes in wastewater treatment plants are classified as preliminary treatment, primary treatment, secondary treatment, tertiary treatment and sludge treatment. The conventional operations involved in waste water treatment have been summarised by Willmott (1997), as shown in Table 1.4.1.

The preliminary stage involves flow balancing and reduces any variation in temperature or concentration, thereby helping to enhance the performance of the downstream processes. This preliminary stage is followed by primary, secondary and tertiary treatments that combine physical, chemical and biological processes to remove suspended solids, metal ions, organics, colour and odour from the

wastewater. The purpose of primary treatment is to remove settleable suspended solids.

Table 1.4.1 Conventional Wastewater Treatment Processes (Source: Willmott, 1997)

<table>
<tr><td>Preliminary treatment</td><td colspan="2">Screening
Grinding
Equalisation
Oil separation</td><td>Regulates and concentration</td><td rowspan="4">Sludge
↓
Incineration, landfill or ocean</td></tr>
<tr><td rowspan="2">Primary treatment</td><td>Chemical</td><td>Neutralisation
Coagulation
Flocculation</td><td>Removals nutrients and metals</td></tr>
<tr><td>Physical</td><td>Sedimentation
Floatation</td><td>Removals suspended solids</td></tr>
<tr><td>Secondary Treatment</td><td colspan="2">Activated sludge
Aerobic lagoon
Trickling filter
Anaerobic lagoon
Stabilisation basin</td><td>Removals soluble biodegradable organics</td></tr>
<tr><td>Tertiary treatment</td><td colspan="2">Ultrafiltration
Reverse osmosis
Carbon adsorption
Ion exchange
Sand filtration
Denitrification
NH3 stripping
Coagulation
Sedimentation</td><td>Removes non-biodegradable organic ions, macromolecules, nutrients, colour and odour</td></tr>
<tr><td>Disinfection</td><td colspan="2">Chlorination
Ozonation</td><td colspan="2">Eliminates living organisms</td></tr>
<tr><td>Ultimate disposal</td><td colspan="4">Receiving waters, Surface/Land, reuse</td></tr>
</table>

The primary treatment removes finer solids by settling and/or by flotation and may also lower the oxygen availability requirements of subsequent treatments. This stage may also involve chemical neutralisation of the effluent stream or enhance the removal of small suspended solid particles through coagulation and flocculation (Willmott, 1997).

The role of secondary treatment is to remove the colloidal material and the dissolved material remaining after the preliminary and primary treatment stages. Secondary treatment can include acid-cracking, chemical coagulation/flocculation – particularly using polyelectrolytes, biological degradation (aerobic and/or anaerobic), membrane separation (e.g. ultra-filtration), evaporation and/or incineration for wool scour effluents (HMIP, 1995).

Tertiary treatment is a "polishing" step. The tertiary operations include the removal of phosphorus compounds, nitrogen compounds, dissolved solids, residual organic compounds and coloured compounds (Willmott, 1997).

The final stages involve the discharge of the treated wastewater to the receiving water system and the removal of any resulting sludge for landfill. This method for the removal of colour of textile wastewaters falls into three categories, chemical methods, physical methods and biological methods (Slokar and Le Marechal, 1998; Chiavola, 2003). To achieve optimised colour removal, a combination of the different technologies is required, as much research into effective alternatives has shown (Bes-Piá *et al.*, 2002; Kim *et al.*, 2004; Chakraborty *et al.*, 2005; He *et al.*, 2007).

The wastewaters from textile dyeing facilities are difficult to treat satisfactorily because of their high compositional variability and their possible (likely) high colour intensity. The colour of water that is polluted with organic colorants reduces following the cleavage of –C=C– or –N=N– double bonds or following heterocyclic and aromatic ring cleavage. Also, the colour of such species can be reduced as the absorption of light by associated molecular shifts from the visible region to the

ultraviolet (UV) or infrared regions of the electromagnetic spectrum (Joshi and Purwar, 2004).

Table 1.4.2 Advantages and Disadvantages of Physico-chemical Treatment Processes for Colour Removal from Textile Wastewaters (Source: Pearce, 2004)

Physico-chemical processes		Advantages	Disadvantages
Advanced oxidative processes	Fenton's reagent	Effective decoloration of both soluble and insoluble dye	Sludge generation and reduced dye degradation in presence of salt
	Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 minutes), no reduction in COD
	Photochemical	No sludge production	Formation of by-products
	Irradiation	Effective oxidation of azo dyes	Requires a lot of dissolved O_2
Reductive processes	Zero-valent iron metal	Rapid reduction of azo dyes	Aromatic amines produced
Electrochemical processes		No addition of chemicals	High cost of electricity
Coagulation/ flocculation	Electrokinetic coagulation	Economically feasible	High sludge production
	Magnesium chloride	Effective decoloration of reactive dyes and industrial waste	Requires addition of alkali and produces sludge
Adsorption	Activated carbon	Good removal of a wide variety of dyes	Very expensive
Ion exchange		Regeneration: no adsorbent loss	Not effective for all dyes
Membrane	Reverse osmosis	Effective dye removal	High capital costs and sludge production
	Membrane filtration	Removes all type of dyes	Concentrated sludge production

1.4.1 Physico-chemical processes for the treatment of coloured wastewater

Some of the dyes that require disposal are of a non-biodegradable nature. Thus, direct biological treatment of the coloured effluents is neither appropriate nor effective (Torrades *et al.*, 2004). Consequently, chemical and/or physical techniques have to be applied to achieve complete or partial degradation of the dyes. These techniques could be coupled with amendable secondary biological treatment, if required. The most commonly used physico-chemical treatment techniques for the treatment of coloured wastewaters are summarised in Table 1.4.2 (Slokar and Le Marechal, 1998; Robinson *et al.*, 2001; Pearce, 2004; Husain, 2006; dos Santos *et al.*, 2007).

1.4.1.1 Advanced oxidative processes (AOPs)

Advanced oxidative processes are the more commonly used chemically based decoloration methods. Chemical oxidation degrades the dye in the dye-containing effluent by oxidation, resulting in aromatic ring cleavage of the dye molecules. Advanced oxidative processes use either the hydroxyl radical ($^{\bullet}OH$) or the primary oxidant and include systems such as combinations of O_3, H_2O_2 and UV radiation (Ledakowicz *et al.*, 2001). Such systems have significant advantages over conventional treatment methods since this type of chemical oxidation does not result in large amounts of either chemical components or biological sludge. Also, almost complete demineralisation of organic species is possible (Azbar *et al.*, 2004). However, chemical oxidative processes have disadvantages in the high cost of the reagents and of the energy sources. They also have problems in that partial oxidation could generate species that may be more harmful to the environment than those in the original effluent (Southern, 1995). The chemical decoloration methods may differ in the way in which hydrogen peroxide is activated. It has been shown that the advanced oxidative treatment of textile wastewaters enhanced the subsequent

biodegradation by increasing the bioavailability of the by-products to the degradative enzymes of the activated sludge. Also, a significant decrease in the inhibition of microbial growth has been observed (Ledakowicz *et al.*, 2001).

Fenton's reagent (Fe^{2+}/H_2O_2)

Oxidation using Fenton's reagent (hydrogen peroxide, activated with Fe^{2+} salts) is based on the generation of hydroxyl radicals from Fenton's reagent, when Fe(II) irons are oxidised by hydrogen peroxide and the hydroxyl radical, $HO^{\bullet}$ is produced. This free radical attacks organic compounds and decomposes them chemically (van de Zee, 2002; Meriç *et al.*, 2005). These redox reactions can be expressed as follows:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

$$RH + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O$$

$$R^{\bullet} + Fe^{3+} \rightarrow R^{+} + Fe^{2+}$$

$$R^{+} + H_2O \rightarrow ROH + H^{+}$$

The Fenton process occurs primarily in the first stage of hydroxyl radical oxidation. The treatment is very effective in the decoloration of textile wastewaters (Kang *et al.*, 2002). The Fenton process also offers advantages in the reduction of COD and in the reduction of toxicity (Slokar and Le Marechal, 1998). Compared with other oxidation processes, Fenton oxidation offers a cheaper means of coloured effluent treatment with advantages of easy operation, simplicity of the system and the possibility to work over a wide range of temperatures (Kim *et al.*, 2004). The Fenton's treatment process consists of a combination of chemical oxidation and chemical coagulation of organic compounds. The drawback of this process is that the excessive generation of sludge causes problems in sludge separation during the subsequent treatments (Robinson *et al.*, 2001).

Ozonation

Ozone is a strong chemical oxidant with a high oxidation potential (2.07 V), which allows ozone to degrade most organic compounds (Assalin *et al.*, 2004). Ozone may

attack pollutants via two different reaction pathways. These are direct ozonation by the ozone molecule and radical ozonation by hydroxyl free radical ($^{\bullet}OH$) species, generated in aqueous media (Shang *et al.*, 2006). The decomposition of ozone in water has been well interpreted by Staehelin and Hoigné (1985) and can be summarised by the following reaction sequence (Selcuk, 2005):

$$O_3 + OH^- \rightarrow HO_2^{\bullet} + O_2^{-\bullet}$$

$$O_3 + O_2^{-\bullet} \rightarrow O_2 + O_3^{-\bullet}$$

$$O_3^{-\bullet} + H^+ \leftrightarrow HO_3^{\bullet}$$

$$HO_3^{\bullet} \rightarrow HO^{\bullet} + O_2$$

$$O_3 + HO^{\bullet} \rightarrow HO_4^{\bullet}$$

$$HO_4^{\bullet} \rightarrow O_2 + HO_2^{\bullet}$$

$$2HO_4^{\bullet} \rightarrow H_2O_2 + 2O_3$$

$$HO_4^{\bullet} + HO_3^{\bullet} \rightarrow H_2O_2 + O_2 + O_3$$

Ozone and/or hydroxyl free radicals are able to break the double bonds (-N=N-, -C=C-) in the long conjugated chains of the dye molecule. They were also reported to be able to open aromatic rings (Leshem *et al.*, 2006; Assalin *et al.*, 2004). During the ozonation process, dyes lose their colour by the oxidative cleavage of the chromophores. The cleavage of double bonds and/or other functional groups will shift the absorption spectra of the molecule out of the visible region (Sevimli and Kinaci, 2002). Ozonation is usually coupled with other oxidants such as hydrogen peroxide (H_2O_2) or UV irradiation, to enhance the formation of hydroxyl radicals in the aqueous phase.

Ozonation can be used alone as a treatment process for the decoloration of textile wastewaters. Ozone converts the organic compounds into smaller (often biodegradable) molecules such as dicarboxylic acids and aldehydes. The reduction of the COD is, therefore, slight. Some of the ozonation products (especially the aldehydes) are highly toxic (van der Zee, 2002). The ozonation process has been

used more widely as a potential alternative for decoloration and improvements to the biological degradation of textile effluents (Alvares *et al.*, 2001; Mantzavinos and Psillakis, 2004).

One of the major advantages in using ozone is that it can be applied in the gaseous state and, therefore, does not increase the volume of wastewater and sludge. No chemical sludge is left in the treated effluent after the ozonation process is completed. The process has the potential to accomplish both colour removal and organic compound removal in one step (Soares *et al.*, 2006). It can also remove many toxic chemicals from wastewaters to facilitate the decomposition of detergents, chlorinated hydrocarbons, phenols, pesticides and aromatic hydrocarbons (Slokar and Le Marechal, 1998). Another reason for the increased interest in the ozonation process is that recent, improved ozone generation systems require lower energies to generate ozone. Consequently, costs are reduced (Assalin *et al.*, 2004).

The main disadvantage of the ozone oxidising species is their short half-life, typically 20 minutes. This time can be further shortened if dyes are present. Their stability is also affected by the presence of salts and by the pH and temperature. In alkaline conditions, ozone decomposition is accelerated and a careful monitoring of the effluent pH is required (Robinson *et al.*, 2001; Assalin *et al.*, 2004). It is known that continued ozonation generally leads to an increase in biodegradability of the treatment wastewaters (Liakou *et al.*, 2003). Ozonation is an effective approach to the destruction of a broad range of recalcitrant organic compounds and of inorganic compounds. However, their degradation in the form of complex mixtures results in a large organic load that remains, as represented by high COD and total organic carbon (TOC) residuals, even at high doses of ozone. Thus, processing may require elevated detection times, which will increase the cost of treatment considerably (Ledakowicz and Gonera, 1999; Arslan-Alaton and Seremet, 2004).

A comparison of the decoloration of an azo dye, Orange II, was carried out involving wet oxidation processes and ozonation. It was found that ozonation provides good results in decoloration but requires coupling with biological processes, due to the

persistence of intermediates that tend remain in the solution (Kurnik and Gobec. 2003). The decoloration of three dyes (C.I. Acid Blue 113, C.I. Reactive Red 241 and C.I. Basic Red 14) was studied by the means of ozonation. The wet oxidation process decolorised all of the solutions but the removal of TOC was not satisfactorily achieved (Faria *et al.*, 2005).

Photochemical AOPs

Photochemical AOPs are ultraviolet radiation (200–280 nm) driven, Advanced Oxidation Processes that are primarily based on the generation of powerful oxidising species either through direct photolysis or via photo-induced processes or photocatalysis (Stefan, 2004). The major photochemical AOPs involve UV/O_3 (Azbar *et al.*, 2004; Shu, 2006), photo-Fenton's (Aplin and Waite, 2000; Huang *et al.*, 2007), UV/H_2O_2 and UV/TiO_2 processes. The mechanisms of the UV/O_3 process and of the photo-Fenton's process are similar to those mentioned in the references to the use of Fenton's reagent and ozonation.

The UV/H_2O_2 process, using ultraviolet radiation (UV) in the presence of hydrogen peroxide is a very promising AOP technology for the decoloration of textile wastewaters. UV wavelengths in the range of 200–280 nm lead to cleavage of H_2O_2, with a mercury lamp emitting at 254 nm being the most commonly used (Bali, 2004). This process involves photolysis of H_2O_2 and yields reactive and non-selective hydroxyl radicals ($^{\bullet}OH$) by a direct process, with a yield of two radicals formed per photon absorbed, as shown in the scheme below:

$$H_2O_2 + hv \rightarrow 2HO^{\bullet}$$

The UV/H_2O_2 technique is able to eliminate residual dyestuffs in the dyeing wastewaters with high colour removal efficiency. It has been claimed that no sludge is produced (Shu *et al.*, 1994; Fung *et al.*, 2001; Georgiou *et al.*, 2002). It has been suggested that this process can be the basis of a suitable pre-treatment method for systems that are aimed at the complete decoloration and detoxification of effluents from textile dyeing and finishing processes, once the optimum operating conditions

are established (Ince and Gőnenç, 1997). An advantage of this process is that no additional disposal problems are involved after treatment. This is because the organic components in the water are almost completely destroyed while removing the colour (Kurbus *et al.*, 2003). However, in the decoloration of reactive azo dyes, it was reported that, with an increasing number of azo groups, the decoloration efficiency of the UV/H_2O_2 process decreased (Fung *et al.*, 2001). The kinetics and the mineralisation of reactive azo dyes using UV/H_2O_2 are well documented (El-Dein *et al.*, 2001; Neamtu *et al.*, 2002). The mechanisms are summarised in the following reactions:

$$Dye + hv \rightarrow Intermediates \qquad \text{(Equation 1)}$$

$$H_2O_2 + hv \rightarrow 2HO^{\bullet} \qquad \text{(Equation 2)}$$

$$Dye + HO^{\bullet} \rightarrow Products\ (P) \qquad \text{(Equation 3)}$$

$$P + HO^{\bullet} \rightarrow final\ products \qquad \text{(Equation 4)}$$

$$HO^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O \qquad \text{(Equation 5)}$$

It was reported that the direct photolysis of the dye (Equation 1) is not negligible but is very slow. The decoloration is enhanced by the presence of H_2O_2 due to the hydroxyl radicals that are generated during the photochemical reactions. According to Neamtu *et al.* (2002), the experimental results show a dependence of the decolorising degree on the initial concentration of the hydrogen peroxide. Excess H_2O_2 consumes the reactive $HO^{\bullet}$ radicals to give less reactive hydroperoxy ($HO_2^{\bullet}$) radicals, as illustrated above in (Equation 5). It was also found that the UV/H_2O_2 process is very dependent on the pH and on the dye concentration (Schrank *et al.*, 2007). The UV/H_2O_2 method has been successfully used for the decoloration of acid dyes, direct dyes, basic dyes and reactive dyes but has proven to be inadequate for the decoloration of vat dyes and some disperse dyes (Forgacs *et al.*, 2004).

In recent years, effort has been devoted to the study of photochemical processes using semiconductor oxides, *e.g.* the UV/TiO_2 system, for the treatment of textile wastewaters (Fernández *et al.*, 2004; Liu and Chiou, 2005; Alinsafi *et al.* 2007). The

photocatalytic TiO_2 process involves the formation of conduction-band electrons (e^-) and valence-band holes (h^+). The mechanisms are summarised below:

$$TiO_2 + hv \rightarrow [TiO_2\,(e^-) + (h^+)] \qquad \text{(Equation a)}$$

$$O_2 + TiO_2\,(e^-) \rightarrow TiO_2 + O_2^{\bullet -} \qquad \text{(Equation b)}$$

$$TiO_2\,(h^+) + H_2O \rightarrow TiO_2 + HO^{\bullet} + H^+ \qquad \text{(Equation c)}$$

$$TiO_2\,(h^+) + Dye \rightarrow Products \qquad \text{(Equation d)}$$

$$HO^{\bullet} + Dye \rightarrow Degradation\ intermediates \qquad \text{(Equation e)}$$

$$\rightarrow CO_2 + H_2O + Salts$$

It has been shown that the use of TiO_2 in such oxidations could provide decoloration and increases the biodegradability of the textile wastewater in colour removal treatments. It was observed that the effectiveness was strongly dependent on the chemical structure of the dyes (Alinsafi *et al.*, 2007). TiO_2 photocatalysis can be considered to be an emerging wastewater treatment technology. The key advantages include the lack of mass transfer limitations, the feasibility of operation under ambient conditions and the possible use of solar irradiation (Pekakis *et al.*, 2006). The photocatalytic removal of colour from textile effluents using TiO_2 suspension has been studied (Prieto *et al.*, 2005). Efficient decoloration of the effluent was established, though the flow rate affected the colour removal rate. Two reactive azo dyes C.I. Reactive Black 5 and C.I. Reactive Yellow 145 were degraded using the UV/TiO_2 system. It was concluded that the solutions of the two selected azo dyes were successfully mineralised using TiO_2 media that were coated on paper substrates to an optimal initial loading (Aguedach, 2005).

Other AOPs and the application of combinations of AOPs

Gamma irradiation has been applied to the decoloration and degradation of reactive dyes in aqueous solution (Şolpan and Güven, 2002). The dye solutions can be decolorised with addition of an oxidising agent and a decolorising agent. Ultrasonic

irradiation of synthetic dyes has been studied as a support to alternative advanced oxidation processes. Tezcanli-Guyer and Ince (2003) studied the degradation of reactive dyes and of basic dyes in deionised water solutions using 520 kHz ultrasonic irradiation. The degradation of azo dyes and oxazine dyes is indicated by colour decay, followed by destruction of the organic dye molecule by the ultrasonically generated hydroxyl radicals. Decoloration of six vinylsulphone reactive dyes, each in aqueous solution, was evaluated using ultrasound and ultrasound/H_2O_2 degradation (Vončina and Majcen-Le-Marechal, 2003). The application of ultrasound/H_2O_2 provided superior decoloration relative to the use of ultrasound or sonolysis alone.

An investigation of the decoloration of C.I. Reactive Black 5 using a combination of ultrasound and ozone has been made. The dye solution was decolorised but without satisfactory removal of TOC (He *et al.*, 2007). A drawback in the use of ultrasound options is the high energy input in the application. It has been claimed that the technique might also produce toxic compounds during the treatment (Husain, 2006).

The chemical oxidation of coloured wastewaters can be induced by the use of sodium hypochlorite (NaOCl). This reactive species attacks the dye molecule, initiating and accelerating the subsequent azo bridge cleavage. Treatments involving the use of NaOCl in the decoloration of reactive dyes required longer times, while solutions of metal-complex dyes remained partially coloured. Disperse dyes cannot be decolorised by this method. The use of this approach has become less frequent due to the impact of chlorine in the environment and the possible release of carcinogenic compounds (Slokar and Le Marechal, 1998; Robinson *et al.*, 2001).

A comparison of various advanced oxidation processes, (O_3, O_3/UV, H_2O_2/UV, O_3/H_2O_2/UV, Fe^{2+}/H_2O_2), in the colour removal from a polyester and acetate fibre dyeing effluent was undertaken (Azbar *et al.*, 2004). The use of the Fe^{2+}/H_2O_2 system seemed to achieve a satisfactory COD and colour removal performance and to be an economically viable choice. The application of advanced oxidative processes, prior to biodegradation, showed that the most advisable advanced oxidative process was that of ozonation combined with UV radiation or the

combination of $O_3/H_2O_2/UV$, giving a similar result to that which Ledakowicz and Gonera (1999) found. Processes such as UV/TiO_2, electro-Fenton (EF), wet-air oxidation (WAO) and UV/electro-Fenton (UV/EF) have been applied to the degradation of a solution of C.I. Reactive Red 120. The decoloration efficiency was observed to be in the order of WAO > UV/TiO_2 = UV/EF > EF, while the total organic carbon (TOC) removal was in the order of WAO > UV/TiO_2 > UV/EF > EF (Kusvuran *et al.*, 2004). Mantzavinos and Psillakis (2004) have reviewed developments and addressed some important aspects of chemical oxidation as a pre-treatment before the biological post-treatment of industrial wastewaters is undertaken.

1.4.1.2 Reductive processes

The primary degradation and decoloration of dyes with azo-based chromophores can be achieved by the reduction of the azo bond (-N=N-), using strong reducing agents such as sodium hydrosulphite, thiourea dioxide, sodium formaldehyde sulphoxylate and sodium borohydride (Delée *et al.*, 1998). Such processes have received less attention, perhaps due to cost factors, difficulties in handling and problems associated with the degradation by-products.

1.4.1.3 Electrochemical processes

Electrochemical treatments principally concern the passage of an electric current resulting in different chemical situations. The resultant reactions following/during the electrochemical treatment of wastewater are electro-coagulation, electro-flotation, electro-oxidation and electro-reduction (Hao *et al.*, 2000; Fongsatitkul *et al.*, 2006). Electrochemical decoloration could be achieved either by reduction or by oxidation. In the case of the decoloration of azo dyes, the chromophore of azo dyes is broken to generate two amino groups by the means of electrochemical reduction (López-Grimau and Gutiérrez, 2006). It has been proposed that dye molecule degradation by electrochemical oxidation occurs in two stages. In

the first stage, anodic electrolysis of water results in the formation of hydroxyl radicals that are adsorbed onto the active site of the electrode. In the second stage, the dye molecules are oxidised by the adsorbed hydroxyl radicals that are continually formed at the anode (Leshem *et al.*, 2006).

Electrochemical oxidation is claimed to be a relatively easy process to operate. The process does not require the use of extra oxidants or other chemicals, as is required by the chemical pre-treatment for industrial effluents (Mantzavinos and Psillakis, 2004). Electrochemical oxidation provides certain advantages that include almost complete decoloration, operation under moderate pH ranges, low final temperatures, significant COD and BOD reductions as well as no sludge formation (Sakalis *et al.*, 2005). However, in solutions such as textile wastewaters that typically contain chloride ions, the dominant process is through indirect oxidation by various forms of chlorine. The possibility of the production of high levels of total organic halides and the high cost of electricity contribute to the disadvantages for this technique (Robinson *et al.*, 2001; Sakalis *et al.*, 2005).

An electrochemical method that employs Ti/Pt as the anode and Stainless Steel 304 as the cathode, with NaCl as the electrolyte solution, has been used for the treatment of both total effluent and effluent from the dyeing stage only from cellulosic reactive azo dyeing processes. The data obtained showed that the COD, biochemical oxygen demand reduction and colour reduction exceeded 80% in almost all cases (Vlyssides *et al.*, 2000). Moreover, the electrochemical oxidation of dyes can also be assisted by photocatalysis (Osugi *et al.*, 2005; López-Grimau and Gutiérrez, 2006).

1.4.1.4 Coagulation/ flocculation processes

Advanced oxidation processes are effective in wastewaters that contain very low concentrations of organic dyes. Thus, significant dilution or balancing is necessary as a facility requirement. One difference between coagulation/flocculation processes and AOPs is that the coagulation and flocculation processes are in extensive use for the pre-, main or post- treatments. Also, full decoloration is possible by such

processes (Papić *et al.*, 2004). The use of the coagulation and flocculation in textile wastewater treatment is centred on the decoloration of the waste stream through the removal of dye molecules from the effluents, and not through a partial decomposition of dyes. A disadvantage that comes with this process is the high production of sludge.

Surfactants and dyes with high molecular weights can be successfully removed by a coagulation/flocculation processes followed by sedimentation, flotation and filtration, or use prior to biological treatment, respectively. The coagulation/flocculation method has been examined as a wastewater treatment technique for the decoloration of residual dyebath effluents after the dyeing of cotton/polyamide blends, using reactive and acid dyes, has been carried out. Almost complete decoloration has been achieved. Thus, the total organic carbon (TOC), chemical oxygen demand (COD), adsorbed organic halides (AOX), biochemical oxygen demand (BOD) and anionic surfactants loads were reduced and the biodegradability was increased (Golob *et al.*, 2005). Coagulant dose and coagulation pH are important factors in influencing the mechanism and the extent of coagulation. Also, the type and chemical structure of the dye plays an important role in the effectiveness of the coagulation process.

Chemical coagulation has been used to remove disperse dyes and reactive dyes. Compared with reactive dyes, disperse dyes have lower solubility, higher suspended solids concentrations and lower (soluble COD)/ (total COD) ratios. In one study, it was concluded that disperse dye dispersions were more easily decolorised by chemical coagulation than were reactive dye solutions (Kim *et al.*, 2004).

A polymer coagulant was synthesised from cyanoguanidine and formaldehyde and the product applied to the treatment of reactive dyes. C.I. Reactive Black 5, C.I. Reactive Blue 2, C.I. Reactive Red 2 and C.I. Reactive Yellow 2 were nearly completely removed from solution using the inorganic coagulants and the polymeric coagulants, in combination, within required pH range. The combination system resulted in much better colour removal than was provided by the inorganic coagulant

system alone. The colour removal rate from real dyeing wastewater was not as great it was in model wastewater systems, up to 62% (Joo *et al.*, 2007).

The processes often use conventional coagulants such as potassium alums and aluminium chloride as the coagulant of choice. The use of alum, aluminium chloride and other required chemicals for conventional water treatment may be considered as a high cost approach in some countries. There is also the possibility that a secondary pollution problem will arise because of the excessive chemical use (Sanghi *et al.*, 2006). Magnesium chloride has been shown to be an effective alternative to conventional coagulants. The magnesium based by-products can be removed from the precipitated sludge and recycled. Therefore, there is a reduction in the chemical costs and sludge disposal problems that are associated with the use of many conventional coagulants (Tan *et al.*, 2000; Gao *et al.*, 2007). Several natural, economic and environmentally friendly materials have been identified and applied in the coagulation and flocculation processes (Sanghi *et al.*, 2006).

Electrocoagulation has been successfully applied to colour removal from wastewaters (Alinsafi *et al.*, 2005). This procedure offers some advantages over the conventional coagulation methods. These include the fact that many of the processes can be simple and easy to operate, have no requirement of addition of chemicals, operate with a shorter reaction time, and also give a lower sludge production. However, several parameters such as, the shape and distance between electrodes, the cell potential, the conductivity, the pH and the fact that the system should be handled with care to optimise the efficiency of the process (Alinsafi *et al.*, 2005; Cañizares *et al.*, 2006), need to be given attention.

1.4.1.5 Adsorption treatments

Physical adsorption is widely used in the treatment of wastewaters because of its simplicity and the fact that the processes are economically feasible. Activated carbon has been used as an adsorbent extensively in textile wastewater treatment, either independently or coupled with biological degradation (Abu-Salah *et al.*, 1996). This

popularity is mainly due to the well-developed porous internal structure of the activated carbons and their various surface functional groups. Hence, they have remarkable adsorption properties. Activated carbon provides one of the more efficient techniques of dyehouse wastewater treatment, especially as a final polishing step before discharging or recycling the treated wastewater (Yang and Al-Duri, 2005). Despite its importance, activated carbon is an expensive material and is difficult to regenerate after use. This has led to a search for low-cost materials as alternatives. These include fly ash (Eren and Acar, 2006), coal-based bottom ash (Dinçer *et al.*, 2006), chitin (Akkaya *et al.*, 2007), narrow-leaved cattail (Inthorn *et al.*, 2004), coir pith activated carbon (Santhy and Selvapathy, 2006), titania-silica mesoporous materials (Messina and Schulz, 2006), and many other effective adsorbents (Hu, 1996; Tsui *et al.*, 2003; Dinçer *et al.*, 2006). These authors have shown that the approaches have considerable potential.

Chitosan offers a cheaper and better adsorbent in comparison with many adsorbents particularly in the adsorption of heavy metals and acidic dyestuffs from aqueous solution. Effective colour removal of C.I. Reactive Black 5 has been achieved with control of the chitosan dosage and the pH of the treating system at high temperatures (Sakkayawong *et al.*, 2005; Guibal and Roussy, 2007). Adsorbents, such as pine sawdust and polyamide-epichlorohydrin-cellulose polymers, have been applied successfully in removal of metal-complex dyes from aqueous solutions (Hwang and Chen, 1993; Özacar and Şengýl, 2005).

1.4.1.6 Ion exchange treatments

Ion exchange is a process in which ions are exchanged between a solution and an ion exchanger, an insoluble solid or gel. Typical ion exchangers are ion exchange resins, zeolites, montmorillonites, clays, and humus. Ion exchangers are either cation exchangers for positively charged cations or anion exchangers for negatively charged anions. Ion exchange is a reversible process and the ion exchanger can be regenerated or loaded by washing with an excess of the ions to be exchanged. Ion

exchange has not been widely used for the treatment of dye-containing effluents. A major disadvantage is the cost, since the system requires the use of organic solvents for the regeneration of the ion-exchanger which are expensive (Slokar and Le Marechal, 1998). Advantages of this method include the fact that there is no loss of adsorbent on regeneration, reclamation of solvent after use and the removal of soluble dyes (Robinson *et al.* 2001).

1.4.1.7 Membrane processes

The membrane techniques employed in textile effluent treatment involve one or more of reverse osmosis, ultrafiltration, nanofiltration and microfiltration. The classification of membranes is largely based on their pore size and their ability to retain solutes with different molecular weights cutoff (Hao *et al.*, 2000).

Reverse osmosis membranes are effective for most types of ionic compounds and produce a high quality of permeate. Decoloration and the elimination of chemical auxiliaries in dye-house wastewater can be carried out in a single step. (Allègre *et al.*, 2006). Reverse osmosis permits the removal of all mineral salts, hydrolysed reactive dyes and chemical auxiliaries. The problem involved is that the higher the concentration of salt, the more important the osmotic pressure becomes and, therefore, the greater is the energy that is required. High osmotic pressure differences limit the applicability of reverse osmosis membranes. Reverse osmosis membrane filtration has problems with fouling, which result in low fluxes and poor separation efficiency (Tang and Chen, 2002; Kim *et al.*, 2005).

Nanofiltration membranes have applications in the separation of the soluble dye residues, e.g. nanofiltration membranes are able to reject dyes and other organic molecules, while allowing NaCl and other monovalent salts pass through the membrane at the permeate stream (Koyuncu *et al.*, 2004). Compared with reverse osmosis membranes, nanofiltration membranes possess a looser structure. Because of this, nanofiltration membranes can be operated at a lower pressure, while still providing a high flux (Jiraratananon *et al.*, 2000).

Ultrafiltration has not been widely accepted by the textile industry since it makes direct water reuse impossible and requires further filtration by either nanofiltration or reverse osmosis (Tang and Chen, 2002). Ultrafiltration enables the elimination of macromolecules and particles but the elimination of polluting substances, such as colour is never complete. The possibility of retaining bifuntional reactive dyes by ultrafiltration was studied by Petrov and Stoychev (2003). Poly(acrylonitrile) and related poly(acrylonitrile)-co-polymeric membranes were used. It was found out that the membrane itself affects the process both through its pore size and the characteristics of the co-monomers in the polymer. The micellar-enhanced ultrafiltration of C.I. Reactive Black 5 and C.I. Reactive Orange 16 dyes in solutions containing cetylpyridinium chloride, using 10,000 thin-film composite membranes was studied. The reactive dyes were removed under a suitable operating pressure (Ahmad, 2006).

Microfiltration is suitable for concentrating colloidal dyes such as sulphur dyes, vat dyes and azoic dyes as well as subsequent rinsing baths while the auxiliary chemicals remain in the permeate (Muhammad *et al.*, 1997; Akbari *et al.*, 2006). Both ultrafiltration and microfiltration have an advantage in the low pressure required compared with reverse osmosis and nanofiltration. However, the drawback for these two methods is the insufficient quality of treated wastewater.

Filtration technologies require a high initial setup cost. These are overweighed by the significant cost saving achieved through reuse of permeate (Ahmad, 2006). One severe limitation of the application of filtration technologies is the disposal of the retentate or concentrate stream. At the moment, the retentate stream is disposed of by evaporation, incineration or discharging into the ocean (Tang and Chen, 2002). Kim *et al.* (2004) have applied a membrane bioreactor using white-rot fungi for the decoloration of reactive dye solutions. It was found that most of the dye was decolorised by the white-rot fungus, *Trametes versicolor*, while most of the TOC was removed by the reverse osmosis membrane process.

In all of the processes of colour removal, four facts need to be considered. These are:

1. The fact that the dye effluent will quite likely contain more than one chromophoric type. Thus, non-specificity of action of favoured.
2. Whatever treatment process is chosen, it must be capable of delivering rapid rates of decoloration so that large throughputs can be catered for. This allows for more efficient and more cost effective operations.
3. Biomass creation should be controllable (steady state in effect) and limited.
4. Adsorption related processes can still present problems that are associated with the handling and the treatment of coloured species. even though these species can be quite lightly bound to the support.

1.4.2 Biological processes for the treatment of coloured wastewater

Willmott *et al.*, 1998 have concluded that only biotechnological solutions can offer complete destruction of the dyestuff, with a co-reduction in the biological oxygen demand (BOD) and chemical oxygen demand (COD). Biological treatments in the decoloration of textile wastewaters offer potential advantages over the other treatments that were introduced in Section 1.4.1. Biological treatments tend to "destroy" the pollutants rather than concentrate them. In addition, the biotechnological approach makes feasible the efficient use of the limited development space available in many traditional dyehouse sites. As a result, less sludge is created. Lower operational costs and less health and safety risks are also advantages of biological treatments over other physical and chemical treatments. Moreover, microbes have the ability, in colour removal, of adaptation, i.e. they can be used in the treatment of a wide range of new synthetic compounds.

Many microorganisms including bacteria, fungi, yeast and algae are capable of decolorising a wide range of dyes via the appropriate processes. Applications of fungi and bacteria in colour removal of textile wastewaters are considered below.

1.4.2.1 Colour removal of textile wastewaters involving fungi

The most extensively studied dye-decolorising microorganisms are the white-rot fungi. *Phanerochaete chrysosporium* and *Trametes versicolor* are the major white-rot fungal strains that have been used that are able to degrade a variety of complex aromatic compounds, such as dyes, that usually would be recalcitrant to biodegradation, all the way to carbon dioxide (Aksu *et al.*, 2007). The ability of white-rot fungi to degrade a wide range of recalcitrant dyes has generally been associated with their production of ligninolytic enzymes that are highly oxidative and substrate non-specific. The lignin-modifying enzymes typically include lignin peroxidase, manganese peroxidase and laccase (Máximo and Costa-Ferreira, 2004).

Phanerochaete chrysosporium produces extracellular lignin peroxidase and manganese peroxidase during the degradation of lignin or xenobiotic compounds. Lignin peroxidase catalyses the oxidation of non-phenolic aromatic compounds directly. Manganese peroxidase oxidises Mn(II) to Mn(III), which is responsible for the oxidation of many phenolic compounds. Lignin peroxidase has higher oxidation potentials than manganese peroxidase (Harazono *et al.*, 2003). There are two major problems in the application of *Phanerochaete chrysosporium* in the treatment of real wastewaters (Rai *et al.*, 2005). Firstly, the lignin peroxidase, for dye degradation, is released by fungal cells following a strict secondary metabolism under either carbon or nitrogen limitation. This means that the presence of a carbon or a nitrogen nutrient in the industrial effluent would prohibit the release of this enzyme by the fungal cells. Secondly, dye degradation by lignin peroxidase consumes considerable amounts of hydrogen peroxide and veratryl alcohol as reagents. Although veratryl alcohol is a metabolite released by the fungus, a large amount of hydrogen peroxide, veratryl alcohol and lignin peroxidase may not be produced simultaneously in most industrial effluents (Rai *et al.*, 2005). Moreover, since fungi decolorise dyes in secondary metabolism and dyes cannot be utilised as the primary carbon source for fungi growth, a primary carbon source such as glucose is necessary in fungal dye

decoloration. In the absence of glucose, almost all fungi have no decoloration capability (Sanghi, 2006).

Compared to *Phanerochaete chrysosporium*, *Trametes versicolor* offers some distinct advantages. *Trametes versicolor* can produce the oxidative enzyme, laccase, an extracellular oxidase, even in the presence of nitrogen and carbon nutrients. More importantly, laccase can catalyse the oxidation of organic pollutants even in the absence of hydrogen peroxide or other secondary metabolites. Laccase needs O_2 to catalyse the oxidation reaction. Various redox mediators have been reported to play an important role in the oxidation phenolic compounds (Rai *et al.*, 2005). The single and combined effects of chromium (VI) and Remazol Black B reactive dye on the chromium (VI) and dye removal properties of adapted *Trametes versicolor* was investigated in a batch system, at different levels of chromium (VI) and dye. The growing white-rot fungus *Trametes versicolor* was shown to be capable of accumulating chromium (VI) and decolorising the Remazol Black B reactive dye, both singly and in combination in a batch process. Special nutrients and low pH (4-4.5) requirements for the optimum activity of the enzymes and long biodegradation time are the disadvantages of fungal bioremediation (Aksu *et al.*, 2007).

Intensive studies using white-rot fungi in the decoloration of textile wastewaters have been carried out. Unlike *Phanerochaete chrysosporium*, *Irpex lacteus* produces high levels of lignin-modifying enzymes in media that contain high nitrogen levels. It has been shown that when *Irpex lacteus* was grown in a N-limited medium, no lignin peroxidase was formed and low levels of manganese perosidases and laccase were found (Máximo and Costa-Ferreira, 2004). The selected reactive dyes were decolorised successfully with *Irpex lacteus*. 18 fungal strains that were able to degrade lignocellulosic material or lignin derivatives were tested with the azo dyes C.I. Reactive Orange 96, C.I. Reactive Violet 5 and C.I. Reactive Black 5. Only the white-rot fungal strains of *Bjerkandera adusta*, *Trametes versicolor* and *Phanerochaete chrysosporium* were able to decolorise all of the azo dyes (Heinfling

et al., 1997). The white rot fungus *Coriolus versiclolor* could decolorise the reactive dye Remazol Brilliant Violet to almost 90%. The fungal mycelia removed colour as well as COD up to 95% and 75%, respectively, in a batch reactor (Sanghi, 2006). Three types of reactive dyes, C.I. Reactive Blue 19, C.I. Reactive Blue 49 and C.I. Reactive Black 5 were decolorised with a membrane bioreactor which contained the white-rot fungus, *Trametes versicolor* KCTC 16781, and reverse osmosis membrance filtration were combined. The decoloration efficiencies were 99.1%, 76.9% and 100.0% respectively, for C.I. Reactive Black 5, C.I. Reactive Blue 19 and C.I. Reactive Blue 49 (Kim *et al.*, 2004). The white-rot fungus *Phanerochaete sordida* strain YK-624 was isolated from decayed wood obtained from a forest that has been found to secrete manganese peroxidase. The decoloration of an azo-reactive dye, C.I. Reactive Red 120, by a white-rot basidiomycete, *Phanerochaete sordida* strain YK-624, was investigated. 90.6% of dye was decolorised after 7 days. (Harazono *et al.*, 2003). Decoloration of 1:2 metal-complex azo dye, C.I. Acid Blue 193, was carried out by a white rot fungus *Cladosporium Cladosporioides* and 80% of colour removal was achieved (Vijaykumar, 2006).

1.4.2.2 Colour removal of textile wastewaters involving bacteria

Long growth cycles and moderate decoloration rates limit the performance of fungal decoloration systems. In contrast, colour removal of textile wastewaters using bacterial cells has been considered as a faster, more cost-effective and more environmental friendly technique. Such approaches have received extensive interest (Chen, 2002; Pearce *et al.*, 2003). The biological removal of the dyes from effluents from the textile and dyestuff manufacturing industries can be broadly classified into three categories: aerobic treatments, anaerobic treatments and combined anaerobic-aerobic treatments (Rai *et al.*, 2005). Comparisons between the aerobic treatments (processes using oxygen) and the anaerobic treatments (processes that do no use oxygen) are summarised in Table 1.4.3.

Table 1.4.3 Comparisons of Aerobic and Anaerobic Biolgical Treatments

Aerobic treatment	Anaerobic treatment
• Presence of O_2 • Widely used for effluent treatment • Colour is usually not destroyed • Might be adsorbed onto biomass • Degradation of organic pollutants	• Absence of O_2 • Production of methane, CO_2 and water • Requires less energy • Can break down compounds that aerobic process cannot • Toxicity of dye-degradation products • Systems operate effectively between 25 and 37°C

Aerobic treatment processes

The conventional activated sludge treatment of wastes offers effective and highly economic systems for reducing organic pollutants in wastewater. Much research has been conducted using activated sludges in the colour removal from textile effluents (Gulnaz *et al.*, 2006). Wang *et al.* (1999) have decolorised up to 94% of methyl violet waste water using cattle dung slurry as the source of activated sludge and coal powder as the biological carrier. However, the aerobic treatment of azo dye wastes has proven to be ineffective in most cases. None-the-less, this is often the typical method of treatment used today (Wallace, 2001). Textile dyeing wastewater cannot be readily degraded by the activated sludge process. This is because the structures of most commercial dye compounds are generally very complex. Many dyes are non-biodegradable due to their chemical nature and their molecular size. Thus, sludge bulking occurs (Kim *et al.*, 2004). The adsorption of various hues on activated sludge has been examined (Skelly, 2003). The highly soluble acid dyes and reactive dyes have a very low degree of adsorption. Basic dyes typically give high levels of adsorption. Disperse dyes undergo adsorption in the high to medium range and direct dyes have a high degree of adsorption.

The decoloration of dyes by bacteria can be due to adsorption to microbial cells and to biodegradation (Ren *et al.*, 2006). In adsorption, bacterial cells become coloured

because of the adsorbing dyes. In biodegradation they remain their original colour. Several bacterial cultures have been applied aerobically, in the decoloration of the dye-containing systems, as adsorbents in biosorption. Three Gram-negative bacteria: *Aeromonas sp.* (isolated from soil), *Pseudomonas luteola* (isolated from an activated sludge that had been acclimated to the dyeing wastewater) and *Escherichia coli*, and two Gram-positive bacteria: *Bacillus subtilis* and *Staphylococcus aureus* and a mixed biomass of activated sludge are the tested biosorbents have been reported to be capable of removing colour from aqueous solutions of the reactive dyes by bio-adsorption (Hu, 1996). Not all of the Gram-negative genera have the dye adsorption capability, the biosorption is specific. Moreover, the dead cells of Gram-negative bacteria have a higher specific adsorption capacity than occurs with the living cells.

Several bacterial strains have been reported to be capable of decolorising dyes aerobically, by reductive mechanisms (Chang *et al.*, 2001; Senan and Abraham, 2004; Kodam *et al.*, 2005; Olukanni *et al.* 2006; Ren *et al.*, 2006). A newly isolated *Aeromonas hydrophila* strain has been found that can decolorise triphenylmethyl dyes and anthraquinone dyes aerobically (Ren *et al.* 2006). Olukanni *et al.* (2006) have examined the aerobic decoloration of stimulated textile effluents of reactive dyes using eighteen organisms belonging to the genera, *Bacillus, Acinetobacter, Legionella, Staphylococcus and Pseudomonas* that were isolated from the textile effluents and six organisms belonging to the genus *Balics* that were isolated from a landfill site. The effluent-adapted stains have shown a better potential in bioremediative uses, with an average decoloration extent of ~40%, which is not very efficient. Some aerobic bacteria are able to reduce azo compounds and produce aromatic amines. Organisms such as *Bacillus subtillis, Aeromonas hydrophilia* and *Flavobaterium* can reduce aerobically, specific azo dyes (Kamilaki, 2000). Bacterial strains have been described that can aerobically decolorise azo dyes and have been considered by Banat *et al.* (1996) and by Stolz (2001).

Anaerobic treatment processes

The efficacy of anaerobic treatment applications in the colour removal from textile dye effluents has been many times demonstrated. Biological anaerobic treatment has shown superior abilities in colour removal compared to conventional physical/ chemical processes. Anaerobic biological processes provide rapid decoloration of a wide range of dyestuffs and also offer cost-effective and environmentally safe options. Anaerobic treatment is considered as the cheaper alternative because expensive aeration is omitted and problems with bulking sludge are avoided. There is a wide range of organisms that are able to reduce the dyes under anaerobic conditions.

Various organisms have been applied anaerobically to the decoloration of textile dye effluents. This has been shown for single cultures, mixed cultures and in the application of anaerobic sludge. Twenty selected azo dyes were decolorised completely by anaerobic granular sludge due to non-specific extracellular reactions (van der Zee *et al.* 2001). C.I. Acid Orange 7 and C.I. Reactive Black 8 have been decolorised by seed sludge under anaerobic conditions (Manu and Chaudhari, 2002). The feasibility of the anaerobic sludge use, in this context, has been also proven by Méndez-Paz *et al.* (2005). C.I. Acid Orange 7 can be degraded as a sole carbon source by methanogenic granular sludge. The decoloration rate becomes even higher when an additional carbon source is added. Song *et al.* (2003) have investigated some selected azo dyes for decoloration by *Rhodobacter sphaeroides.* The results have shown that the selected azo dyes can be decolorised by more than 90% under anaerobic conditions and with addition of a carbon source such as peptone. *Rodopseudomonas palustres* is capable of possessing azoreductase activity to decolorise azo dyes under strict anaerobic conditions (Liu *el al* 2006). *Aeromonas hydrophila* strain has been applied to the decoloration of azo dye solutions under anaerobic conditions. Rapidly effective decoloration of azo dye solutions has been observed (Ren *et al.* 2006). Handayani *et al.* (2007) have used *Enterococcus faecalis*

to treat the dye solutions of C.I. Acid Red 27 and C.I. Reactive Red 2. High decoloration efficiencies, ~95-100%, can be achieved. Xu *et al.* have isolated a highly efficient dye decolorising bacterium, *Shewanella decolorationis* S12, and have proven that the bacterium can remove more than 99% of selected anthraquinone dyes, in solutions, under anaerobic conditions (Xu *et al.*, 2005; Xu *et al.*, 2006). Willmott and Guthrie (1997) have isolated *Shewanella* strain J18 143. This culture has been effectively applied to the decoloration of reactive azo dyes and selected pigments (Willmott, 1997). More literature concerning the application of *Shewanella* species will be given in Section 1.4.3.

The decoloration of dyes using pure cultures is often impractical as most of the isolated cultures are dye specific. Hence, their application on a large scale is impractical. This is because to maintain the pure form in a wastewater treatment plant is difficult (Manu and Chaudhari, 2002). Mixed bacterial cultures offer better prospects of serving as an excellent biosystem for use in reactive dye removal from wastewaters, under anaerobic conditions (Çetin and Dönmez, 2006). A selected reactive monoazo dye (Everzol Red RBN), a reactive disazo dye (Drimaren Orange KGL) and a direct disazo dye (Everdirect Supra Yellow PG) have been decolorised effectively by a mixed anaerobic bacterial culture (Kapdan *et al.*, 2000). However, the mixed culture was not effective for all of the selected dyes. Khehra *et al.* (2005) have studied the decoloration of the effluents of six dyes, C.I. Acid Red 88, C.I. Acid Red 119, C.I. Acid Red 97, C.I. Reactive Red 120, C.I. Acid Blue 113 and C.I. Acid Brown 100 using single cultures of *Stenotrophomonas acidaminiphila*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Bacillus cereus*, and a mixed culture of these four isolates. The individual isolates only completely decolorised C.I. Acid Red 119 and C.I. Acid Brown 100. The consortium was able to decolorise all of the dyes at a higher decoloration rate compared to that achieved by the individual species. The anaerobic biological decoloration of an anthraquinone dye (C.I. Reactive Blue 19) and that of a phthalocyanine dye (C.I. Reactive Blue 21) using a suspended-growth, halophilic mixed culture fed with glucose, under hypersaline conditions, was

achieved to the extent of 87% and 37% respectively (Lee *et al.*, 2005). Information concerning other research that has been done, relating to the anaerobic decoloration of textile dye effluents by single and mixed bacterial culture can be found in the literature (Banat *et al.*, 1996; Delée *et al.*, 1998; Stolz, 2001; Pearce *et al.*, 2003; Pearce, 2004).

Anaerobic azo dye reduction has been studied intensively. It is now well documented that it is a non-specific, microbially mediated process. Under anaerobic (highly reductive) conditions, azo-reactive dyes undergo fission, yielding colourless aromatic amines, compounds that are generally biodegradable using aerobic biological methods (Georgiou *et al.*, 2005). Moreover, the process involved is an enzymatic reaction, which requires reducing agents (e.g. riboflavin), acting as electron shuttles between the dyes and cellular-reducing enzymes (Georgiou and Aivasidis, 2006). The exact mechanism of such azo dye reduction, whether occurring intracellularly or extracellularly, is still a subject of investigation. Intracellular azo dye reduction cannot be responsible for the conversion of all types of azo dyes, especially for sulphonated azo dyes that have limited membrane permeability. The current hypothesis is that azo dye reduction mostly occurs by extracellular or membrane-bond enzyme interaction/involvement (Stolz, 2001; dos Santos *et al.*, 2007).

Anaerobic-aerobic sequencing processes

The anaerobic reduction of azo dyes is generally a more efficient process than is aerobic degradation. The intermediate products, aromatic amines, produced by the reduction process are colourless. However, they are very resistant to further degradation under anaerobic conditions. The amines produced by the reduction of the azo dyes are much less toxic to the methanogens than are their parent compounds, but are still potentially carcinogenic (Forgacs *et al.*, 2004). Under aerobic conditions, the mineralisation of these amines can be accomplished. Complete decoloration can, thus, be obtained by a sequenced anaerobic/aerobic treatment (Delée *et al.*, 1998). A sequential anaerobic packed column reactor and an activated sludge unit have been

applied continuously for treatment of a textile wastewater. Over 85% decoloration and about 99% of COD removal efficiencies have been obtained (Kapdan and Alparslan, 2005). A sequential anoxic-aerobic bioreactor was designed for the decoloration and degradation of C.I. Acid Red 88, a sulphonated, azo group-containing textile dye that is commonly used in the textile dye industries (Khehra *et al.*, 2006). The aromatic metabolic intermediates that were produced under anaerobic conditions have been transformed to non-aromatic metabolites during aerobic treatment. 98% of colour removal and 95% COD removal have been achieved. This two-stage biological treatment has been carried out in the colour removal of C.I. Reactive Black B in synthetic wastewaters (Libra *et al.* 2004; Mohanty *et al.* 2006). It was confirmed that the dye molecules were reduced and that aromatic amines were generated under anaerobic conditions. The aromatic amine metabolite was partly removed in subsequent aerobic treatment.

1.4.3 Metabolic diversity of *Shewanella* species

Members of the genus *Shewanella* have been studied extensively since 1931 with regard to a variety of topics of relevance to both applied and environmental microbiology (Venkateswaran *et al.*, 1999). A notable feature of members of this genus is their ability to use a variety of different electron acceptors such as manganese oxide minerals and iron oxides, uranium ions, thiosulphate ions and elemental sulphur (Pearce, 2004). Most of the recognised species of the genus have the potential to mediate the co-metabolic bioremediation of halogenated organic pollutants and to reduce heavy metal ions (Xu *et al.*, 2005). *Shewanella putrefaciens* was one of the earlier recognised microorganisms that enzymatically reduced U(VI) (Abdelouas *et al.*, 1998). This microorganism uses U(VI) as the sole electron acceptor. It has been demonstrated that in both aerobic and anaerobic cultures. *Shewanella oneidensis* cells are capable of reducing chromate ions in aqueous solutions (Neal *et al.*, 2002). *Shewanella oneidensis* MR-1 is a facultative.

gram-negative non-fermenting γ-proteobacterium whose respiratory versatility involves its ability to reduce a variety of compounds, including iron(III), manganese (IV), nitrate ions, thiosulphate ions, sulphite ions, trimethylamine N-oxide, dimethyl sulphoxide, fumarate ions, uranium(VI) ions, technetium (VII) ions, chromium (VI) ions, elemental sulphur and carbon tetrachloride (Bencheikh-Latmani *et al.*, 2005). *Shewanella oneidensis* MR-1 may be useful for the in-situ immobilisation of uranium ions and chromium ions in sub-surface environments because of its ability to reduce toxic and soluble hexavalent uranyl ions (UO_2^{2+}) and chromate ions (CrO_4^{2-}) to less soluble and less toxic forms [U(IV) mineral and Cr(III) mineral]. *Shewanella decolorationis* S12, from the activated sludge of a textile-printing wastewater treatment plant, can decolorise the anthraquinone dye, Reactive Brilliant Blue K-GR, with flocculation first (Xu *et al.*, 2006). Pearce (2004) has reviewed the reduction of a range of electron acceptors by various *Shewanella* strains that had been reported in the literature. Table 1.4.4 gives the research that has been done, on this theme, in recent years.

Table 1.4.4 Reduction of a Range of Electron Acceptors by Various *Shewanella* Strains: Review of Studies Reported in the Literature

Electron acceptors	Organisms	Comments	References
Uranium (VI)	*Shewanella putrefaciens* 200R; *Shewanella oneidensis* MR-1	U(VI) can be used as the electron acceptor instead of Fe(III) by some of the *Shewanella* strains. U(VI) can be reduced to U(IV).	Abdelouas *et al.* (1998); Bencheikh-Latmani *et al.* (2005)
Ferric (III)	*Shewanella putrefaciens* 200R; *Shewanella putrefaciens* CN32; *Shewanella* sp. HN-41	Microbal reduction of structural Fe(III) to Fe(II) using Fe(III) as electron acceptor. Fe(III) can be used as a sole electron acceptor in the systems.	Bonneville *et al.* (2006); Jaisi *et al.* (2007); Lee *et al.* (2007)

Fe(III)-cyanide complex Prussian Blue	*Shewanella alga* strain BrY	The insoluble and colloidal Fe(III)-cyanide complex. Prussian Blue can be reduced and utilised as electron acceptor by the dissimilatory iron-reducing bacteria *Shewanella alga* stain BrY.	Jahn *et al.* (2006)
Chromium ions (VI)	*Shewanella oneidensis* MR-1	*Shewanella oneidensis* can reduce Cr(VI) containing mineral to Cr(III) ions under anaerobic cultures where the Cr(III)is the sole terminal electron acceptor. The final microbial pathway for Cr(VI) reduction is intracellularly localised.	Daulton *et al.* (2007)
Selenite	*Shewanella oneidensis* MR-1; *Shewanella* sp. HN-41	The bacteria can reduce Se(IV) to its elemental form Se(0) under anaerobic conditions. The Se(0) deposits are located extracellularly.	Klonowska *et al.* (2005): Lee *et al.* (2007)
Tellurite	*Shewanella oneidensis* MR-1	Tellurite can be reduced from Te(IV) to its elemental form of Te(0) under anaerobic conditions. The Te(0) precipitates intracellularly.	Klonowska *et al.* (2005)
$AuCl_4^-$	*Shewanella algae*	The intracellular recovery of insoluble gold from soluble $AuCl_4^-$ ions using the anaerobic bacterium *Shewanella algae* with H_2 as the electron donor. The $AuCl_4^-$ ions cannot be used as sole electron acceptor. Fe(III) need to be added.	Konishi *et al.* (2006)
Naphthylamine-sulphonic azo dye amaranth (C.I. 16185)	*Shewanella decolorationis* S12	Under anaerobic conditions, azo dye amaranth (C.I. 16185) can be reduced by strain S12. It has been established that the mechanism is one of azo dye reduction via the reductive cleavage of the azo bond to form corresponding aromatic amines.	Hong *et al.* (2007)

Reactive azo dyes	*Shewanella putrefaciens*	The bacterium acts as a biocatalyst that is capable of removing colour from the industrial textile wastewater. The colour removal process involved the transfer of electrons from the cell to the dye molecule, via a dye reductase to produce significantly less coloured solution-containing amines.	Willmott (1997); Kamilaki (2001); Pearce (2004)
Pigment dispersions	*Shewanella* strain J18 143	The study shows the potential of the *Shewanella* species in reducing extracellular insoluble azo/ketohydrozone pigments.	Pearce *et al.* (2006)
Anthraquinone dye	*Shewanella decolorationis* S12	Reactive Brilliant Blue K-GR, an anthraquinone dye, can be decolorised by *S. decolorationis* S12 under anaerobic conditions. Lactate ions were used as the carbon source for the dye decoloration.	Xu *et al.* (2006)

1.4.3.1 *Shewanella* strain J18 143

Coloured wastewater from dye works is a problem in all industrialised countries. It is not surprising, therefore, that scientists work worldwide on dyework wastewater remediation. As discussed in previous sections, the decoloration of azo dye effluents is a more difficult task than that of the decoloration of several other dye types. Azo dye decolorising *Shewanella* cells were isolated from dyework wastewaters by Willimott and Guthrie in Leeds (UK, 1997), von Canstein et al. in Kyoto (Japan, 2002) and Xu et al. in Guangzhou (China, 2005), or from river water by Kishimoto et al. (Japan, 2002) in Mara (Group meeting minutes). These *Shewanella* strains were all distinct from each other, suggesting that the decoloration of azo dyes, i.e. the reduction of the xenobiotic azo bond, is a common feature of the genus *Shewanella* and is most likely based on the same mechanism.

Shewanella strain J18 143 is a gram-negative, facultative anaerobic bacterium that was isolated from an industrial textile effluent (Willmott, 1997). The bacterium shows an ability to decolorise selected reactive dyes on a solid medium, under aerobic conditions. The presence of a supplemental carbon source is essential to sustaining bacterial growth and to obtaining an acceptable level of decoloration. Decoloration of synthetic textile effluents, containing a commercial Remazol Black B dye has been achieved under anaerobic conditions by Willmott (1997), using free cells and immobilised cells of *Shewanella* strain J18 143. These studies were continued further by Kamilaki (2000) and by Pearce (2004). *Shewanella* strain J18 143 cells are also capable of using the azo/ketohydrazone chromophores in solid coloured species, such as pigment dispersions and dyed cotton fibres and fabrics, as electron acceptors for anaerobic respiration (Pearce, 2004; Pearce *et al.*, 2006).

1.5 Immobilisation of microbial cells and applications in the decoloration of textile wastewaters

An early industrial application of immobilised microbial cells was realised in 1969 in the continuous production of L-aspartic acid (Chibata and Tosa, 1983). Since then, immobilised microorganisms have been the subject of increased interest, and have been developed rapidly for application in wastewater treatments (Kudlich *et al.*, 1996).

Immobilised cells have offered several advantages over the free cells in industrial and analytical applications (Chibata and Tosa, 1983; Gil, 1983). The stability of immobilised cells can be improved, relative to the stability of the non-immobilised equivalents. A better control of reaction is achievable. The immobilised cells can be easily removed from the reaction system and, under appropriate conditions, can be used repeatedly and flexibly to make the continuous processes practical. Less space is required for reaction using immobilised cells and a high cell concentration can be introduced in the system. In this way, expensive enzymes can be used economically

for industrial applications. Furthermore, the immobilisation supports can provide a protective environment for the cells against denaturants, proteolysis and reduced susceptibility to contamination, so that the cells are less affected by any fluctuations in the characteristics of their surroundings (Kamilaki, 2000; Kulshrestha and Husain, 2006).

1.5.1 Methods for immobilisation of microbial cells and enzymes

Methods for immobilising microbial cells and enzymes have been reported in a number of texts (Chibata and Tosa, 1977; Gil, 1983; Klein and Wagner, 1983; Willmott, 1997).

Adsorption of microbial cells and enzymes on insoluble supports is one of the more inexpensive and simple immobilisation techniques available (Khan *et al.*, 2006). The immobilisation can be achieved by mixing an aqueous solution/dispersion of an enzyme, or a cell suspension with a support material for a given period of time, after which the excess biological species is washed away (Willmott, 1997). The adsorption of microbial species can be carried out in two ways, by physical adsorption onto the carrier or by ionic binding to the support (Gil, 1983). This method may be disadvantaged by the leakage of enzymes from cells owing to autolysis of cells during continuous enzyme reaction (Chibata and Tosa, 1977). Another disadvantage is the fact that diffusion problems may occur.

Entrapment and encapsulation of microbial cells or of enzymes have also been used in physical immobilisations. Entrapment may be achieved by entrapping the microbial cells or enzymes into a polymer matrix where they are physically restrained (Chibata and Tosa, 1977). This method involves the formation of a highly cross-linked network-polymer. The drawback of entrapment is that such systems may easily suffer from problems of diffusion. The entrapment of microorganisms can be achieved by entrapping microoganisms in gels, such as agar gels and alginate gels (Chen and Cheng, 1996; Lu *et al.*, 1996; Chen and Liu 2007). In the method of

encapsulation, the microbial cells or the enzymes are immobilised in microcapsules that have either permanent or non-permanent semipermeable membrane character (Gil, 1983).

Chemical methods involve the formation of covalent bonds between the support and the biological species (cells, enzymes). For chemical methods, cells can be immobilised by reaction with a reactive polymer without pre-activation, by activation of a polymeric support by conversion of a functional group of the polymer and by activation of the polymer using selected coupling agents. However, covalent immobilisation of enzymes often resulted in the loss of activity (Khan and Husain, 2007).

More detailed information relating to the techniques of immobilisation of microbial cells and enzymes is given in many texts (Chibata and Tosa, 1977; Gil, 1983; Klein and Wagner, 1983; Willmott, 1977).

1.5.2 Applications of immobilised microbial cells and enzymes in the decoloration of textile wastewaters

Oxspring *et al.* (1996) have studied the decoloration of Remazol Black B solutions by an immobilised microbial consortium. In one system, the decoloration of the dye solutions by gravel-substratum immobilised cells was built with an upflow anaerobic filter. The ability of the microbial consortium to decolorise the selected dye was enhanced by the developed immobilisation system.

Juang *et al.* (2002) have studied the immobilisation of enzymes onto glutaraldehyde-cross-linked chitosan beads via the method of physical adsorption. The process was carried out by keeping the cross-linked chitosan beads contacted with the enzymes, in a shaker, for 18 hours at 4°C. The activity and the stability of the immobilised enzymes or biological cells were affected by the immobilisation substrates. Therefore, the utilisation of the immobilisation support or the modification of the

chosen substrate is an important factor in the applications of the immobilised biological species.

Peralta-Zamora *et al.* (2003) and Domínguez *et al.* (2005) have immobilised laccase, which was produced by *Trametes versicolor*. The immobilised laccase was used in the decoloration of aqueous solutions of selected textile dyes. Chemically modified silica, with imidazole groups, and alginate beads were efficient adsorption supports in the studies. However, in most cases, the enzymatic decoloration was masked by a simple adsorption process of the dyes onto the support surface. Thus, colour removal was not totally by biological degradation.

Khan and Husain (2007) have studied the decoloration and removal of selected reactive textile dyes from polluted wastewaters and dyeing effluents using a system of celite-bound, potato polyphenol oxidase. The immobilised potato polyphenol oxidase was found significantly to be more effective in decolorising the individual dye solutions of complex mixtures of dyes than was the equivalent soluble enzyme.

Lu *et al.* (2007) have also applied immobilised laccase in dye decoloration studies. The ligninolytic enzymes of laccase were immobilised by entrapment in alginate-chitosan microcapsules. The immobilised laccase was less active and had a lower substrate affinity than did the free enzyme. However, the stability and the reusability of the enzymes were enhanced compared with the performance of the free enzyme equivalents.

Pseudomonas luteola cells have been immobilised by entrapment in alginate-silicate sol-gel beads for the decoloration of the azo dye, C.I. Reactive Red 22 (Chen and Lin, 2007). The immobilised cells gave a lower specific decoloration rate than did the free cells. The advantages of the immobilised cells were that the cells became less sensitive to changes in pH values and were more stable during repeated use. Also, they showed better thermal stability during storage and operation than did the free cells.

1.5.3 The options of support materials for immobilising microbial cells and enzymes in the decoloration of textile wastewaters

Various materials have been explored and investigated for their suitability for use as cell immobilisation substrates. The support materials need to meet the following criteria: non-biodegradability, high mechanical stability, high diffusivity, simple immobilisation procedure, high biomass retention, minimal attachment of other organisms and, preferably, a low cost price (Leenen, 1996).

The use of a cheaper source of enzyme and support will definitely minimise the cost of immobilisation of the enzyme. There is a need to provide a suitable system for the treatment of large volume of wastewater in batch processes as well as in continuous reactors. Microbial cells that have been immobilised on natural polymers (alginates, carrageenan, chitosan or cellulose derivatives) have found more applications during continuous operation in different processes.

Several studies have employed different materials for the attachment procedures. These materials include polyurethane foams (Mielgo *et al.*, 2002), textile strips and straw (Kaluskar *et al.*, 1999), nylon cubes, polystryrene foams and stainless steel sponges (Rodríguez-Couto *et al.*, 2004). Some studies have been conducted with cells that have been immobilised on sodium alginate or calcium alginate. Calcium alginate, as an immobilisation substrate for the biological cells, offers some advantages. These include biodegradability, hydrophilicity, presence of functional carboxylic groups, natural origin, low density, mechanical stability and stability over an experimental pH range of 3.0-8.0 (Domínguez *et al.*, 2005). Chitosan has been used as a support material for enzyme immobilisation because of its many suitable characteristics such as hydrophilicity, biocompatibility, biodegradability and anti-bacterial properties. It appears to be economically attractive since chitin is the second most abundant biopolymer in nature, after cellulose. Highly swollen chitosan beads, prepared from cuttlefish wastes, have been cross-linked with glutaraldehyde or glyoxal. Enzymes have been immobilised onto these glutaraldehyde-cross-linked

chitosan beads (Juang *et al.*, 2002). An attempt at the direct immobilisation of polyphenol oxidase on an adsorbent, Celite 545, has also been made (Khan *et al.*, 2006).

Cellulose and cellulose derivatives have been used in many applications as a support in microbial cell immobilisation studies. Many workers have chosen cellulose or cellulose derivatives as a support because they are inexpensive, have good physical shape, are chemically stable, and are resistant to microbiological contamination (Chen and Tsao, 1977). Also, cellulose has three hydroxyl groups on each anhydro-glucose unit providing high versatility as well as a large capacity for the immobilisation of a desired substance.

Willmott (1997) has regenerated cellulose beads as an immobilisation support for the bacterial cells of *Shewanella* strain J18 143 and had applied this system to the removal of colour from reactive dye effluents. Sawayama *et al.* (1998) have used cellulose beads for immobilising bacterial cells of *Rhodobacter capsulatus* in water treatment applications. The use of the cellulose beads as the immobilisation support has been applied due to the inherent high porosity of the beads and also their improved mechanical strength. Direct immobilisation of peroxidases on activated diethylaminoethyl cellulose from ammonium sulphate-fractionated proteins of bitter gourd has been investigated (Kulshrestha and Husain, 2006).

The application of chemically modified cellulose in the immobilisation of microbial cells and of enzymes is very effective. The modified cellulose possesses advantages of cellulose or cellulose beads. Furthermore, the modified cellulose can provide a higher capacity for forming a large number of bonds. Also, surface adsorption can be readily achieved. Gil (1983) has studied the immobilisation of the proteins, enzymes and cells onto graft copolymeric substrates, since systems containing hydroxyl groups are good as supports for the immobilisation of biocatalysts. Grafting copolymerisation is a very convenient way of modifying the surface properties of polymers. Graft copolymerisation onto cellulose can be achieved in several ways based on radiation treatment and on chemical reaction routes. Grafting via chemical

reaction has offered a major approach to the creation of active groups on the cellulose copolymer or derivatives. The grafting of monomers or mixture of monomers can greatly increase the number of reactive groups that can be used for cell immobilisation. The extent of incorporation of hydrophilic groups or of hydrophobic groups can be controlled. creating a microenvironment that could improve the viability and stability of the biological materials that have been immobilised.

2 Materials and Methods

In this chapter, the experimental procedures of the decoloration of coloured textile wastewaters using *Shewanella* strain J18 143 are described.

Section 2.1 describes the materials and the methods that were used for the preparation of the immobilisation substrate, that involved for the preparation of the bacterial cells of *Shewanella* strain J18 143 and that used for the immobilisation of the bacterial cells. A graft cellulose copolymer was prepared as the substrate for immobilisation. The prepared bacterial cells, in a cell suspension, were immobilised onto the substrate by three methods namely "growing-in", coupling and adsorption.

Section 2.2 gives the materials and the methods used for the decoloration of Remazol Black B dye solutions using the immobilised cells. Several standards were established for a comparison of the decoloration effect.

Studies relating to the colour reduction of metal-complex azo dyes, using free resting cells of *Shewanella* strain J18 143, are given in Section 2.3. Four Irgalan series of metal-complex azo dyes were chosen. These are commercially named Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL.

Factors such as the dye concentration, the temperature and the pH, which might have influence on the colour reduction of the metal-complex azo dyes, are concentrated in Section 2.4.

Several analytical techniques, gravimetric analysis, scanning electron microscopy, UV-visible spectrophotometry, particle size analysis and protein assay, were used as described in Section 2.5. These methods were employed to investigate the modification of the cellulose, the cell preparations, the colour reduction of the coloured compounds and the cell concentrations used in the decoloration systems.

2.1 Materials and methods for the preparation of the immobilisation substrate and for the immobilisation of biological cells

All of the chemicals used were of analytical grade, obtained from Sigma-Aldrich Gillingham (Dorset, UK), unless otherwise stated.

2.1.1 Materials and methods for the preparation of the cellulosic copolymer-based immobilisation substrate

Modified cellulose (a grafted cellulose copolymer) was chosen as immobilisation substrate. This section covers the preparation and the characterisation studies of the cellulosic graft copolymer.

2.1.1.1 Materials used in preparation of the cellulosic graft copolymer

The cellulose-g.co-monomer system was chosen as the immobilisation substrate for biological cells in the decoloration systems involving immobilised cells. The cellulose used was in the form of bleached cotton fabric, and was supplied by Whaleys (Bradford) Ltd. (Bradford, UK). The cellulose was cut into strips (20 cm × 30 cm). The strips were washed with detergent and then water before being dried at 60 °C, thoroughly. All of the dried strips were kept in desiccator before use.

Potassium persulphate was chosen as the initiator. The monomer grafted onto the cellulose was methacrylic acid. The methacrylic acid was purified by passage through an aluminium oxide column before use. Removal of inhibitor was ensured by spectrophotometric analysis of the eluent monomer.

2.1.1.2 Grafting copolymerisation

The pre-washed cellulose strips were cut into smaller pieces, about 2 cm × 5 cm.

Initial swelling of the cellulose was achieved by stirring the cotton fabric slivers at 28 °C in distilled water for 2 hours, followed by soaking in the system overnight.

The graft copolymerisation treatment of the swollen cotton fabrics was carried out in 500 cm^3 round-bottomed reaction vessels.

The swollen cotton fabrics were stirred gently for 60 minutes in an aqueous solution containing an appropriate amount of the initiator (0.075 mol dm^{-3}), potassium persulphate, at 28 °C. After one hour of impregnating of the pre-treated fabrics, the purified monomer, methacrylic acid (0.5 mol dm^{-3}), was added into the reaction system, dropwise. The treatment was allowed to proceed at 60 °C in a water bath, with the use of thermostirrer and condenser, for 5 hours. At the end of the desired reaction period, the fabrics were washed thoroughly with distilled water in Soxhlet extractor for 6 hours, to dissolve any produced extractable homopolymer. Afterwards, the grafted fabric was washed with distilled water again, and dried at 35 °C in the oven until a constant weight was achieved. The process is shown in Figure 2.1.1.

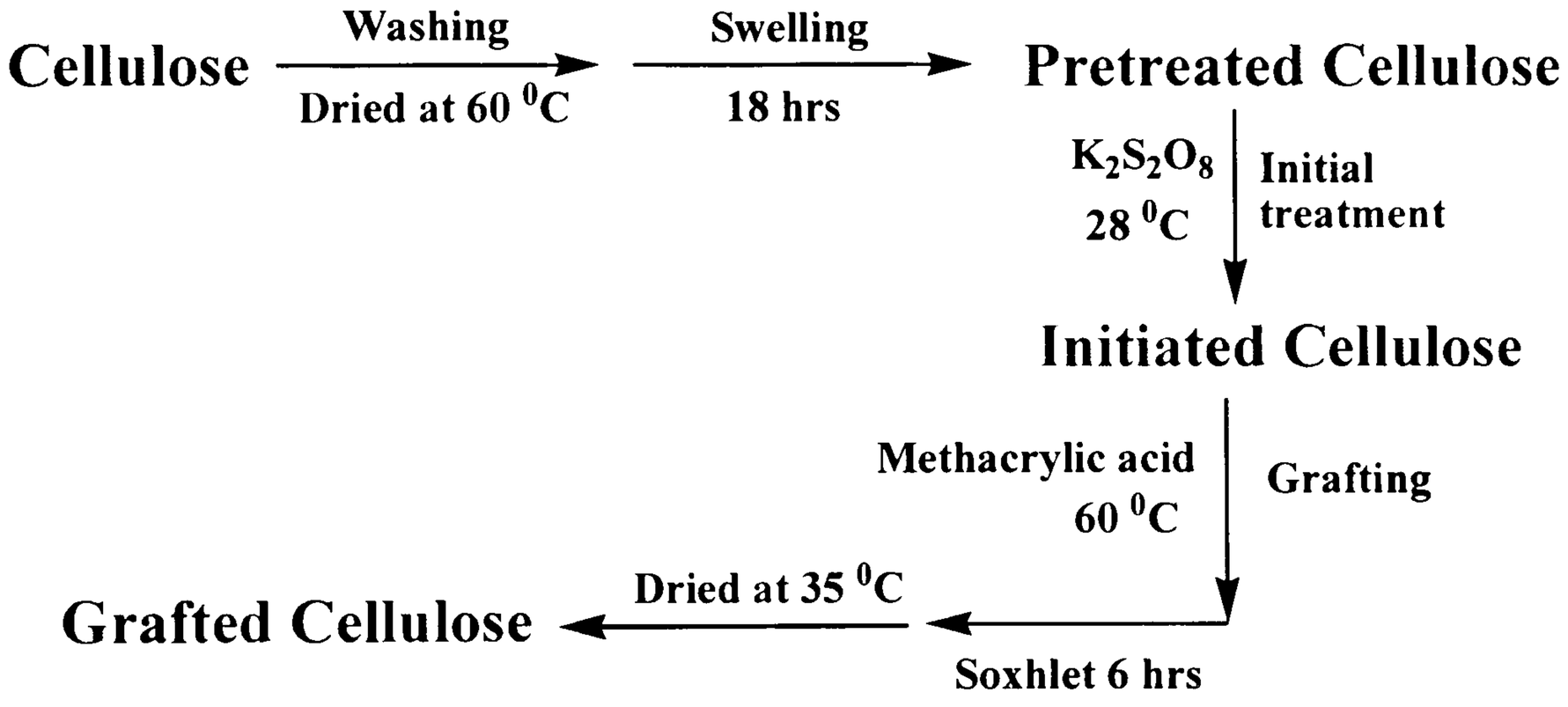


Figure 2.1.1 Scheme of Process of Grafting Copolymerisation

The grafting yield was quantified by the percentage increase in weight of the samples after grafting. The calculation of the grafting yield was based on the scheme expressed below.

$$\%Grafting\ Yield = \left(\frac{W_{GraftedCellulose} - W_{OriginalCellulose}}{W_{OriginalCellulose}} \right) \times 100$$

A series of tests was set up to control the grafting yield. This was carried out by optimising the amount of the monomer and of the initiator. The concentration of the monomer used was 0.25 mol dm^{-3} while a series of solutions of the initiator was prepared, with a final concentration in the reaction system at 0.025 mol dm^{-3}, 0.05 mol dm^{-3}, 0.06 mol dm^{-3}, 0.075 mol dm^{-3}, 0.10 mol dm^{-3} and 0.15 mol dm^{-3}. The same procedures were repeated with the final concentration of the monomer in the reaction system at 0.50 mol dm^{-3}, 0.75 mol dm^{-3} and 0.10 mol dm^{-3}.

To determine the amount of –COOH groups that were available for subsequent immobilisation reactions, the method of titrimetric analysis was employed. The titration began with the preparation of a standard solution of potassium hydrogen phthalate (0.1 mol dm^{-3}) and a solution of sodium hydroxide (0.1 mol dm^{-3}). The prepared solution of sodium hydroxide was titrated using phenolphthalein as indicator. In analysing the copolymer, a known amount of grafted cellulose (by weight) was immersed into certain known amount of NaOH solution and was left for 18 hours. 20 cm^{-3} of NaOH solution was then extracted from the solution containing the immersed cellulosic graft copolymer and was titrated with the standard potassium hydrogen phthalate solution (0.1 mol dm^{-3}). The concentration of the NaOH solution before and after the immersion of the grafted cellulose was then determined. The concentration of the –COOH existed on the grafted cellulose then can be calculated.

2.1.2 Materials and methods for the preparation of biological cells – *Shewanella* strain J18 143

The bacterial strain, *Shewanella* J18 143, is a facultative anaerobic bacterium. It was originally isolated from soil that had been contaminated with textile wastewater (Willmott, 1997).

2.1.2.1 Preparation of growth medium

The bacteria were grown in a tryptone soy broth (T.S.B.) medium. The T.S.B. powder was obtained from Lab M (International Diagnostics Group plc, Bury, UK). The composition of tryptone soy broth powder is shown in Table 2.1.1. To prepare a sterile culture medium, the 30 g dm^{-3} tryptone soy broth solution was made up with distilled water and was autoclaved using a pressure cooker (Section 2.1.2.4) obtained from Breville (Oldham, UK), for 15 minutes at 121 °C.

Table 2.1.1 Composition of Tryptone Soy Broth and Tryptone Soy Agar

Component	Concentration (g dm^{-3})	
	Tryptone Soy Broth	Tryptone Soy Agar
Tryptone Casein digest U.S.P.	17.0	15.0
Soy peptone	3.0	5.0
Sodium chloride	5.0	5.0
Dipotassium hydrogen phosphate	2.5	–
Dextrose	2.5	–
Agar No.2	–	12.0

2.1.2.2 Streak-plate technique

Two types of agar plates were used in the experiments. One type was the tryptone soy agar plate that was used for short-term storage of bacterial culture and for purification checks of the bacterial cultures. Another type was the dye-agar-broth plate that was used for the evaluation of decoloration ability of the biological cells on the contained dye.

The tryptone soy agar (T.S.A.) that was used was obtained from Lab M (International Diagnostics Group plc, Bury, UK). The components of the agar powder are shown in Table 2.1.1 as above.

To prepare an agar plate, tryptone soy agar powder was stirred and heated in distilled water in an Erlenmeyer conical flask (Fisher Scientific, Manchester, UK) to the boil, to make up a 37 g dm^{-3} solution. The solution was then transferred immediately from the conical flask to a Duran bottle which was obtained from Adelphi [Adelphi (Tubes) Ltd., Haywards Heath, UK]. It was then autoclaved in the pressure cooker for 15 minutes at 121°C. The sterilised agar was cooled to 50°C before pouring into a Petri dish (Fisher Scientific, Manchester, UK). The Petri dishes used were triple-vented. 20 cm^3 of agar was poured, just enough to cover the bottom of the dish. The dish was left inverted to dry over weekend, after which time the agar was solidified. Prepared plates were sealed with parafilm, supplied by Alcan packaging (Neenah, Wisconsin, USA). The prepared plates were pre-incubated before use to check for any contaminants. The incubator used was a Binder general purpose incubator obtained from Phillip Harris Scientific Ltd. (Hull, UK).

The dye-agar-broth plates were prepared by dissolving the target dye powder in the agar solution before sterilisation. The same procedures were followed as that used in preparing the agar plates.

To streak a plate, a platinum transfer loop was sterilised by flaming until it glowed red. It was then cooled by stabbing it into sterile agar medium from the plate. The cooled loop was used to pick up bacteria for streaking. A single, well-isolated bacterial colony, grown on the surface of solid medium was touched by the loop, or the bacterial culture was picked up by dipping the loop into growing bacterial starter culture (Section 2.1.2.5). The bacteria adhering to the loop were streaked onto a segment of a plate that contained the agar medium. The loop was sterilised by flaming and cooled by stabbing into a region of the agar medium that was free of bacterial cells. The loop was passed once across one end of the primary streak and the bacteria that adhered to the loop were spread into a fresh region of the agar medium. The loop was sterilised and cooled again and was streaked from one end of the secondary streak. This process was repeated triply or more, serially. Again, the loop was sterilised and cooled and streaked from one end of the secondary streak to

spread a third direction on the plate, and repeated triply or more. The lid was replaced on the plate and the plate was sealed. The bottom of the plate was labelled and the plate incubated in an inverted position at the appropriate temperature (usually 30 °C) for 16-24 hours.

2.1.2.3 Culture storage

The bacterial cultures were stored in three types of storage arrangements, short-term storage on agar plates, medium-term storage on agar slopes and long-term storage involving cryogenic freezing.

Colonies of the strains of bacteria could be maintained up to a period of a week on the surface of agar media of the plates (Section 2.1.2.2) that were tightly wrapped in parafilm and stored inverted in incubator at 30°C.

The bacterial strains stock was also stored in agar slopes that were normally kept in a refrigerator for one month. The agar slopes were prepared in screw-capped, universal bottles [25 cm^3, obtained from Adelphi (Tubes) Ltd., Haywards Heath, UK] containing 10 cm^3 of tryptone soy agar medium (37 g dm^{-3}). The bottles that contained boiled agar solution were sterilised at 121°C for 15 minutes and then left to dry at an angle of 45° overnight, with the caps loosely opened. The prepared agar slopes were wrapped tightly in parafilm to prevent desiccation and contamination of the medium.

To prepare the medium-term storage bacterial stock, 0.3 cm^3 of starter culture (Section 2.1.2.5) were streak-spread onto the surface of the agar slope. The slope was left in the incubator (Economy Incubator with fan model QCD 35.) at 35°C with the cap loosely opened for 24 hours. The prepared stock was wrapped tightly in parafilm and kept in a refrigerator.

Shewanella strain J18 143 cultures were cryogenically frozen for long term storage. The long-term storage stock was prepared and stored at Manchester University (Department of Geomicrobiology, School of Earth, Atmospheric and Environmental

Sciences, The University of Manchester UK). 0.9 cm^3 of inoculum were added to 2.0 cm^3 of cryogenic vials (Corning Inc., UK) together with 0.6 cm^3 of sterile glycerol solution (50% in distilled water). The prepared vials were flash frozen with liquid nitrogen and stored at -80°C (Pearce, 2004).

2.1.2.4 Aseptic techniques

Three types of aseptic technique were used in the experimental work, flaming, autoclaving and the use of chemicals.

Rapid sterilisation for loops, bottles and flask necks was carried our by flaming the relevant sections with the use of Bunsen burner. The loop was flamed until it appeared red and kept for five seconds before and after it was used. The bottle and flask necks were flamed before each opening and each closing.

A Commander SES benchtop steriliser (Eschmann Equipment, West Sussex, UK) and a Breville electric express pressure cooker (Oldham, UK) were used for autoclaving procedures.

All of the pipette tips, bottles and microtubes etc., were autoclaved using the Commander SES steriliser. The steriliser was used for unwrapped instruments and utensils according to manufacturer instructions. In use, all of the media, bacterial cultures, and glassware were autoclaved using the pressure cooker. This pressure cooker was also used to sterilise liquids.

Aqueous ethanol (75% w/w), was used for sterilising the apparatus and the work bench.

Hands were washed with anti-microbial hand soap supplied by Day-Impex Ltd (Essex, UK) and all of the apparatus used was washed with Hibitane concentrate 5% (Astra Zeneca UK Ltd, Hertfordshire, UK).

2.1.2.5 Preparation of planktonic *Shewanella* strain J18 143 cells

To prepare free cells of *Shewanella* strain J18 143, 50 cm^3 of the prepared bacterial growth medium (Section 2.1.2.1) were measured in 250 cm^3 Erlenmeyer conical flasks. The flasks were sealed with foam bungs (38 mm × 50 mm. Fisher Scientific. Manchester, UK) and covered with foil. The flasks were autoclaved in the pressure cooker for 15 minutes at 121 °C. A loop of inoculum, from the culture stock. *Shewanella* strain J18 143, was put into the cooled sterile growth medium. The flasks were then left overnight in a water-shaker bath (Grant Instruments Ltd, Cambridge, UK), incubated at 30 °C with shaking at 200 rpm overnight.

The following day, the aerobica starter culture of *Shewanella* strain J18 143 was suitable for use in experimentation.

To prepare the anaerobically grown *Shewanella* strain J18 143 cells, 100 cm^3 universal bottles, containing 90 cm^3 of the prepared growth medium, loosely covered with butyl rubber stoppers (20 mm), were sterilised in a pressure cooker for 15 minutes at 121 °C. The universal bottles and the butyl rubber stoppers were obtained from Adelphi (Tubes) Ltd. (West Sussex, UK). The bottles were sealed immediately out of the pressure cooker. The bottles then were cooled to room temperature and inoculated with 9 cm^3 of the prepared starter culture. The anaerobic growth cultures were incubated in a Binder general purpose incubator obtained from Phillip Harris Scientific Ltd. (Hull, UK). To obtain sufficient anaerobic cell growth, the bottles were incubated for 4 hours at 35 °C.

The cells were harvested by centrifugation at 3000 rpm for 10 minutes, using a Jouan Centrifuge C3-12 (Jouan Ltd., UK) and washed twice in phosphate buffer saline (P.B.S., 10 mM, pH 7) medium solution (see Section 2.2.1). The cells supernatant in P.B.S was prepared to make the cell suspension. The cell suspension was diluted (10 times) such that the diluted suspension gave an absorbance of 0.5 at 600 nm, to ensure that the absorbance of the monitored reduction reactions was maintained below 1 – 1.5, using a Specord S100 UV–Visible spectrophotometer (Spectronic

Analytical Instruments, Leeds, UK). This prepared cell suspension was used immediately.

2.1.3 Immobilisation of *Shewanella* strain J18 143 cells

Three immobilisation methods were used in the study (Figure 2.1.2), the "growing-in" method, the physical adsorption method and the chemical coupling method. The immobilisation method of "growing-in" was aimed at growing the biological cells within the texture of the cellulose copolymeric substrate for immobilisation. The method of adsorption was aimed at allowing the biological cells to be adsorbed onto the immobilisation substrate physically. The biological cells were also immobilised by chemical coupling. Thus, a chemical coupling agent was used to couple the cells onto the cellulose copolymer substrate (Gil, 1983). The coupling agent used was CMC, 1-cyclohexyl-3-[2-morphilinyl-4-ethyl]carbodi-imide metho-p-toluene sulphonate (Sigma-Aldrich Co Ltd., Dorset, UK).

The immobilisation methods are summarised in Figure 2.1.2.

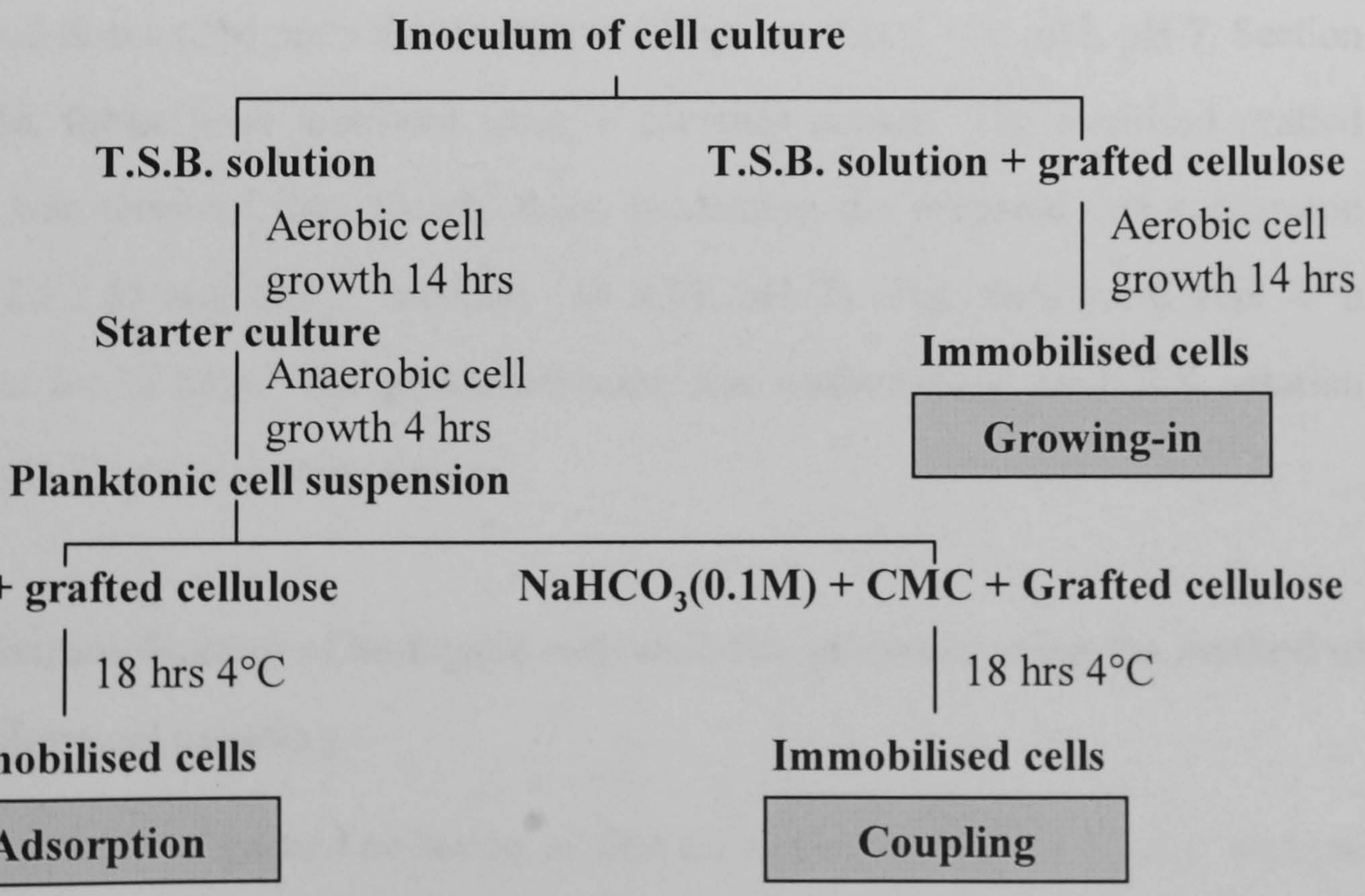


Figure 2.1.2 Scheme of Methods of Biological Cells Immobilisation

2.1.3.1 Immobilisation of biological cells onto the substrate using the method of "growing-in"

To start the process of cell immobilisation using the method of "growing-in", a known amount (approximately 1 g) of grafted cellulose was put into 50 cm^3 of the prepared tryptone soy broth growth medium (30 g dm^{-3}) in 250 cm^3 conical flasks. The flasks were covered with foam bungs and aluminium foil and were autoclaved for 15 minutes at 121 °C. A loop of inoculum was put into the cooled flasks. The flasks were sealed and were left overnight in the water shaker bath, incubated at 30 °C, with shaking at 200 rpm.

The grafted cellulose samples were then removed from the flasks and washed thoroughly in phosphate buffer saline solution (P.B.S., 10 mM, pH 7, Section 2.2.1). These grafted cellulose samples, together with the immobilised cells, were prepared after washing, for the decoloration experiments.

2.1.3.2 Immobilisation of biological cells onto substrates using the method of physical adsorption

The same amount of grafted cellulose as that mentioned in Section 2.1.3.1, was put into conical flasks (250 cm^3) that contained 50 cm^3 of P.B.S. (10 mM, pH 7, Section 2.2.1). The flasks were sterilised using a pressure cooker. The sterilised grafted cellulose was removed into 10 cm^3 vials, containing the prepared cell suspension (Section 2.1.2.5) and P.B.S. solution (10 mM, pH 7). The vials were kept in a refrigerator for 18 hours. The grafted cellulose was washed using the P.B.S. solution (10 mM, pH 7) and was ready for use.

2.1.3.3 Immobilisation of biological cells onto the substrate using the method of chemical coupling

The same amount of grafted cellulose, as that mentioned in Section 2.1.3.1, was put into conical flasks (250 cm^3) that contained 50 cm^3 of P.B.S. (10 mM, pH 7, Section

2.2.1). The flasks were sterilised at 121 °C for 15 minutes. The sterilised grafted cellulose was removed into conical flasks (100 cm^3) containing 30 cm^3 of $NaHCO_3$ (0.1 M), 0.15 g of carbodiimide (1-cyclohexyl-3-[2-morphilinyl-4-ethyl]carbodi-imide metho-p-toluene sulphonate, CMC) and 2.0 $cm^3$of the prepared cell suspension (Section 2.1.2.5). The flask was left in the water bath at 4 °C, and shaken for 4 hours. Then the flask was refrigerated for 18 hours. The grafted cellulose, with the coupled cells, was taken out from the flask, after coupling, and washed with P.B.S. solution (10 mM, pH 7).

2.1.4 Protein assay of immobilised biological cells

Protein assays of immobilised cells were employed. The protein assay method used by Ramachandran (2004) was followed. Unfortunately, the procedure was not suitable for this work. Protein analysis based on the method for the planktonic resting cells, as used by Pearce (2004), was suggested (von Canstein, 2005, personal communication). The method is given in detail in Section 2.5.5.2.

2.2 Decoloration of Remazol Black B dye solution using immobilised *Shewanella* strain J18 143 cells

Decoloration of Remazol Black B dye solution using immobilised bacterial cells was studied. The bacterial cells used were immobilised onto the cellulosic graft copolymer by the three methods described in Section 2.1.3.

2.2.1 Materials

The Remazol Black B dye used was obtained from Dystar UK Ltd. (Huddersfield, UK). Pearce (2004) previously compared a commercially synthesised dye sample with a laboratory synthesised dye sample of the Remazol Black B. The conclusion

was that the commercial Remazol Black B dye had a greater purity than the laboratory synthesised dye. Furthermore, the additives that were presented in the commercial dye increased the colour intensity of the dye. The selection of the dye solution was based on the wish that it should present a "real" textile effluent for the colour removal treatment study. Therefore, it was decided that the commercial dye would be used for all of the subsequent experiments.

All of the decoloration experiments based on Remazol Black B dye solutions were carried out in the phosphate buffer saline (P.B.S., 10 mM, pH 7) solution. The P.B.S. solution was prepared by mixing 4 g of sodium chloride, 30.5 cm^3 of potassium monohydrogen phosphate (K_2HPO_4, 0.1 M) and 19.5 cm^3 of potassium dihydrogen phosphate (KH_2PO_4, 0.1M) and diluting to 500 cm^3 with distilled water.

Addition of an organic acid electron donor increases the rate of dye reduction and gives an indication of the enzyme pathway that is responsible for the reduction reaction (Pearce, 2004). Sodium formate was chosen and was used in all of the subsequent decoloration experiments after comparing its efficiency with that of other possible electron donors (Pearce, 2004). The addition of an exogenous extracellular electron shuttle can accelerate the reaction rate of the dye decoloration. Anthraquinone-2,6-disulphonic acid (AQDS, 0.01M) was used as the electron shuttle in the decoloration systems.

All of the decoloration experiments were carried out in autosampler vials (size 10 cm^3, cap 23 × 46 mm) obtained from Kimble Glass Inc. (Vineland, New Jersey, USA), sealed with 20 mm gold/alumina top seals with PTFE-faced, butyl rubber liners, obtained from Alltech Associates/Applied Sciences (Carnforth, Lancashire, UK).

2.2.2 Calibration curve of Remazol Black B dye solutions

A calibration curve of absorbance versus dye concentration of the Remazol Black B solution was constructed. A series of Remazol Black B solutions, with concentrations

ranging from 10 mg dm^{-3} to 60 mg dm^{-3}, were prepared in volumetric flasks. The absorbance of the dye solutions was measured at 597 nm using a Specord S100 UV-Visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). The absorbance, measured at the same wavelength for the same dye solution, i.e. with the same absorption coefficient, is proportional to the concentration of the absorbing medium. The calibration is affected by means of linear regression. The calibration curve was used to calculate the amount of Remazol Black B that had been reduced in each experiment. The UV-visible spectroscopy analysis method, used in preparation of calibration curves, is given in Section 2.5.3.

2.2.3 Establishment of standards

The decoloration system was designed to remove the colour from Remazol Black B dye solutions, using immobilised *Shewanella* strain J18 143 cells with the addition of sodium formate solution and anthraquinone-2,6-disulphonic acid (AQDS) solution. To have an appreciation of the colour removal that has been brought by the immobilised cells, as prepared in Section 2.1.3, standards needed to be prepared, for comparisons. All of the standards were prepared in 10 cm^3 autosampler vials. The total volume of liquid in each of the decoloration systems was 9.5 cm^3. The very small amounts of liquid that were transferred into the systems with the immobilised cells were negligible in their influence. The concentration of the sodium formate solution used was 1 M, and the final concentration in the system was 21 mM. The concentration of the AQDS solution used was 0.01 M, and the final concentration in the system was 100 μM. The concentration of the phosphate buffering saline solution used was 10 mM, pH 7. All of the prepared vials were sealed with gold/alumina top seals with PTFE-faced butyl rubber liners and the headspaces were filled with nitrogen.

To have a better understanding of the colour removal effect of the immobilised biological cells on the targeted dye solutions, several standard controls were

established as the immobilised cells standard, the dye solution standard, the cell growth medium solution standard, the grafted cellulose standard and the planktonic cells standard. All of the standards had been treated under the same conditions and same procedures as those for the colour removal samples. The standards were incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at 597 nm using a Specord S100 UV–visible spectrophotometer. At the same time, photographic representations were taken using a digital camera (Nikon COOLPIX2500). Each experiment was carried out in triplicate.

2.2.3.1 Immobilised cells standard

A control of immobilised cells was established for evaluating the effect of the immobilised cells and that of the existing of the immobilisation substrate in the absorbance measurement. The absorbance of the prepared systems, without the addition of dye solution, was measured as one of the colour removal controls. The vials were prepared with sodium formate (21 mM), AQDS (100 μM), with prepared immobilised cells and P.B.S. solution. As mentioned in Section 2.1.3, the bacterial cells were immobilised by the three different methods. However, there was no need to establish the individual standard for each of the methods. The aim of establishing this control was to investigate the effect of the immobilised cells on the measurement of the adsorption of the analysed solution.

2.2.3.2 Dye solution standard

As the decoloration of the targeted dye solution occurred, the colour of the treated samples would be expected to change. As the durations of the treatment varied, a control of targeted dye solution without any addition of biological cells was needed throughout the process.

The dye solution standards were prepared with dye solution (final concentration in the system was 50 μM), sodium formate (final concentration in the system was 21

mM), AQDS (final concentration in the system 100 μM) and P.B.S. solution (10 mM, pH 7) to make 9.5 cm^3 in total volume.

2.2.3.3 Cell growth medium solution standards

The cell growth medium used was tryptone soy broth (Section 2.1.1.2). The effect of any cell growth medium that might incidentally be brought into the system from the immobilisation process was investigated.

The vials prepared for these standards contained dye solution (final concentration in the system was 50 μM), sodium formate (final concentration in the system was 21 mM), AQDS, (final concentration in the system was 100 μM), the "treated" graft cellulosic copolymer (the graft cellulosic copolymer underwent the procedures of immobilisation of the method of "growing-in" with the absence of bacterial cells) and a specified amount of P.B.S. solution (10 mM, pH7) to make the system 9.5 cm^3 in total volume.

2.2.3.4 Graft cellulosic copolymer standard

The effect of the immobilisation substrate, the cellulosic graft copolymer, was investigated. The vials prepared for the grafted cellulose standards were prepared with dye solution (final concentration in the system was 50 μM), sodium formate (final concentration in the system was 21 mM), AQDS (final concentration in the system was 100 μM), appropriate amounts of grafted cellulose and specified amounts of P.B.S. solution (10 mM, pH7) to make 9.5 cm^3 of the system.

2.2.3.5 Free cells standard

The effect of the planktonic *Shewanella* strain J18 143 cells on the colour removal of Remazol Black B dye solutions has been well investigated (Pearce, 2004). The same concentration of the biological cells was used in this control. The vials contained dye solution (final concentration in the system was 50 μM), sodium formate (final

concentration in the system was 21 mM), AQDS (final concentration in the system was 100 μM), prepared cell suspension (0.5 cm^3) and known amounts of P.B.S. solution (10 mM, pH 7) to make the final up to 9.5 cm^3.

2.2.4 Colour removal from Remazol Black B dye solutions using immobilised *Shewanella* strain J18 143 cells

Remazol Black B dye was chosen as the model azo dye with respect to a typical dye existing in textile effluents, to evaluate the decoloration efficiency of the immobilised cells. All of the reduction investigations were carried out in 10 cm^3 autosampler vials. Each vial contained the Remazol Black B dye solution, sodium formate, anthraquinone-2,6-disulphonic acid disodium salt (AQDS), prepared cell suspension (0.5 cm^3) and certain, known amounts of buffer saline solution to make up 9.5 cm^3 in total volume, unless otherwise stated.

The grafted cellulose samples, with the immobilised cells, for each of the immobilisation methods, were put into the vials at same weight, respectively. The concentration of the Remazol Black B dye solution was 1 mM, and the final concentration in the decoloration system was at 50 μM. The concentration of the sodium formate solution used was 1 M, and the final concentration in the system was 21 mM. The concentration of the AQDS solution used was 0.01 M, and the final concentration in the system was 100 μM. The concentration of the phosphate buffering saline solution used was 10 mM at pH 7. All of the prepared vials were sealed with gold/alumina top seals with PTFE-faced butyl rubber liners. The headspace was filled with nitrogen.

The samples were incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at 597 nm using a Specord S100 UV–visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). The technique was that described in more detail in Section 2.5.3. At the same time as the

absorbance was measured, photographic representations were taken using a digital camera (Nikon COOLPIX2500). Each experiment was carried out in triplicate.

2.3 Colour reduction of metal-complex azo dye solutions using planktonic cells of *Shewanella* strain J18 143

The colour removal of reactive azo dyes using the biological cells, *Shewanella* strain J18 143 has been studied and well developed (Willmott, 1997; Kamilaki, 2000 and Pearce, 2004). The research described in this section is focused on the colour reduction of another large group of azo dyes, i.e. the metal complexes. Metal-complex azo dyes represent an important class of dyes in the textile industries due to their excellent light fastness. Their share is estimated to be approximately 30% in wool dyeing and 40% in polyamide fibre dyeing (Hunger, 2003). In commercial terms, the most important metal-complex dyes are the chromium complexes, the cobalt complexes and the copper complexes with azo ligands. Four representatives of the textile dyes were chosen for the study of colour reduction of metal-complex azo dyes using *Shewanella* strain J18 143 cells. These dyes were the mono-azo metal-complex dyes, Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL respectively. The dyes were obtained from Ciba Specialty Chemicals (Ciba Specialty Chemicals PLC, Macclesfield, UK).

2.3.1 Materials

Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL were chosen with respect to being a typical dyes that exist in metal-complex azo dye-based textile effluents after polyamide and wool dyeing processes. One objective was to evaluate the reduction efficiency of the planktonic cells with respect to these colorants.

Irgalan Grey GLN is a mixture of two metal-complex azo dyes. Irgalan Black RBLN (disodium[3-hydroxy-4-[(2-hydroxy-1-naphthyl)azo]-1-naphthalenesulphonato(3-)[1-[[2-hydroxy-5-[(p-methoxyphenyl)azo]phenyl]azo]-2-naphtholato(2-)] chromate(2-), 70-80%, Figure 2.3.1) and Irgalan Blue 3GL (disodium [6-amino-5-[(2-hydroxy-4-nitrophenyl)azo]-N-methylnaphthalene-2-sulphonamidato(2-)] [6-amino-5-[(2-hydroxy-4-nitrophenyl)azo] naphthalene-2-sulphonato(3-) cobaltate(2-), 5-10%, Figure 2.3.2). Irgalan Black RBLN has a black shade and is a mono-sulphonated 1:2 metal-complex dye that is used for dyeing and printing wool and polyamide fibres. Irgalan Blue 3GL has a colour that in greenish blue. It is a non-sulphonated 1:2 metal-complex dye that is also used for dyeing and printing wool and polyamide fibres.

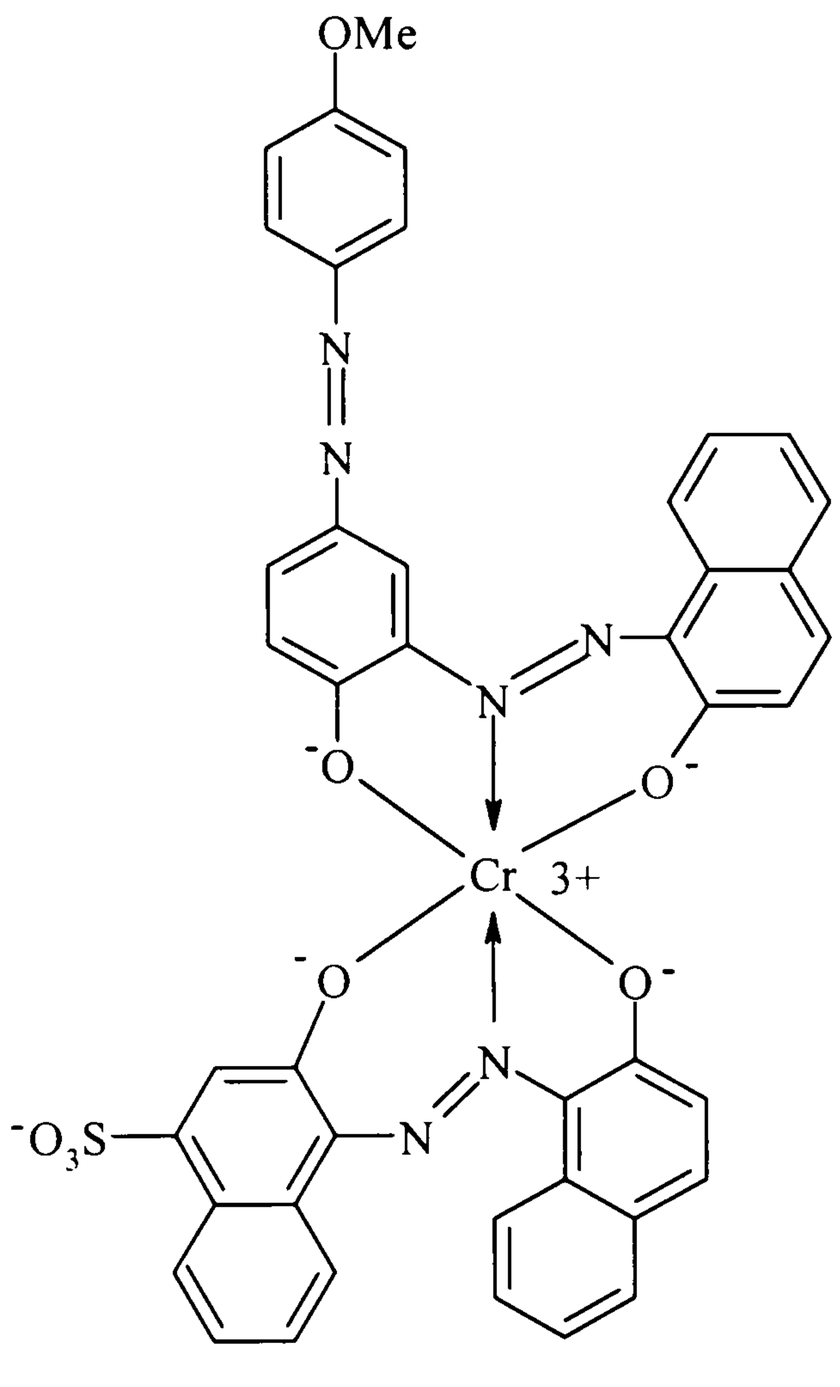


Figure 2.3.1 The Chemical Structure of the Chromophore in Irgalan Black RBLN

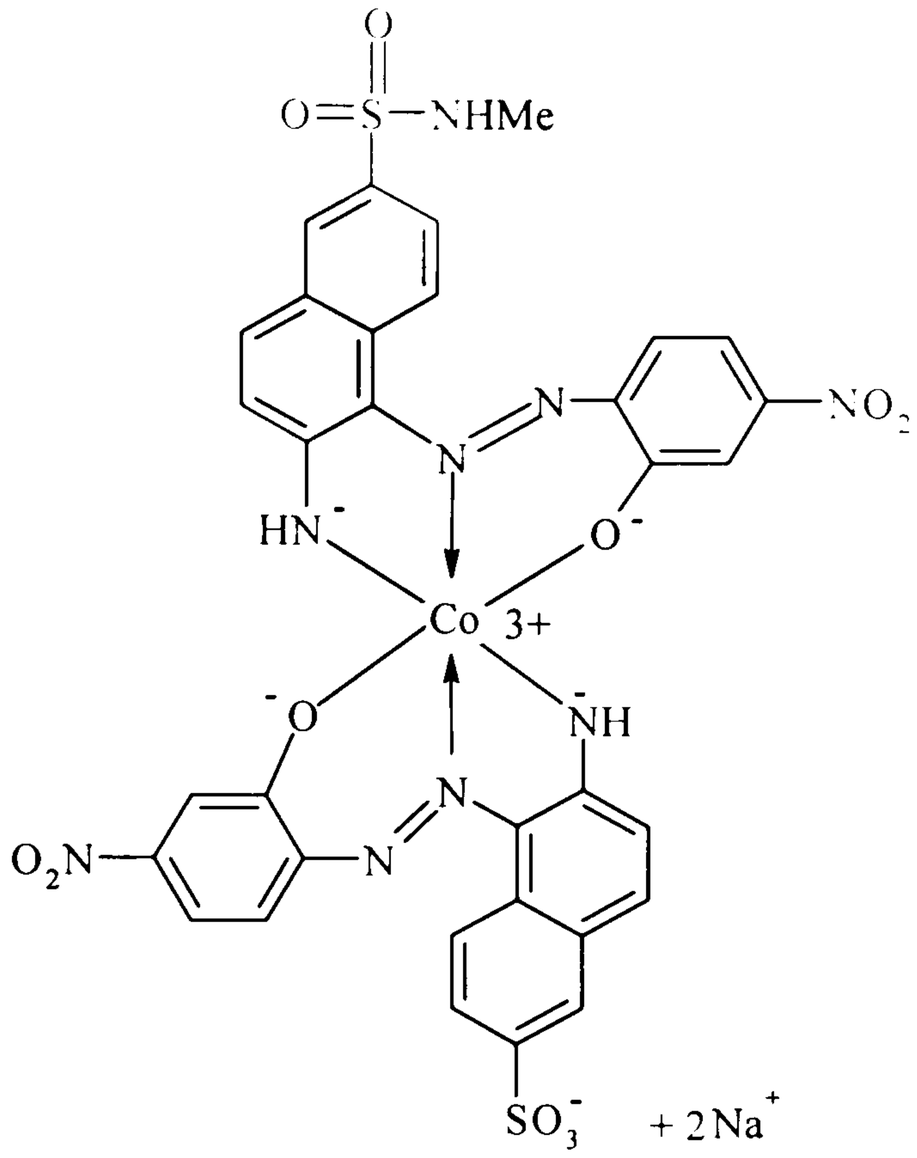


Figure 2.3.2 The Chemical Structure of the Chromophore in Irgalan Blue 3GL

The selected metal-complex dye of Irgalan Yellow 3RL KWL is mainly composed of 2-[methyloleoylamino]ethane-1-sulphonate, 3-8%; sodium bis[3-[[1-(3-chlorophenyl)-4,5-dihydro-3-methyl-5-oxo-1H-pyrazol-4-yl]azo]-4-hydroxybenzene sulphonamidato (2-) cobaltate(1-), 40-50% (Figure 2.3.3).

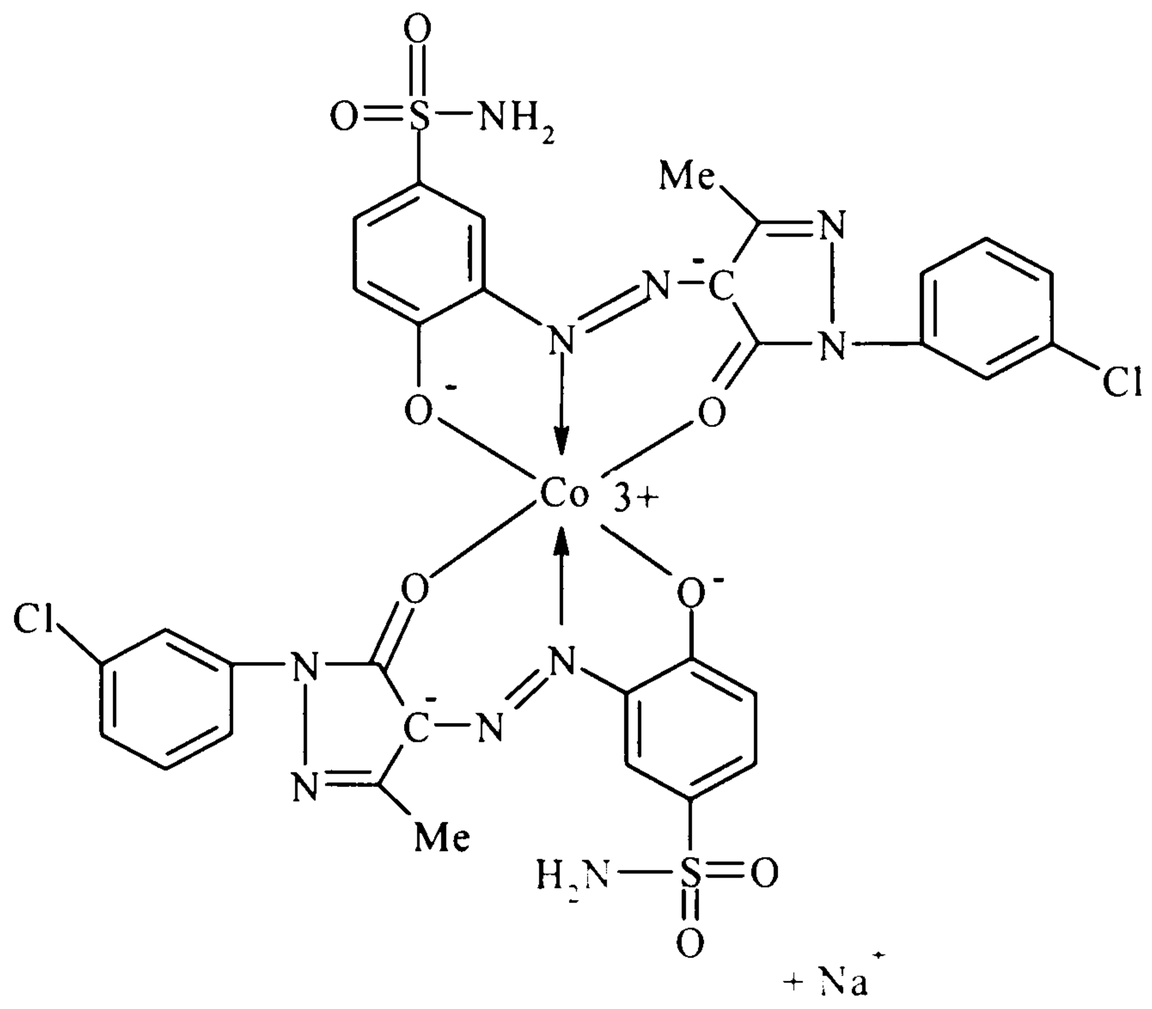


Figure 2.3.3 The Chemical Structure of the Chromophore in Irgalan Yellow 3RL KWL

Information concerning the dyes was provided by Ciba Specialty Chemicals (Macclesfield, UK). All of the dyes were used without further purification.

Colour reductions of solutions of the four selected metal-complex dyes were carried out using the *Shewanella* strain J18 143 cells. All of the colour reductions of the metal-complex dye solutions were carried out in buffered saline media.

A 1 M solution of sodium formate in the phosphate buffer saline (P.B.S., 10 mM, pH 7) was used as electron donor, at a final concentration of 21 mM, in all of the subsequent colour reductions of separate solutions of Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL. A solution of electron shuttle, anthraquinone-2,6-disulphonic acid (AQDS, 10 mM), was prepared in P.B.S. (10 mM, pH 7) and was used in the systems, providing the colour reduction of Irgalan Grey GLN, Irgalan Blue 3GL, Irgalan Black RBLN and Irgalan Yellow 3RL KWL, at a final concentration of 100 μM.

The decoloration experiments were carried out in autosampler vials (size 10 cm^3, cap 23 × 46 mm) that were obtained from Kimble Glass Inc. (Vineland, New Jersey, USA) sealed with 20 mm gold/alumina top seals with PTFE-faced, butyl rubber liners, obtained from Alltech Associates/Applied Sciences (Carnforth, Lancashire, UK). The headspaces of all of the vessels used were degassed with nitrogen gas after being sealed.

All of the general chemicals were of analytical grade and were obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated.

2.3.2 Preparation of the *Shewanella* strain J18 143 cells

The colour reduction of each of the selected metal-complex azo dyes, Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL, was carried out using a suspension of freshly prepared *Shewanella* strain J18 143 cells.

The procedures for preparing the cell suspension of *Shewanella* strain J18 143 were described in Section 2.1.2.5. Each cell suspension was used immediately after preparation.

2.3.3 Colour reduction of solutions of the Irgalan Grey GLN metal-complex azo dye using the planktonic cells of *Shewanella* strain J18 143

Colour reduction from the solutions of Irgalan Grey GLN using planktonic cells of *Shewanella* strain J18 143, was carried out. A calibration curve of the Irgalan Grey GLN dye solution was assembled. Colour reduction criteria for Irgalan Grey GLN dye solutions, using the bacterial cells and the relative standards, were established.

2.3.3.1 Calibration curve of the solutions of Irgalan Grey GLN

A calibration curve of absorbance versus dye concentration for Irgalan Grey GLN solutions was constructed. A series of Irgalan Grey GLN solutions, with concentrations ranging from 7.5 mg dm^{-3} to 120 mg dm^{-3}, was prepared in volumetric flasks. The absorbance values of the dye solutions were measured at λ_{max} for Irgalan Grey GLN (577 nm) using a Specord S100 UV-Visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK).

The absorbance, measured at the same wavelength for the same dye solution, is proportional to the concentration of the absorbing medium. The analysis method used in preparation of calibration curve of the targeted dye solution is described in Section 2.5.3.

The calibration curve was used to calculate the amount of Irgalan Grey GLN that had been reduced in each experiment.

2.3.3.2 Reduction of solutions of the Irgalan Grey GLN metal-complex dye using the planktonic cells of *Shewanella* strain J18 143

All of the reduction investigations were carried out using 10 cm^3 vials. Each vial contained a solution of Irgalan Grey GLN, sodium formate, anthraquinone-2.6-disulphonic acid disodium salt (AQDS), prepared cell suspension (0.5 cm^3) and known amounts of phosphate buffer saline solution to make up 9.5 cm^3 in total volume, unless otherwise stated. The concentration of Irgalan Grey GLN in solution was 1.6 g dm^{-3}. The final concentration of the dye in solution in the decoloration system was 0.08 g dm^{-3}. The concentration of the sodium formate solution used was 1 M, and the final concentration in the system was 21 mM. The concentration of the AQDS solution used was 0.01 M, and the final concentration in the system was 100 μM. The concentration of the phosphate buffering saline solution used was 10 mM and the pH was 7. All of the prepared vials were sealed with gold/alumina top seals with PTFE-faced butyl rubber liners (Alltech Associates/Applied Sciences, Carnforth, UK) and the headspaces were filled with nitrogen.

The samples were incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at wavelength of 577 nm using a Specord S100 UV–visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). At the same time, photographic representations of the samples were taken using a digital camera (Olympus C-370). Each experiment was carried out in triplicate.

2.3.3.3 Establishment of dye solution standards

The colour reduction system was designed to reduce the colour from the Irgalan Grey GLN dye solution, using the planktonic bacterial cells of *Shewanella* strain J18 143. with the existing of sodium formate solution and anthraquinone-2.6-disulphonic acid (AQDS) solution. To have an appreciation of the colour removal that had been brought about by the biological cells, as prepared in Section 2.3.2, standards needed to be prepared. for comparisons. All of the standards had been treated under the same

conditions and same procedures as those for the colour reduction samples. The standards were incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at 577 nm using a Specord S100 UV–visible spectrophotometer. At the same time, photographic representations were taken using a digital camera (Olympus C-370). Each experiment was carried out in triplicate.

On decoloration of the targeted dye solution, the colour of the treated samples would be expected to change with time. The durations of the treatments were varied. Thus, a control based on the targeted dye solution without any addition of biological cells was needed throughout the process. Such vials were prepared with the Irgalan Grey GLN dye solution (final concentration 0.08 g dm^{-3}), sodium formate (final concentration 21 mM), AQDS, (final concentration 100 μM) and P.B.S. solution (10 mM, pH 7) to make 9.5 cm^3 in total volume.

2.3.3.4 Establishment of planktonic cells standard

The biological cells used in the reduction systems were prepared as a suspension in the phosphate buffer saline solutions. 0.5 cm^3 of each cell suspension were added to each of the evaluated systems, contained a total volume of 9.5 cm^3. The effect of the planktonic *Shewanella* strain J18 143 cells on the absorbance of the reduction system, without the addition of any dye solution, needed to be investigated. The vials of the planktonic cells standard contained sodium formate (final concentration 21 mM), AQDS (final concentration 100 μM), prepared cell suspension (0.5 cm^3) and known amounts of P.B.S. solution (10 mM, pH 7) to make the final volume to 9.5 cm^3.

2.3.3.5 Particle size analysis

Particle size analysis using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was carried out to determine the effect of the bacterial cells of *Shewanella* strain J18 143 on the particle size of the "solutions". The "solutions" tested were the Irgalan Grey GLN standard, the dye treated with the bacterial cells

and the cell suspension standard after an incubation of 18 hours. More description relating to the use of particle size analysis is given in Section 2.5.4.

2.3.4 Colour reduction of metal-complex azo dye solutions (Irgalan Black RBLN and Irgalan Blue 3GL) using the planktonic cells of *Shewanella* strain J18 143

The metal-complex azo dye, Irgalan Grey GLN was mainly composed of two chromophores, Irgalan Black RBLN and Irgalan Blue 3GL (Section 2.3.1.1). The chromate complex in Irgalan Grey GLN is the main chromophore, which is 60-70% content of the metal-complex dye of Irgalan Black RBLN (Figure 2.3.1). The cobaltate complex in Irgalan Grey GLN is the main chromophore, which is 60-70% content of the metal-complex dye of Irgalan Blue 3GL (Figure 2.3.2). The studies of the colour reduction of these two dyes were carried out respectively using the planktonic bacterial cells of *Shewanella* strain J18 143.

2.3.4.1 Calibration curve of the solutions of Irgalan Black RBLN and Irgalan Blue 3GL

Calibration curves of absorbance versus dye concentration of Irgalan Black RBLN solutions and of Irgalan Blue 3GL were constructed. A series of Irgalan Black RBLN solutions, with concentrations ranging from 40 mg dm^{-3} to 120 mg dm^{-3}, were prepared in volumetric flasks. The absorbance of the dye solutions was measured at λ_{max} for Irgalan Black RBLN (572 nm) using a Specord S100 UV-Visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). A series of Irgalan Blue 3GL solutions, with concentrations ranging from 20 mg dm^{-3} to 120 mg dm^{-3}, were prepared in volumetric flasks. Absorbance of the dye solutions was measured at λ_{max} for Irgalan Blue 3GL (637 nm) using a Specord S100 UV-Visible spectrophotometer. The absorbance, measured at the same wavelength for the same dye solution, i.e. with the same absorption coefficient, is proportional to the

concentration of the absorbing medium (Section 2.5.3). The calibration curve was used to calculate the amount of dye that had been reduced in each experiment.

2.3.4.2 Colour Reduction from solutions of Irgalan Black RBLN and Irgalan Blue 3GL using the planktonic cells of *Shewanella* strain J18 143

The colour reduction of the Irgalan Black RBLN dye solutions and of the Irgalan Blue 3GL dye solutions was carried out at the same conditions for each of the dye solutions. The reduction investigations were carried out in 10 cm^3 vials and in sealed, anaerobic, precision cuvettes (10 mm) made of Quartz SUPRASIL® (Hellma UK Ltd., Southend-on Sea, Essex, UK). Each vial/cuvettes contained the metal-complex azo dye solution (Irgalan Black RBLN or Irgalan Blue 3GL), sodium formate, anthraquinone-2,6-disulphonic acid disodium salt (AQDS), prepared cell suspension (0.5 cm^3 in vial or 0.16 cm^3 in cuvettes) and known amounts of phosphate buffer saline solution to make up 9.5 cm^3 (in vial) or 3.0 cm^3 (in cuvettes) in total volume, unless otherwise stated. The concentration of the dye solution prepared (Irgalan Grey GLN or Irgalan Blue 3GL) was 1.6 g dm^{-3}. The final amount of the dye solution in the decoloration system was 0.08 g dm^{-3}. The concentration of the sodium formate solution used was 1 M, and the final concentration in the system was 21 mM. The concentration of the AQDS solution used was 0.01 M, and the final concentration in the system was 100 μM. The concentration of the phosphate buffering saline solution used was 10 mM and the pH was 7. All of the prepared vials were sealed with gold/alumina top seals with PTFE-faced butyl rubber liners (Alltech Associates/Applied Sciences, Carnforth, UK). The headspaces of the vials and of the cuvettes were filled with nitrogen.

The samples were incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at 572 nm for the investigation of the colour reduction of the Irgalan Black RBLN dye solutions and at 637 nm for the investigation of the colour reduction of the Irgalan Blue 3GL dye solutions, using a Specord S100 UV–visible spectrophotometer. At the same time, photographic

representations were taken by a digital camera (Nikon COOLPIX2500). Each experiment was carried out in triplicate.

2.3.4.3 Establishment of dye solution standards

The colour reduction system was designed to reduce the colour from the selected metal-complex azo dye solutions using the bacterial cells of *Shewanella* strain J18 143 in the presence of the sodium formate solution and anthraquinone-2,6-disulphonic acid (AQDS) solution. To have an appreciation of the colour removal that was brought by the biological cells, standards needed to be prepared.

All of the standards were treated under the same conditions and same procedures as those used for the colour reduction samples. The standards were incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at 572 nm for the Irgalan Black RBLN standards and at 637 nm for the Irgalan Blue 3GL standards, using a Specord S100 UV–visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). At the same time, photographic representations were taken using a digital camera (Nikon COOLPIX2500). Each experiment was carried out in triplicate.

If decoloration treatment of the targeted dye solution occurred, the colour density of the treated samples would be expected to change in a controlled way. The duration of the treatment were varied. Thus, a control of targeted dye solution of each dye solution (Irgalan Black RBLN and Irgalan Blue 3GL) without any addition of biological cells was needed respectively throughout the process. The vials were prepared on the basis of dye solution (final concentration 0.08 g dm^{-3}), sodium formate (final concentration 21 mM), AQDS, (final concentration 100 μM) and P.B.S. solution (10 mM, pH 7) to make 9.5 cm^3 (in the 10 cm^3 vial) or 3.0 cm^3 (in the anaerobic cuvettes) in total volume.

2.3.4.4 Establishment of planktonic cells standard

The biological cells used in the reduction systems were prepared as a suspension in the phosphate buffer saline solutions. 0.5 cm^3 of cell suspension was added to each of the evaluated systems, contained to total volume of 9.5 cm^3. The effect of the free *Shewanella* strain J18 143 cells on the absorbance of the reduction system without the addition of any dye solutions needed to be investigated. The vials of the planktonic cells standard contained sodium formate (final concentration 21 mM), AQDS, (final concentration 100 μM), prepared cell suspension (0.16 cm^3) and certain amounts of P.B.S. solution to make the final up to 9.5 cm^3.

2.3.5 Colour reduction from solutions of Irgalan Yellow 3RL KWL dye using the planktonic cells of *Shewanella* strain J18 143

Irgalan Yellow 3RL KWL was one of the metal-complex azo dye selected for the colour reduction study. The chemical structure of the main chromophore in this dye was given in Section 2.3.1.1 (Figure 2.3.3). The calibration curve of the Irgalan Yellow 3RL KWL was established. Colour reduction of the dye solution of this yellow dye and the related standards were features of this section of the total study.

2.3.5.1 Calibration curve for solutions of Irgalan Yellow 3RL KWL

A calibration curve of absorbance versus dye concentration for Irgalan Yellow 3RL KWL solution was constructed. A series of Irgalan Yellow 3RL KWL solutions, with concentrations ranging from 10 mg dm^{-3} to 80 mg dm^{-3}, was prepared using volumetric flasks. The absorbance of the dye solutions was measured at λ_{max} for the Irgalan Yellow 3RL KWL (456 nm) using a Specord S100 UV-Visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). The calibration curve was used to calculate the amount of Irgalan Yellow 3RL KWL that had been reduced in each experiment (Section 2.5.3).

2.3.5.2 Reduction of solutions of Irgalan Yellow 3RL KWL using the planktonic cells of *Shewanella* strain J18 143

All of the reduction investigations were carried out in 10 cm^3 vials. Each vial contained the Irgalan Yellow 3RL KWL dye solution, sodium formate, anthraquinone-2,6-disulphonic acid disodium salt (AQDS), prepared cell suspension (0.5 cm^3) and certain amounts of phosphate buffer saline solution to make up 9.5 cm^3 in total volume, unless otherwise stated. The concentration of the solution of Irgalan Grey Yellow 3RL KWL 250% was 2.4 g dm^{-3}, and the final concentration in the decoloration system was at 0.12 g dm^{-3}. The concentration of the sodium formate solution used was 1 M, and the final concentration in the system was 21 mM. The concentration of the AQDS solution used was 0.01 M, and the final concentration in the system was 100 μM. The concentration of the phosphate buffering saline solution used was 10 mM and pH 7. All of the prepared vials were sealed with gold/alumina top seals with PTFE-faced butyl rubber liners and the headspace were filled with nitrogen.

The samples were incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at 456 nm using a Specord S100 UV–visible spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). At the same time, photographic representations were taken using a digital camera (Olympus C-370). Each experiment was carried out in triplicate.

2.3.5.3 Establishment of dye solution standards

The colour reduction system was designed to reduce the colour of the Irgalan Yellow 3RL KWL dye solution using the bacterial cells of *Shewanella* strain J18 143 with the assistance of sodium formate solution and anthraquinone-2,6-disulphonic acid (AQDS) solution. To have an appreciation of the colour removal that has been brought by the biological cells, prepared as described in Section 2.3.2, standards were established. All of the standards were treated under the same conditions and same procedures as those for the colour reduction samples. The standards were

incubated at 30 °C, unless otherwise stated, without shaking, for 7 days. Absorbance readings were taken daily at 456 nm using a Specord S100 UV–visible spectrophotometer. At the same time, photographic representations were taken using a digital camera (Olympus C-370). Each experiment was carried out in triplicate.

If decoloration treatment of the targeted dye solution occurred, the colour density of the treated samples would be expected to change. The duration of the treatment varied. Thus a control of targeted dye solution without any addition of biological cells was used throughout the study. The vials were prepared with dye solution of Irgalan Yellow 3RL KWL (final concentration 0.12 g dm^{-3}), sodium formate (final concentration 21 mM), AQDS (final concentration 100 μM) and P.B.S. solution (10 mM, pH 7) to make 9.5 cm^3 in total volume.

2.3.5.4 Establishment of planktonic cells standard

The planktonic biological cells used in the reduction systems were prepared as a suspension in the phosphate buffer saline solutions. 0.5 cm^3 of cell suspension was added in each of the evaluated systems contained a total volume of 9.5 cm^3. The effect of the *Shewanella* strain J18 143 cells on the absorbance of the reduction system, without the addition of any dye solutions was investigated. The vials of the planktonic cells standard contained sodium formate (final concentration 21 mM), AQDS (final concentration 100 μM), prepared cell suspension (0.5 cm^3) and certain amounts of P.B.S. solution (10 mM, pH 7) to make the final up to 9.5 cm^3.

2.3.5.5 Particle size analysis

Particle size analysis, using a Zetasizer Nano ZS, was carried out to determine the effect of the bacterial cells of *Shewanella* strain J18 143 on the particle size of the "solutions". The "solutions" were of the Irgalan Yellow 3RL KWL standard, the dye treated with the bacterial cells and the cell suspension standard, after an incubation of 18 hours. Description relating to the use of particle size analysis is given in Section 2.5.4.

2.4 Factors affecting the colour reduction of the metal-complex dye solutions using the planktonic cells of *Shewanella* strain J18 143

There are several factors that might affect the colour reduction of the metal-complex dye solutions using the cells of *Shewanella* strain J18 143. Pearce (2004) studied some of these factors with respect to the reduction of Remazol Black B dye solutions using resting cell suspension of *Shewanella* stain J18 143. Those factors include the type of electron donor, the nitrate ions, dye hydrolysis, the dye concentration, the temperature, the pH and the exogenous extracellular electron shuttle. In the current study, the effect of the dye concentration, the effect of the temperature and the effect of the pH were investigated for the colour reduction of the metal-complex azo dye solutions using biological cells.

2.4.1 Materials

All of the chemicals were of analytical grade and were obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated.

The colour reductions of the four selected metal-complex dye solutions were carried out using the *Shewanella* stain J18 143 cells. All of the colour reductions of the metal-complex dye solutions were carried out in buffered saline solutions. The effect of pH on the colour reduction of the selected metal-complex azo dyes using *Shewanella* strain J18 143 cells was investigated. A series of buffer solutions was prepared at different pH levels as shown in Table 2.4.1. The pH values were determined using a Corning 250 pH meter with a BDH Gelplus general-purpose combination probe.

Table 2.4.1 Buffer Solutions (Source: Pearce, 2004)

Sodium acetate – acetic acid buffer solutions		
pH at 18 °C	0.1 M CH_3COONa solution (cm^3)	0.1 M CH_3COOH solution (cm^3)
4.4	37.0	63.0
5.6	91.0	9.0
Sodium phosphate, dibasic – sodium phosphate, monobasic buffer solutions (P.B.S.)		
pH at 25 °C	0.1 M Na_2HPO_4 solution (cm^3)	0.1 M NaH_2PO_4 solution (cm^3)
6.8	61.0	39.0
8.0	94.7	5.3
Sodium carbonate – sodium bicarbonate buffer solutions		
pH at 20 °C	0.1 M Na_2CO_3 solution (cm^3)	0.1 M $NaHCO_3$ solution (cm^3)
9.2	10.0	90.0
100 cm^3 + NaCl (8 g) diluted to 1 dm^3 with distilled water		

A 1 M solution of sodium formate in the phosphate buffer saline (P.B.S., 10 mM, pH 7) was used as electron donor, at a final concentration of 21 mM, in all of the subsequent colour reductions of separate solutions of Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL. A solution of electron shuttle, anthraquinone-2,6-disulphonic acid (AQDS, 10 mM), was prepared in P.B.S. (10 mM, pH 7) and was used in the systems providing the colour reduction of Irgalan Grey GLN, Irgalan Blue 3GL, Irgalan Black RBLN and Irgalan Yellow 3RL KWL, at a final concentration of 100 μM.

2.4.2 Protein assay of planktonic cells of *Shewanella* strain J18 143

Protein assays were carried out for all of the colour reduction studies relating to the metal-complex azo dyes. Each assay was used to calculate the reduction rate of the dye in the reduction system. The reduction of the dye solutions by *Shewanella* strain J18 143 cells was then expressed as a rate, this being the concentration of dye solution reduced per gram of biomass per minute. The detailed operating procedures associated with the protein assay of the resting biological cells are given in Section 2.5.5.1.

2.4.3 Effect of dye concentration on the colour reduction of the metal-complex azo dyes

The concentration of metal-complex azo dye in solution would be expected to have an important effect on the extent and rate of dye reduction using *Shewanella* strain J18 143 cells. At high dye concentrations, the rate of dye reduction could be affected due to the increased toxicity of the dye. In very dilute dye solutions, a low rate of dye reduction may also be experienced, as there would be insufficient dye present to be recognised by the dye reductase enzyme inside the cells (Pearce, 2004).

To study the effect of dye concentration on dye reduction using *Shewanella* strain J18 143 cells, solutions of each Irgalan metal-complex azo dye (Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL) were added to a series of autosampler vials (size 10 cm^3, cap 23 × 46 mm) to prepare a range of known concentrations (40 mg dm^{-3} to 240 mg dm^{-3}) respectively. A sodium formate solution (1M) was added to the vials as the electron donor (final concentration, 21 mM). A solution of AQDS was added (0.01 M), and the final concentration in the system was 100 μM. The assay solutions were diluted to 9.5 cm^3 using P.B.S. (10 mM, pH 7). The vials were sealed with gold/alumina top seals with PTFE-faced butyl rubber liners and the headspace were filled with nitrogen. The samples were incubated at 30 °C, without shaking.

The absorbance of the solutions in the vials was measured periodically at the λ_{max} for each dye, respectively, using a Specord S100 spectrophotometer that was fitted with a temperature controlled cuvette changer. The absorbance of "blank" solutions, containing all the assay solutions other than the colorant, were also measured and used to normalise the absorbance data that were obtained from the samples containing the colorant. The calibration curve for each of the metal-complex azo dye solutions was constructed (Section 2.3.4.1, Section 2.3.4.1 and Section 2.3.5.1). The colour reduction rate against dye concentration was plotted, based on the results from the calibration curve and the protein assay. At the same time, photographic

representations were taken using digital camera (Olympus C-370). The experiment was carried out in triplicate.

2.4.4 Effect of temperature on the colour reduction of the metal-complex azo dyes

It was realised that the temperature of the system might have a significant effect on the viability of the *Shewanella* strain J18 143 cells and on their ability to reduce the colour from the solutions of the metal-complex azo dyes.

To evaluate the effect of temperature on dye reduction using the *Shewanella* strain J18 143 cells, a series of samples was prepared identically in autosampler vials (size 10 cm^3, cap 23 × 46 mm). Each vial contained the metal-complex azo dye solution (1.6 g dm^{-3} of Irgalan Grey GLN, Irgalan Black RBLN and Irgalan Blue 3GL or 2.4 g dm^{-3} of Irgalan Yellow 3RL KWL), and the final concentration of in the decoloration system was at 0.08 g dm^{-3}/0.12 g dm^{-3}. A sodium formate solution (1M) was added to the vials as the electron donor (final concentration, 21 mM). A solution of AQDS was added (0.01 M), and the final concentration in the system was 100 μM. The assay solutions were diluted to 9.5 cm^3 using P.B.S. (10 mM, pH7). The vials were sealed with gold/alumina top seals with PTFE-faced butyl rubber liners and the headspace were filled with nitrogen. The samples were incubated at 24°C, 30°C. 40°C, 50°C and 60°C, respectively, without shaking.

The absorbance of the solutions in the vials was measured periodically at the λ_{max} for each of the dye solutions using a Specord S100 spectrophotometer that was fitted with a temperature controlled cuvette changer. The absorbance of "blank" solutions. containing all of the assay solutions other than the colorant, were also measured and used to normalise the absorbance data that were obtained from the samples containing the colorant. At the same time, photographic representations were taken by digital camera (Olympus C-370). The colour reduction rate against dye

concentration was plotted based on the results from the calibration curve and the protein assay. The experiment was carried out in triplicate.

2.4.5 Effect of pH on the colour reduction of metal-complex azo dyes

It was considered to be important to investigate the pH of the colour removal system that involves an enzymatic process, as denaturation of the relevant enzyme could occur if the required pH range was not maintained. The optimum pH for dye reduction must be determined as it gives an indication of the extent to which the coloured wastewater, which is often strongly alkaline, must be diluted and/or buffered prior to treatment (Pearce, 2004).

To determine the optimum pH for dye reduction using the *Shewanella* strain J18 143 cells, certain specified amounts of acetate-acetic acid buffered saline solution (10 mM, pH = 5.6), sodium phosphate buffered saline solution (10 mM, pH = 6.8 and 8.0) or sodium carbonate-sodium bicarbonate buffered saline solution (pH = 9.2), to make up 9.5 cm^3 as the total volume, were used respectively (Table 2.4.1). Each vial contained dye solution of the metal-complex azo dye (1.6 g dm^{-3} of Irgalan Grey GLN, Irgalan Black RBLN and Irgalan Blue 3GL or 2.4 g dm^{-3} of Irgalan Yellow 3RL KWL). The final dye concentration in the decoloration system was at 0.08 g dm^{-3}/0.12 g dm^{-3}. A sodium formate solution (1M) was added to the vials as the electron donor (final concentration, 21 mM). A solution of AQDS was added (0.01 M), and the final concentration in the system was 100 μM.

The absorbance of the solutions in the vials was measured periodically at the λ_{max} for each of the dye solutions using a Specord S100 spectrophotometer that was fitted with a temperature controlled cuvette changer. The absorbance of "blank" solutions, containing all the assay solutions other than the colorant, was also measured and used to normalise the absorbance data that were obtained from the samples containing the colorant. At the same time, photographic representations were taken

by digital camera (Olympus C-370). The colour reduction rate against dye concentration was plotted based on the results from the calibration curve and the protein assay. The experiment was carried out in triplicate.

2.5 Analysis and characterisation methods

Several methods were used throughout the experimental work relating to the characterisation and analysis of the stating materials and the resulting products.

2.5.1 Gravimetric analysis

The rate of the graft copolymerisation was monitored as described in Section 2.1.1.2. Since the extent of grafting was designed to be somewhat limited (less than 10%), it was not possible to control the exact grafting rate. The factors affecting the measurement of the grafting process could vary due to the physical properties of the immobilisation substrate. Gravimetric analysis was chosen and was carried out under careful control. The extent of grafting was determined by measuring the weight changes of the treated cellulose related to those of the ungrafted cellulose standard.

2.5.2 Analysis using scanning electron microscopy (SEM)

Scanning electron microscopic evaluations were carried out on the cellulosic substrate, on the cellulose based copolymer and on the biological cells.

To observe the surface morphology of the pre-treated cellulose and the cellulosic graft copolymer, samples were mounted on SEM stubs, using double sided tape, and then coated with gold in a Bio-Rad Microscience Division gold coater (75 seconds at 2 kV).

For the observation of the bacterial cells, inorganic membranes (Whatman Anodisc), with a pore size of 0.02 μm, were used. Diluted cell suspension was dropped onto the

membrane, which was supported by a Millipore sintered glass holder. fitted into a standard filter flask. Excess liquid was extracted that was allowed the bacterial cells lay on the top of the membrane. A small piece of the membrane that contained the bacterial cells was attached onto the SEM stubs using the double sided tape. The rest procedures were the same as those of the cellulosic substrates.

The samples were analysed using a Joel JSM-820 scanning electron microscope. with a 5 kV, 10 kV or 15 kV electron beam. The magnification was varied depending upon the type of sample being analysed (Goldstein *et al.*, 2003).

2.5.3 Analysis using UV-visible spectrophotometry

UV-visible spectrophotometry is often used to analyse solutions or dispersions in the quantitative determination of components that absorb/scatter in the UV or visible regions of the electromagnetic spectrum. The usual region of absorbance which is of interest in the current study is in the range 190 nm to 700 nm. The scattering aspect depends on the nature of the particles, as seen in their loading, their size and their various distributions.

A SPECORD S100 Diode Array UV-visible Spectrophotometer supplied with WinASPECT software (Spectronic Analytical Instruments, Leeds, UK) was used. The spectrophotometer was equipped with a temperature controlled cuvette changer. A halogen lamp provides a basis for detection in the UV region of the spectrum (190 nm – 340 nm). A deuterium lamp is available for detection in the visible region of the spectrum (340 nm – 700 nm). The unit was connected to a PC with AJ-UVVIS software. S100 Device Driver software was used to control the measurement processes and the processing of the measured data.

The UV-visible spectrophotometric technique was used in preparation of planktonic cell suspension (Section 2.1.1.5) to control the amounts of bacterial cells that were used in the evaluating systems. The bacterial cells were harvested by centrifugation and were washed with phosphate buffer saline (P.B.S., 10 mM, pH7) solution to

remove the tryptone soy broth. The cells supernatant in P.B.S. was controlled by UV-visible spectrophotometeric evaluations. Each cells supernatant was diluted 10 times to make it 0.5 in absorbance at 600 nm. This bacterial cell suspension was prepared every time it was required.

UV-visible spectrophotometry was also applied during the preparation of the calibration curves of the dye solutions of Remazol Black B (Section 2.2.2), Irgalan Grey GLN (Section 2.3.3.1), Irgalan Black RBLN (Section 2.3.4.1), Irgalan Blue 3GL (Section 2.3.4.1) and Irgalan Yellow 3RL KWL (Section 2.3.5.1). Series of solutions of each dye, at known concentrations, were prepared. The absorbance of the dye at each known concentration was measured UV-visible spectrophotometrically, with distilled water as a reference. The corresponding absorbance against the known concentrations gave a calibration curve for the respective dye solution. A wavelength at λ_{max} was chosen so that evaluations of the decoloration effects could be carried out. These calibration curves were useful in determining the unknown concentrations of the dye solution or the liquor systems that contained the respective dyes. The UV-visible spectrophotometric method was also used to make calibration curve of the standard protein solution.

The UV-visible spectrophotometric technique was used to monitor the processes of colour reduction of the dye solutions (Section 2.2, Section 2.3 and Section 2.4) that in the quantitative determination of chromophoric compounds in mixtures. The optical densities of the samples, at λ_{max}, were measured regularly for the required periods of time. The change of the absorbance values of the measured solutions against the incubation period was used to monitor the change of the colour of the measured samples. The respective spectra were also used in illustrating the change of the colour of the measured samples.

2.5.4 Particle sizing analysis

The particle size of the studied samples was assessed in those instances in which some precipitation of colorant was observed. The particle size analysis was carried out using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK).

The particle size distribution of the standard dye "solution", the dye "solution" with the biological cells and the cell suspension standard, after incubation of 18 hours at 30°C, was investigated. This analytical method was used in the studies of the colour reduction of the Irgalan Grey GLN and of the Irgalan Yellow 3RL KWL.

2.5.5 Protein assay

The protein assay was applied in two usages in the studies, the protein assay of immobilised biological cells of *Shewanella* strain J18 143 and the protein assay of planktonic resting cell suspension of *Shewanella* strain J18 143.

The protein assay method described by Pearce (2004) was used to measure the protein concentration in both of the situations. It was carried out using a bicinchoninic acid assay kit.

2.5.5.1 Protein assay of planktonic cells

To start a protein assay of resting cell suspension, the standard solutions shown in Table 2.5.1, containing Bovine Serum Albumin and phosphate buffer saline (P.B.S., 10 mM, pH 7), were prepared in disposable cuvettes.

A mixture of bicinchoninic acid-copper sulphate solution (50:1, 1 cm^3) was prepared. 1.0 cm^3 of the mixture was added to each of the disposable cuvettes. The cuvettes were left to stand at room temperature for 30 minutes. After this time, the absorbance at 562 nm, of each of the solutions, with P.B.S. (10 mM, pH 7) as the reference, was measured. A calibration curve of absorbance versus protein concentration was

produced from the absorbance of these standard solutions containing known concentrations of Bovine Serum Albumin protein.

Table 2.5.1 Standard Bovine Serum Albumin (B.S.A.) Solutions of Known Protein Concentration

Solution	Volume of B.S.A. (μ dm^{-3})	Volume of P.B.S. (μ dm^{-3})	Concentration of protein (mg cm^{-3})
1	50	0	1.0
2	25	25	0.5
3	10	40	0.2
4	5	45	0.1
5	0	50	0.0

The Bovine Serum Albumin calibration curve and the absorbance of the solutions that contained the samples of resting cell suspension were used to determine the protein concentration and, therefore, the amount of biomass that was responsible for the reduction of the dye solutions.

2.5.5.2 Protein assay of immobilised biological cells

The grafted cellulose was cut into same sized pieces of 1×2 cm. These pieces then underwent immobilisation procedures (Section 2.1.1.2). The grafted cellulose pieces with immobilised cells were used for protein assay.

The protein assay was performed with in 96-well microtiter plates and an Anthos 2001 plate reader equipped with kinetic software (Jencons Scientific Ltd., Leighton Buzzard, UK). The samples were prepared in the 96-well microtiter plate as indicated in Table 2.5.2. In Table 2.5.2, A1 to A10 contained the standard protein solution (S1 to S5 in duplicate). This approach was used to produce the calibration curve for the standard protein solution. B1 to B12 contained the cellulosic substrates on which the cells were immobilised by the method of physical adsorption (GC Ad).

C1 to C12 contained the cellulosic substrates on which the cells were immobilised by the method of chemical coupling (GC CC). D1 to D12 contained the cellulosic substrates with immobilised cells that the cells were immobilised by the method of "growing-in" (GC GI). The standard protein solution was prepared as same as described in Section 2.5.5.1. The immobilised cells (B1 to D12) were immersed in 50 μ dm^{-3} of P.B.S. solution (10 mM, pH 7). 1.0 cm^{-3} of the mixture (bicinchoninic acid-copper sulphate solution) was added to each of the well. The 96-well plate was then left in incubator at 30°C for 30 minutes.

Table 2.5.2 Preparation of Protein Assay Samples in 24-well Plate

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5		
B	1 GC Ad	1 GC Ad	2 GC Ad	2 GC Ad	3 GC Ad	3 GC Ad	4 GC Ad	4 GC Ad	5 GC Ad	5 GC Ad	6 GC Ad	6 GC Ad
C	7 GC CC	7 GC CC	8 GC CC	8 GC CC	9 GC CC	9 GC CC	10 GC CC	10 GC CC	11 GC CC	11 GC CC	12 GC CC	12 GC CC
D	13 GC GI	13 GC GI	14 GC GI	14 GC GI	15 GC GI	15 GC GI	16 GC GI	16 GC GI	17 GC GI	17 GC GI	18 GC GI	18 GC GI

The absorbance values of the standard protein solution and of the substrates with immobilised cells, after 30 minutes of incubation at 30°C, were measured by the plate reader (at 570 nm).

3 Results and Discussion

In this chapter, the results obtained from the experimental work described in Chapter 2 are presented for discussion. The research work was carried out in two parts. These concern (i) the removal of colour from standard solutions of reactive azo dyes using immobilised biological cells and (ii) the removal of colour from standard aqueous solutions of metal-complex azo dyes using planktonic resting biological cells. *Shewanella* strain J18 143 cells were used throughout the work.

In Section 3.1, the results from the immobilisation of the bacterial cells are presented. These include the results arising from the preparation of the immobilisation substrate, from the preparation of the resting cells and from the immobilisation of the bacterial cells using different immobilising methods.

In Section 3.2, the results from the decoloration of Remazol Black B dye solutions using immobilised cells, created by different immobilising methods, are compared and discussed. The removal of colour from the dye solutions using immobilised bacterial cells is also compared with the results obtained using planktonic whole bacterial cells.

In Section 3.3, the reduction of solutions of metal-complex azo dyes using the *Shewanella* strain J18 143 is characterised. This involved the reduction of four Irgalan dyes, namely, Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL. Irgalan Grey GLN is a mixture of the Irgalan Blue 3GL and Irgalan Black RBLN.

The effect of the dye concentration, the temperature and the pH on dye reduction efficiency is discussed in Section 3.4 with respect to each selected individual metal-complex azo dye. The results obtained from the biological cell-induced dye reductions are expressed with respect to the specific reduction rates, which were calculated from the results of protein assays.

3.1 Preparation of the immobilisation substrate and the immobilisation of biological cells

The first step of the experimental work involved an attempt to immobilise bacterial cells of *Shewanella* strain J18 143 onto a cellulosic copolymer substrate, with the aim of using the immobilised cells in the decoloration of coloured textile wastewater. The results obtained during the work are presented, discussed and analysed.

3.1.1 Preparation of cellulosic copolymer-based immobilisation substrate

Immobilising biological cells onto cellulosic graft copolymer substrates has shown much potential in applications, as mentioned in the introduction (Section 1.5.3). Cellulose is a linear polymer that exhibits a high degree of crystallinity in its native state. There is strong intermolecular hydrogen bonding between adjacent cellulose chains due to the presence of the hydroxyl groups (three per anhydroglucose unit) (Roy *et al.*, 2005). Therefore, not all of these OH groups are accessible to copolymerisation. Thus, a swelling/cleansing pre-treatment of cellulose is required if one wishes to create a reasonably homogeneously grafted copolymeric material. Without such treatment, grafting would be restricted to the surface of the cellulosic fibrils. The cellulose cotton was pre-treated according to the method used by Sabaa and Mokhtar (2002) as descried in Section 2.1.1.1. Scanning electron microscopy (SEM) was employed to "view" the surface of the pre-treated cellulose (Section 2.5.2). An electron beam intensity of 15 k*e*V was shown to be suitable for the samples, giving good resolution. Figure 3.1.1 shows a representation of the corresponding fibres of the pre-treated cellulose.

The pre-treated cellulose underwent graft copolymerisation, with methacrylic acid (MAA) as the monomer and potassium persulphate as the initiator, as described in

Section 2.1.1.2. Although methacrylic acid has been shown to provide a relatively low grafting rate (Zahran *et al.*, 2004), the rate achieved and the grafting characteristics provided were deemed to be suitable for the study.

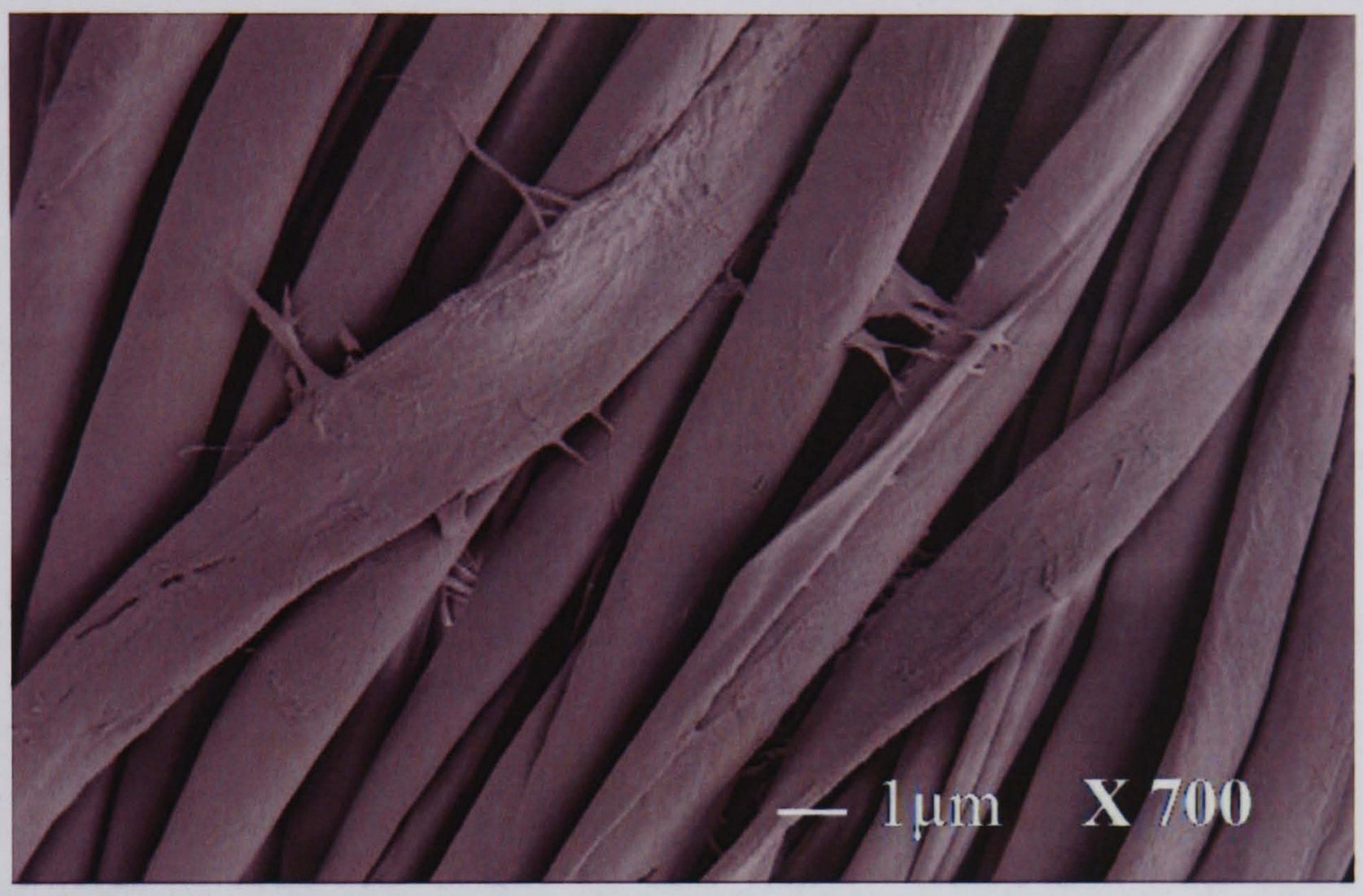


Figure 3.1.1 Scanning Electron Micrograph (SEM) of Pre-treated Cellulose

The grafting of vinyl and acrylic monomers onto cellulose, using persulphate radicals, has been studied extensively (Sabaa and Mokhtar, 2002). The mechanism for the thermal decomposition of persulphate ions in aqueous solution is shown below. The polymerisation of vinyl monomers can be initiated by either the $SO_4^{\bullet -}$ or $HO^{\bullet}$ radicals (Tune, 1989).

$$S_2O_8^{2-} \rightarrow 2SO_4^{\bullet -}$$

$$SO_4^{\bullet -} + H_2O \rightarrow HSO_4^{-} + HO^{\bullet}$$

$$2HO^{\bullet} \rightarrow HO-OH \rightarrow H_2O + \tfrac{1}{2}O_2$$

Methacrylic acid (MAA) is a water-soluble monomer, and poly(methacrylic acid) is also soluble in water. The products from the grafting process comprised of blends of

cellulose-MAA graft copolymer, PMAA homopolymer and any ungrafted cellulose. The removal of as much as PMAA homopolymer as was possible from these products was achieved by extensive Soxhlet extraction with boiling water. However, it was considered possible that not all of the homopolymer was removed. Thus, the resulting products might still have contained small amounts of occluded homopolymer. Since this study was concerned with preparing immobilisation substrates for bacterial cells, it was deemed that the presence of very small amounts of homopolymer would not have serious implications for the graft copolymerisation and the immobilisation aspects.

Studies of grafting rates are commonly based on gravimetric methods. However, this approach can give rise to errors. The primary reason is the ease of moisture uptake by the ungrafted samples, which renders weighing difficult unless carried out under constant temperature and humidity conditions (Guthrie and Haq, 1974). Gravimetry was used in this study, great care being taken with respect to sample drying and to mass transfer issues (Section 2.5.1). The graft yield was calculated using the scheme stated in Section 2.1.1.2. A further consideration was given regarding the weight loss of the cellulose during the processing procedures. Blank tests were undertaken, as described in Section 2.1.1.2.

The pre-treated cellulose was taken run through the whole grafting copolymerisation process without any monomer and any initiator being added. If the weight of the final product (final cellulose blank) was found to be less than that of the initial cellulose (initial cellulose), the weight of the final product (grafted cellulose) after graft copolymerisation with the addition of the monomer and the initiator was modified, based on the weight of the initial cellulose (initial cellulose). There is an assumption that the rate of the weight loss during the grafting copolymerisation, which is unavoidable due to the nature of the cellulosic system (rather broad molar mass distribution), remains the same during the blank test. The acquired information was then used when calculating the grafting extent, as described in the following schemes.

For the blank:

$$Weight\ Loss\,(\%) = \left| \frac{(Final\ Cellulose\ Weight\ (Blank) - Initial\ Cellulose\ Weight) \times 100\%}{Initial\ Cellulose\ Weight} \right|$$

For the copolymer:

$$Weight\ Gain\,(\%) = \frac{(Grafted\ Cellulose\ Weight - Initial\ Cellulose\ Weight) \times 100\%}{Initial\ Cellulose\ Weight}$$

$$Grafting\ Yield\,(\%) = Weight\ Gain\,(\%) + Weight\ Loss\,(\%)$$

The concentration of both the monomer [$CH_2C(CH_3)COOH$] and the initiator ($K_2S_2O_8$) has an effect on the final grafting yield. A series of tests was carried out (Section 2.1.1.2) in this respect. The concentration of the initiator to be used in the experiment was then determined as 0.075 mol dm^{-3}, and the concentration of the monomer used was fixed at 0.5 mol dm^{-3}. The grafting yield averaged 5.5%. This yield of grafting provides sufficient reactive sites to allow the biological cells to be immobilised, since the extent of grafting was designed to be somewhat limited (less than 10%). This largely removes the prospect of creating an environment that would be inherently hostile to the cells that were to be subsequently immobilised. In addition, the fibrous nature of the substrate is maintained. Handling procedures were not compromised.

Figure 3.1.2 represents the image obtained from SEM of the grafted cellulose. An electron beam intensity of 15 k*e*V was used. The image shows evidence of surface change without great changes to the overall form of the fibre matrix. The change should be seen in comparison with the image of the pre-treated cellulose (Figure 3.1.1). The sample of the pre-treated cellulose consists of long, interwoven, orientated fibres. Close examination of the fibre surface reveals smoothness and a good level of homogeneity. With the cellulose-MAA graft copolymer (Figure 3.1.2),

the first indications are of the damage of the fibre surface and "bridging" between the fibres. These changes depict regions for possible grafting.

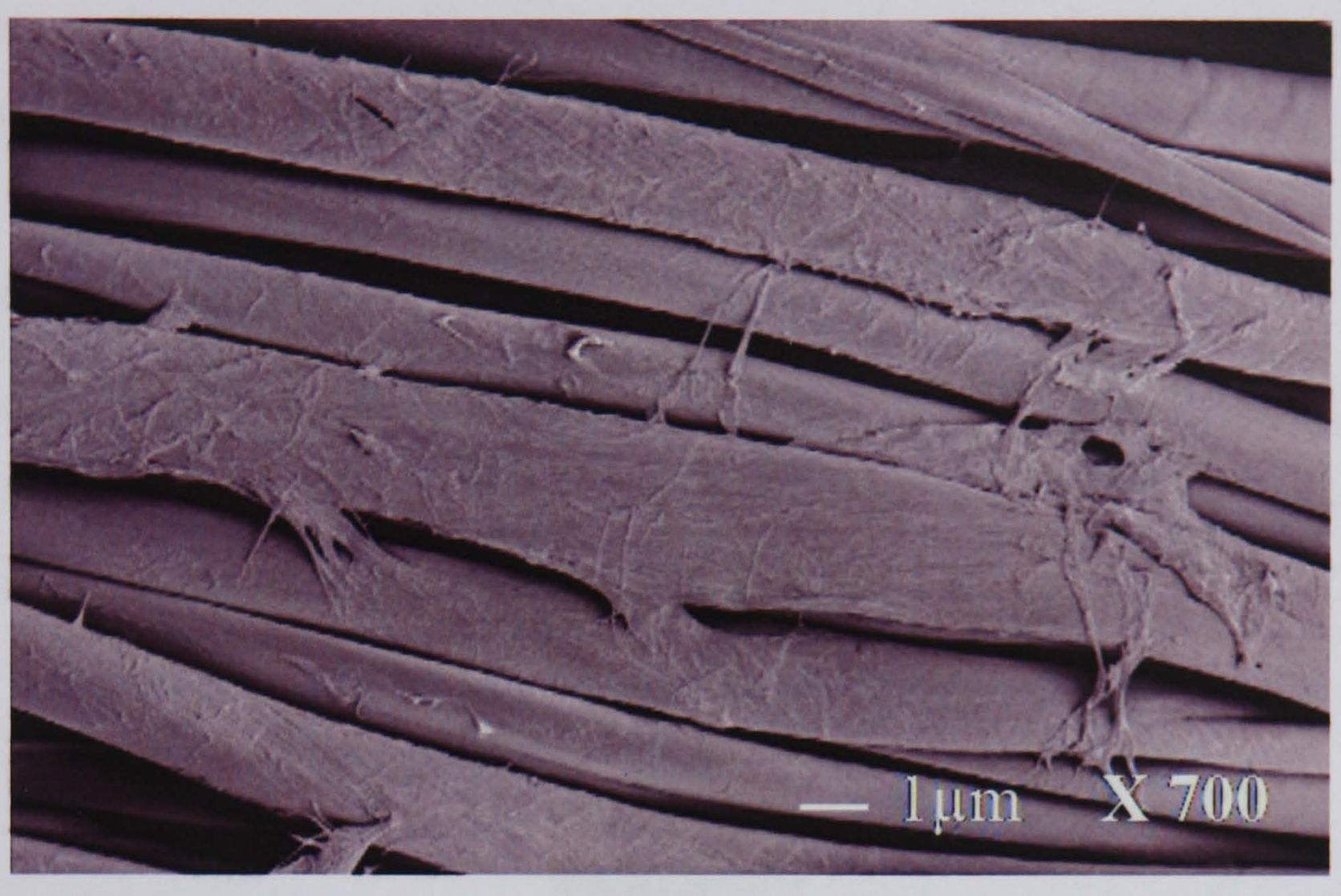


Figure 3.1.2 Scanning Electron Micrograph (SEM) of Grafted Cellulose

To support the conclusion drawn from the comparison of the images (Figure 3.1.1 and Figure 3.1.2), titrimetric analysis was constructed (Section 2.1.1.2). The average concentration of –COOH groups on the copolymer was determined as 0.1±0.01 mol dm^{-3}. Therefore, these would imply that there is sufficient evidence to show that the methacrylic acid was either grafted onto cellulose to a low grafting extent and/or that occluded homopolymer was present. In view of the exhaustive extraction procedures that were used, the view of the occurrence of covalent linking, through grafting, is favoured.

3.1.2 Preparation of biological cells – *Shewanella* strain J18 143

The bacterial culture was isolated and identified as *Shewanella putrefaciens*, a gram-negative motile bacterium. It is a facultative anaerobe that can use the

nitrogen-nitrogen bond in the reactive azo dye as an electron acceptor for anaerobic respiration.

The techniques relating to the aseptic handling of *Shewanella* strain J18 143 in aspects of culture storage, cell growth and preparation of resting cells have been well presented (Willmott, 1997; Pearce, 2004).

3.1.2.1 Culture storage

The bacterial strains used were primarily stored in agar slopes (Section 2.1.2.3) as shown in the circle in Figure 3.1.3. A pink layer of the bacteria of *Shewanella* strain J18 143 can be observed on the surface of the slope.

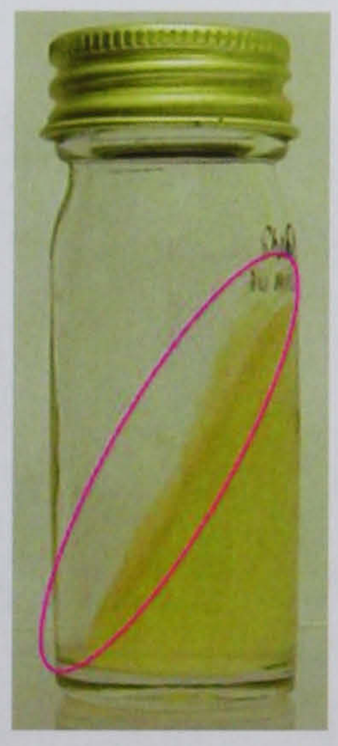


Figure 3.1.3 Bacterial Culture Stored on Agar Slope

Figure 3.1.4 represents the culture of *Shewanella* strain J18 143 that was streaked onto the surface of an agar plate. A pink, streaking layer on the surface of the medium can be seen. Also, colonies of the strains can be recognised from the image. There were two aims in employing the streak-plate technique. One was for short-term storage (see Section 2.1.2.3), whereby the plate can be stored in incubator at 30ºC for a period of up to one week. Another purpose, probably the more important one, was to check for the presence and absence of any contaminants (Section 2.1.2.2).

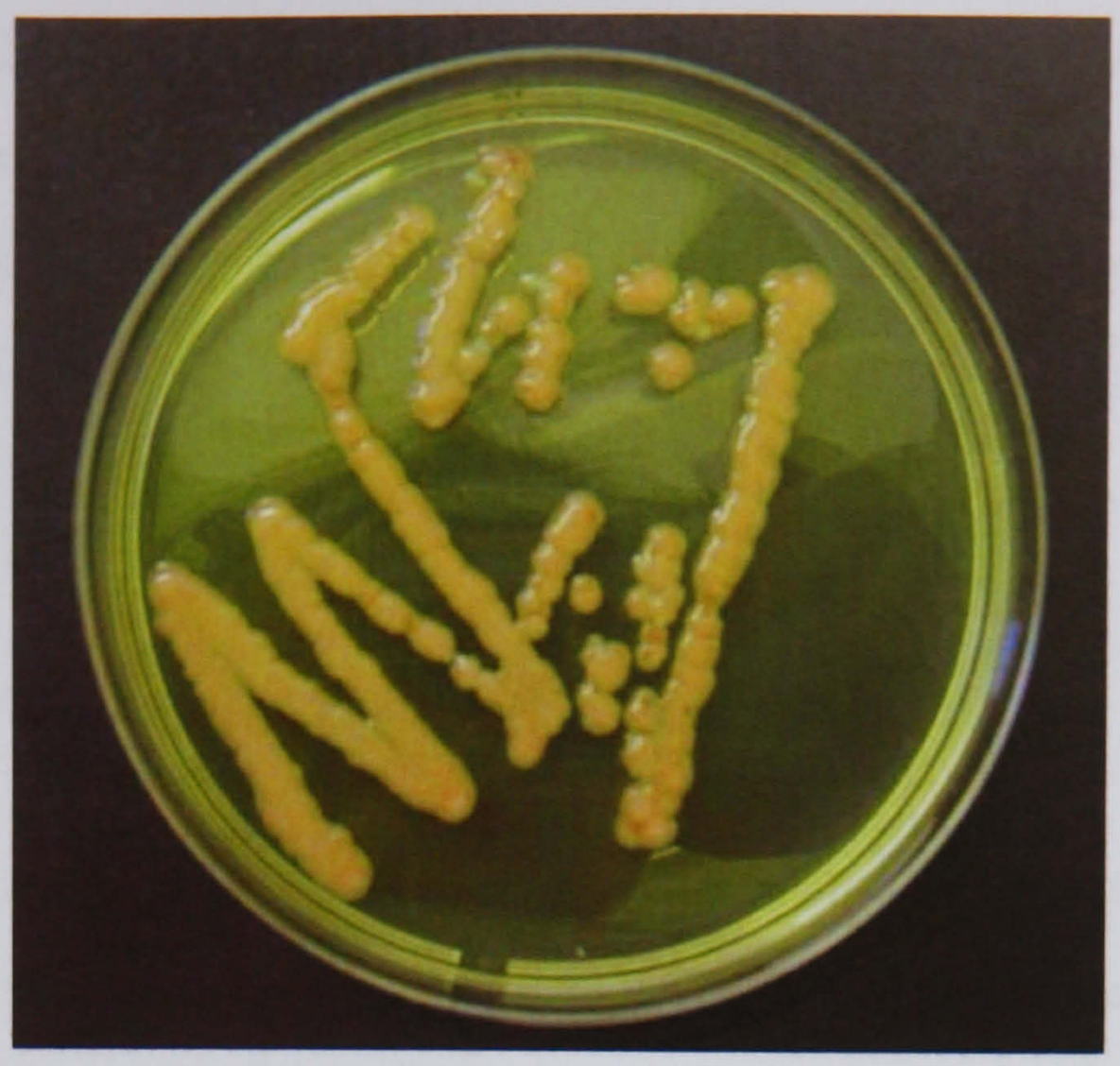


Figure 3.1.4 Bacterial Culture Streaked on a Agar Plate

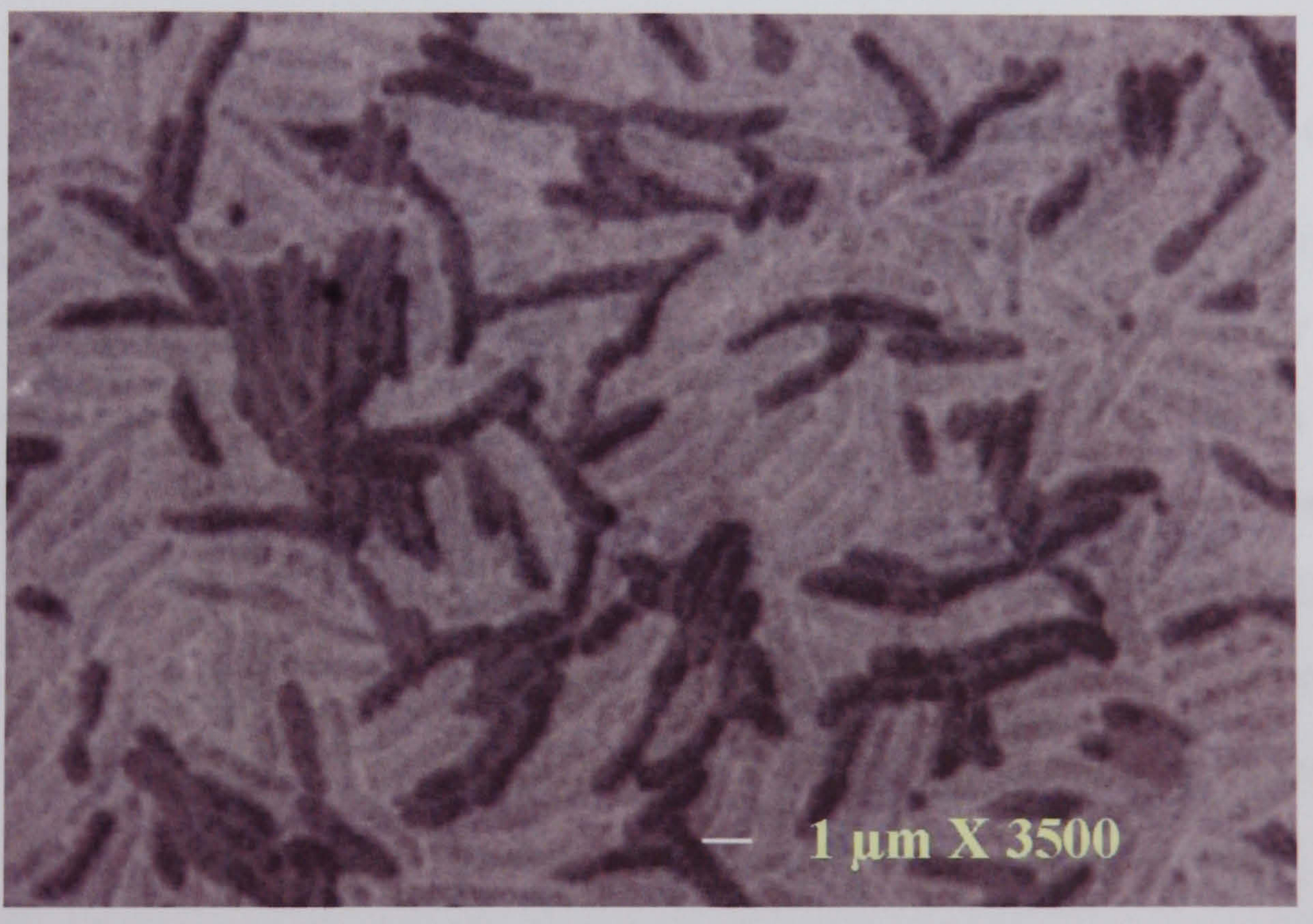


Figure 3.1.5 Scanning Electron Micrograph of Prepared Cell Suspension

The bacterial cells of *Shewanella* strain J18 143 were finally prepared in a cell suspension using aseptic techniques, as introduced in Section 2.1.2.4. The cell suspension prepared was diluted and was investigated using scanning electron

microscopy (SEM). An electron beam intensity of 15 kV was used. Figure 3.1.5 contains the image obtained from the SEM analysis. The image represents the cell suspension that is shown in layers of the individuals, indicating the basic information that the strain is rod shaped. The sharp definitions, shown in the images, suggested that whole cells are being viewed.

3.1.3 Immobilisation of *Shewanella* strain J18 143

Immobilisation of the *Shewanella* strain J18 143 cells was performed using three methods (Klein and Wagner, 1983; Gil, 1983; Willmott, 1997). The results from the immobilisation of the bacterial cells using the method of "growing-in", that of physical adsorption and that of chemical coupling are discussed throughout Section 3.2.

3.1.3.1 Attempts at protein assay of immobilised *Shewanella* strain J18 143

Attempts were made to establish SEM images of cells that had been immobilised on the cellulose copolymer substrate. However, the cells cannot be "seen" under SEM. This was thought, in part to be due to the heterogeneous nature of the matrix of the grafted cellulose, or because of the location of the cells being immobilised. Therefore, an alternative way of the establishing the presence of the immobilised cells was attempted. This was based on a protein assay procedure. A further problem concerned the method of the protein assay of the immobilised cells. Several methods are quoted in the literature (von Canstein, personal communication, 2005; Ramachandran, 2004). Each of these was tried. None of them provided consistently satisfactory results. The focus was then moved from quantitative analysis to qualitative analysis. Thus, protein assays of the immobilised cells were carried out using the method based on the bicinchoninic acid (BCA) assay as used in the protein assay of the planktonic cells.

The principle of the bicinchoninic acid (BCA) assay is similar to that which underpins the Lowry procedure, in that both rely on the formation of a Cu^{2+}–protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+} (www.sigmaldrich.com). BCA forms a purple-blue complex with Cu^{1+} in alkaline environments. The reduction of alkaline Cu^{2+} by proteins giving an absorbance maximum 562 nm was then monitored by UV-visible spectroscopy. The procedures of the protein assay of the immobilised cells were described in Section 2.5.5.2. The calibration plot of protein standard solution is shown in Figure 3.1.6.

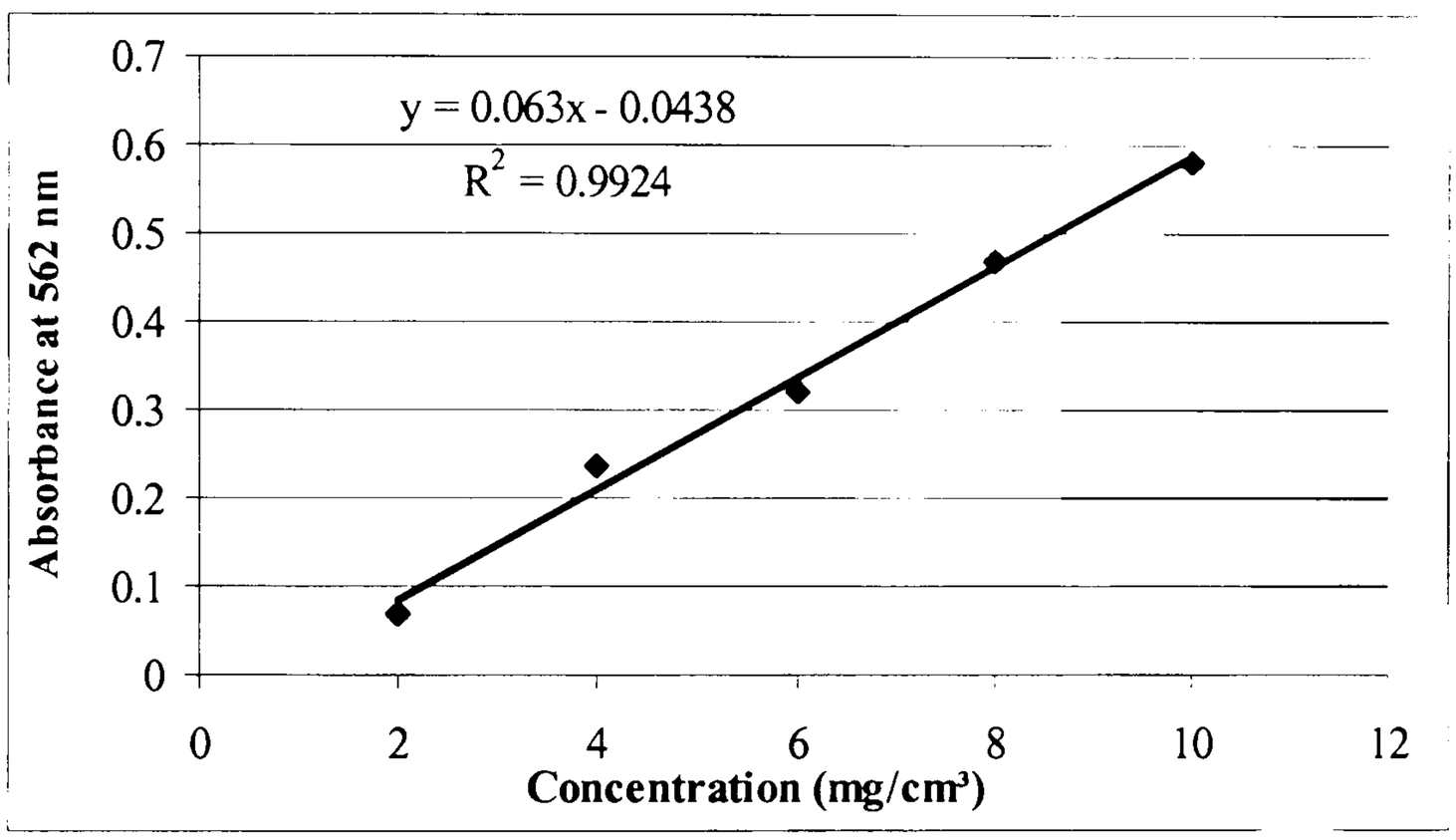


Figure 3.1.6 Calibration Plot of Standard Protein Solution Assay

The measuring results of each unit from the 96-well plates are shown in Table 3.1.1. The data in Table 3.1.1 from A1 to A6 concern the measured results from the analysis of the standard protein solution. The standard protein solutions were prepared in duplicate as five known concentration sets. The measured absorbance values are plotted against the known concentrations of the standard protein solutions. B1 to B6 are the measured results of the protein concentration determination of the immobilised cells in which the cells were immobilised by the method of physical adsorption. C1 to C6 indicate the measured results of the protein concentration of the immobilised cells in which the cells were immobilised by the method of chemical

coupling. D1 to D6 are the measured results of the protein concentration determination of the immobilised cells in which the cells were immobilised by the method of "growing-in".

Table 3.1.1 Results from Protein Analysis of Immobilised Bacterial Cells

Sample		A Standard Protein Solution	B Cells Immobilised by Adsorption	C Cells Immobilised by Coupling	D Cells Immobilised by Growing-in
1	Absorbance	0.056	0.099	0.273	0.468
	Cal.C. (mg/cm^3)	–	2.275	5.087	8.238
	Absorbance	0.081	0.184	0.215	0.488
	Cal.C. (mg/cm^3)	–	3.648	4.149	8.562
2	Absorbance	0.237	0.158	0.238	0.504
	Cal.C. (mg/cm^3)	–	3.228	4.521	8.82
	Absorbance	0.233	0.159	0.251	0.53
	Cal.C. (mg/cm^3)	–	3.244	4.731	9.24
3	Absorbance	0.319	0.139	0.218	0.408
	Cal.C. (mg/cm^3)	–	2.291	4.198	7.269
	Absorbance	0.319	0.149	0.218	0.43
	Cal.C. (mg/cm^3)	–	3.083	4.198	7.624
4	Absorbance	0.414	0.073	0.117	0.238
	Cal.C. (mg/cm^3)	–	1.855	2.566	4.521
	Absorbance	0.467	0.101	0.131	0.255
	Cal.C. (mg/cm^3)	–	2.307	2.307	4.796
5	Absorbance	0.587	0.089	0.156	0.257
	Cal.C. (mg/cm^3)	–	2.065	3.196	4.828
	Absorbance	0.582	0.089	0.156	0.201
	Cal.C. (mg/cm^3)	–	2.113	3.196	3.923
6	Absorbance	–	0.065	0.158	0.277
	Cal.C. (mg/cm^3)	–	1.725	3.228	5.152
	Absorbance	–	0.069	0.165	0.242
	Cal.C. (mg/cm^3)	–	1.79	3.341	4.586

Note: "Cal.C." is the "calculated concentration" obtained from the plate reader.

The samples of the cellulosic graft copolymer that contained the immobilised cells, represented within each column (B, C and D in Table 3.1.1) were dupicately taken

from six identical evaluation sets (1 to 6). However, it can be seen that the concentrations of the protein that was calculated for the immobilised cell systems, as indicated in Table 3.1.1, are not consistent across the two batches. These results imply that the protein concentration of the immobilised cells of each individual sample used in the subsequent colour removal systems, measured using this method, cannot be deemed to be reliable, to a sufficient degree of accuracy. Therefore, the use of the BCA protein assay kit was only used to provide evidence that the bacterial cells, *Shewanella* strain J18 143, were immobilised on the substrate of the cellulosic graft copolymer. Further study will need to be undertaken on the basis of the results obtained, as presented. The assay shows that the cells are obviously present even if their presence cannot be quantified.

3.2 Decoloration of Remazol Black B solutions using immobilised *Shewanella* strain J18 143 cells

Remazol Black B is characteristic of a bifunctional reactive azo dye. It is an azo-ketohydrazone chromophore and two vinyl sulphone reactive centres that can potentially bind with suitable fabrics/substrates. In the area of wastewater treatment, Remazol Black B is a well known surrogate for non-biodegradable reactive azo dyes (Pearce, 2004). Standard solutions of Remazol Black B were chosen as the target of the treatment of colour removal in this instance. Initial studies of the decoloration of solutions of Remazol Black B, involving the immobilised culture, *Shewanella* strain J18 143, were conducted using cellulose-methacrylic acid copolymers as the support system. The procedures of immobilisation of the culture onto this supporting matrix were described in Section 2.1.3.

3.2.1 Calibration curve of the solutions of Remazol Black B

The calibration plot of the Remazol Black B dye solutions was made at known dye concentrations, as shown in Figure 3.2.1. The concentrations of the dye solutions were prepared at 0.01, 0.02, 0.03, 0.05 and 0.06 g dm^{-3}. Figure 3.2.1 is an example of a calibration curve for the dye solution of Remazol Black B that was constructed. A calibration curve was made every time a fresh Remazol Black B dye solution was made up.

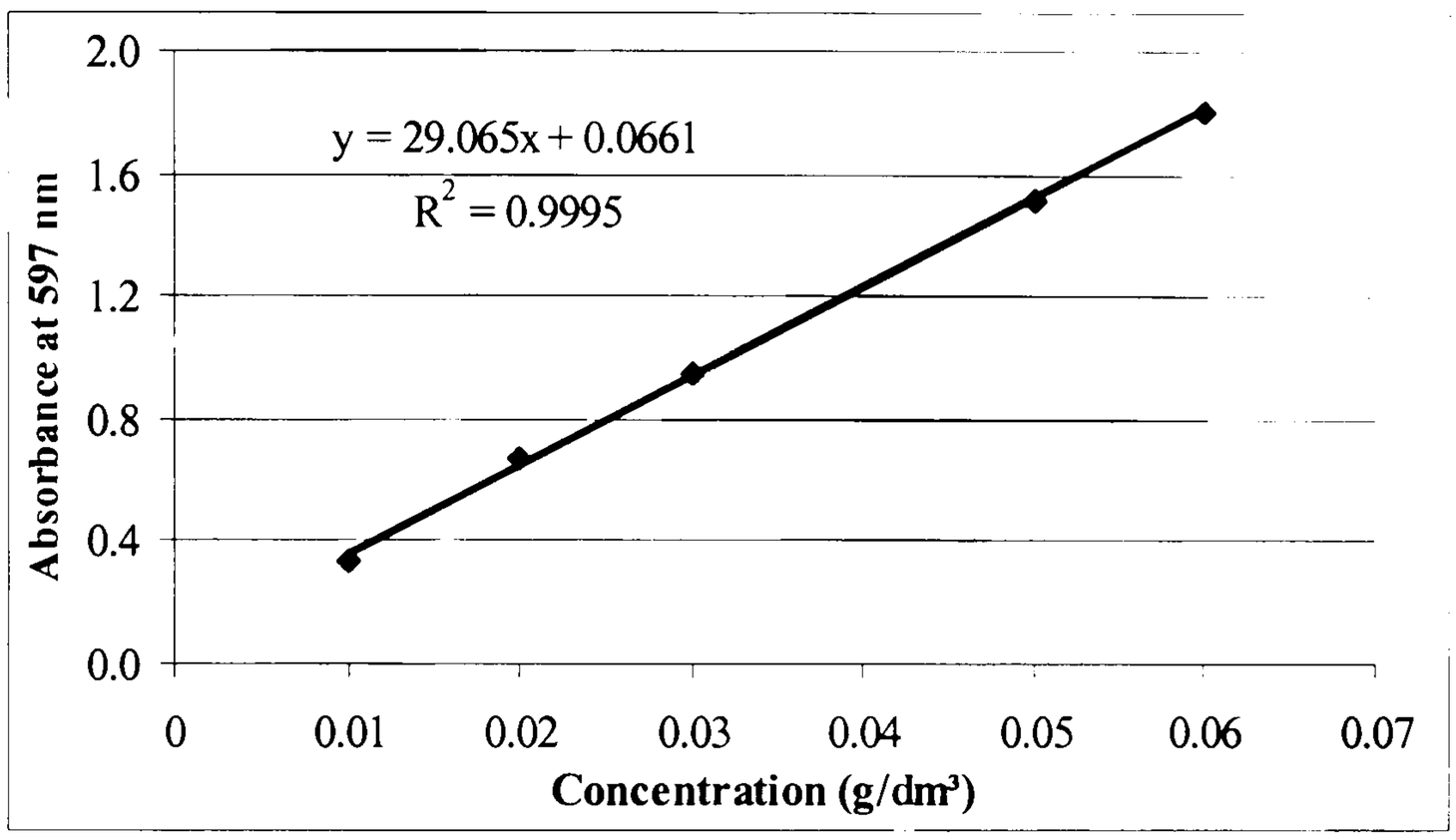


Figure 3.2.1 Calibration Curve of Dye Solutions of Remazol Black B

The Lambert-Beer law was applied that describes the relationship between the absorbance value for the colorant in solution, the path length of the colorant solution and the concentration of colorant in solution:

$$A = \log_{10}^{(I_0/I_T)} = \varepsilon \cdot c \cdot l$$

Here, I_0 – Intensity of incident light at a particular wavelength

I_T – Intensity of transmitted light at a particular wavelength

A – Absorbance of colorant at a particular wavelength

ε – Absorbation coefficient

c – Concentration of the colorant

l – Path length of the light travels through colorant solution

The absorpation coefficient, ε, is constant. For standard cuvettes used in the experiments to give constant l values, the Beer-Lambert law may be expressed as follows:

$$c \alpha A$$

It can be seen that the Lambert-Beer law applies to the calibration curve of the solutions of Remazol Black B, as indicated by the linear plot shown in Figure 3.2.1. This calibration plot can then be used to determine unknown concentrations of solutions containing the same colorant sample, after measurement of absorbance.

3.2.2 Visual appraisal arising from the decoloration of the Remazol Black B solutions by the immobilised cells of *Shewanella* strain J18 143

Investigations of colour removal of dye solutions of Remazol Black B, using cellulosic copolymers with immobilised bacterial cells, have been carried out following the procedures described in Section 2.2.4. Relative standards were established as controls for evaluating the effects of the decoloration of the dye solutions. The effects of the decoloration of the dye solutions and of the relative standards, by the immobilised cells were recorded photographically by digital camera means.

The samples used in investigating the decoloration of Remazol Black B and the relative established standards were photographed, and are given as Figure 3.2.2.

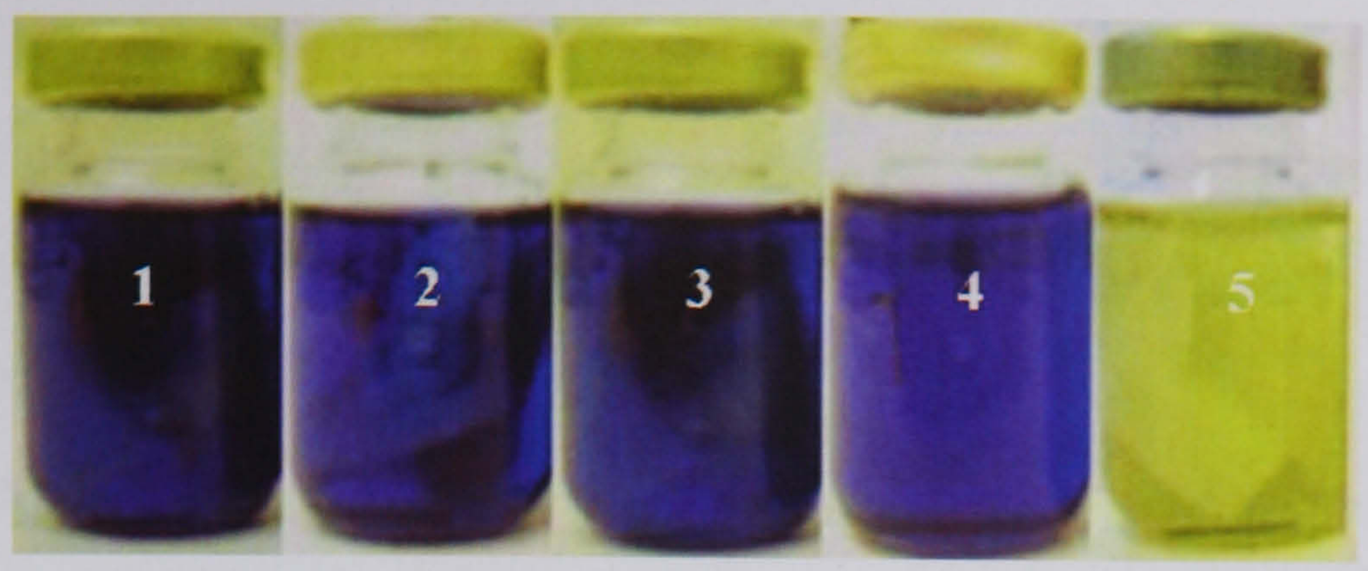


Figure 3.2.2 Visual Results from the Decoloration of Dye Solutions of Remazol Black B. The images of each evaluated sample were taken over different periods of incubation: (a) 20 minutes; (b) 24 hours; (c) 96 hours; (d) 168 hours respectively. "Sample 1" contained the dye solution treated with immobilised cells by the method of "growing-in"; "Sample 2" contained the dye solution treated with immobilised cells by the method of coupling; "Sample 3" contained the dye solution treated with immobilised cells by the method of adsorption; "Sample 4" is the dye solution standard of Remazol Black B and "Sample 5" is the immobilised cells standard.

In "Sample 1", the vial contained the standard dye solution together with the immobilised cells. The cells were immobilised as "growing-in" (Section 2.1.3.1) within the matrix of the cellulosic graft copolymer in the growth medium, i.e. if the cells had become attached, they were fixed/immobilised by the method of adsorption/entrapment. "Sample 2" contained the dye solution and the graft copolymer support linked via chemical coupling of the immobilised bacterial cells (Section 2.1.3.3). "Sample 3" contained the dye solution and the grafted cellulose with the physically adsorbed cells (Section 2.1.3.2). "Sample 4" represents the control of the standard dye solution that contained the sodium fomate and the AQDS without any addition of biological cells, as stated in Section 2.2.3.2. "Sample 5" was made up as the standard of the immobilised cells that contained the cellulosic graft copolymer immobilised cells, the sodium formate and the AQDS with the absence of the dye solution, as described in Section 2.2.3.1.

All of the vials contained the Remazol Black B dye solution (50 μM), the electron donor solution of sodium formate (21 mM), the electron shuttle of solution of anthraquinone-2,6-disulphonic acid (AQDS, 100 μM) and a specified amount of phosphate buffer saline (P.B.S., 10 mM pH7) solution, as stated in Section 2.2.4. It can be seen from the images that the standard of the immobilised cells appeared a colour of yellow and was not colourless. The yellow coloration in the solution was supposed to be due to the presence of cytochromes in the solution. The cytochromes are more likely released by a certain amount of cell lysis that occurred during the treatment (Pearce, 2004) that was used in preparing the compositions.

As can be seen from Figure 3.2.2, all of the samples treated with immobilised cells were decolorised to some degree. Comparing the decoloration results of "Samples" of 1, 2 and 3 visually, the cells immobilised onto the grafted cellulose by the method of "growing-in" seem to provide a faster and more efficient method of colour removal. The colour of the sample that was placed with the cells that were immobilised by adsorption was not completely removed. The process of decoloration by the cells that were immobilised by coupling was slower than that obtained with

the cells that were located by "growing-in" adsorption/entrapment. One problem needs to be recognised in that although these three immobilisation processes were undertaken with the same concentration of the cell suspension and the same amounts of grafted cellulose were used, the biomasses of the immobilised cells would be unlikely to have been identical, despite the precautions that were taken. This could explain the differences described above. It should be noted that, despite these differences, effective colour removal was observed.

3.2.3 Results from the established standard controls

As shown in Figure 3.2.2, the immobilised cells have a positive, desired effect on decoloration of the azo dye solutions. There are some factors that need to be considered with respect to the influence of each factor or component on the decoloration of the dye solution. Therefore, several controls, relating to the decoloration through the utilisation of immobilised cells, were established for interpreting the results obtained (Section 2.2.3).

The established standards were named as, the immobilised cells standard, the dye solution standard, the cell growth medium standard, the grafted cellulose standard and the free cells standard. The photographic results from the dye solution standard (Sample 4) and the immobilised cells standard (Sample 5) were shown in Figure 3.2.2. The photographic results of the rest of the established standards are given in the following sections.

3.2.3.1 Standard of the cell growth medium solution

The immobilisation of bacterial cells by the "growing-in" method (Section 2.3.1.1) was carried out by immersing the grafted cellulose samples, together with the cell culture, into cell growth medium. There could have been a small amount of residual T.S.B. solution in the sample, after washing. Thus, the influence, if any, of the T.S.B. on the decoloration of the dye solutions of Remazol Black B needed to be considered.

"Sample 6" relates to the standard control of Tryptone Soy Broth (T.S.B.) solution in which the composition is corresponding to the parallel control of "Sample 1" (see Section 3.2.1) as described in Section 2.2.3.1. The system contained the "treated" graft cellulosic copolymer (the graft cellulosic copolymer underwent the procedures of immobilisation of the method of "growing-in" with the absence of bacterial cells), Remazol Black B solution (50 μM), the electron donor solution of sodium formate (21 mM), the electron shuttle solution of anthraquinone-2,6-disulphonic acid (AQDS, 100 μM) and a certain known amount of phosphate buffer saline (P.B.S., 10 mM pH7) solution.

It can be concluded that the T.S.B. solution has no effect on the colour removal from the solutions of Remazol Black B, as seen from Figure 3.2.3. However, the tryptone soy broth is a very rich medium that can be used for growing a wide range of bacteria. Thus, strict aseptic environments for the experiments need to be applied with careful handling during the processes. These conditions were applied in the current work.

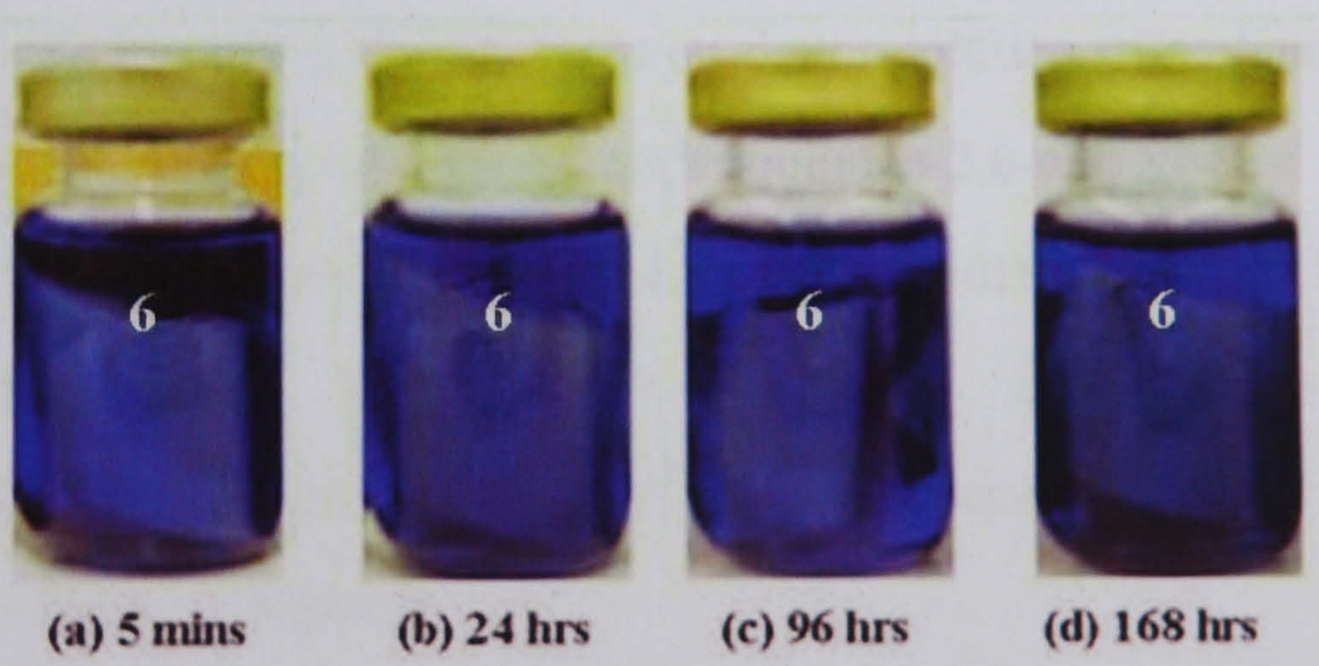


Figure 3.2.3 Established Standard of Immobilisation Substrate-Contains Solutions of Tryptone Soy Broth (Sample 6). Figure above gives the images of the standards that were taken in different periods: (a) 5 minutes; (b) 24 hours; (c) 96 hours; (d) 168 hours after assembly.

3.2.3.2 Standard of graft cellulosic copolymer

A point needs to be considered as to whether or not the immobilisation substrate has effect on the decoloration of the Remazol Black B dye solution. This point was

investigated by establishing the standard of graft cellulosic copolymer, as illustrated in Section 2.2.3.4.

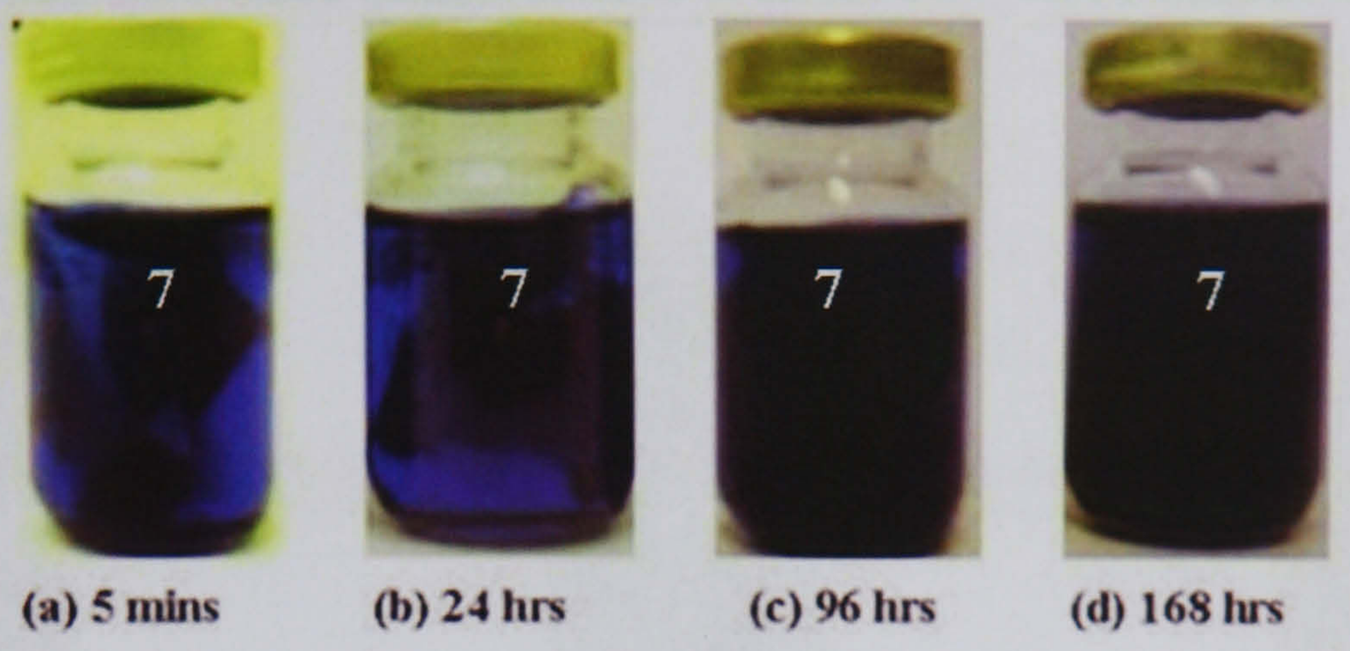


Figure 3.2.4 Established Standard of Immobilisation Substrate (Sample 7). The above figure represents the images of the standards, which they were taken in different period: (a) 5 minutes; (b) 24 hours; (c) 96 hours; (d) 168 hours respectively.

"Sample 7" represents the grafted cellulose standard that contained the same amount of graft copolymer support as those samples represented in Figure 3.2.2, the Remazol Black B dye solution (50 μM), the electron donor solution of sodium formate (21 mM), the electron shuttle solution of anthraquinone-2,6-disulphonic acid (AQDS, 100 μM) and a certain known amount of phosphate buffer saline (P.B.S., 10 mM, pH7) solution. Figure 3.2.4 shows that the immobilisation substrate, cellulose-methacylic acid graft copolymer, has no influence on colour removal from the solution of Remazol Black B.

3.2.3.3 Decoloration of solutions of Remazol Black B using free resting *Shewnaella* strain J18 143 cells

The removal of colour from the Remazol Black B solutions using free resting cells of *Shewanella* strain J18 143 has been well documented (Willmott, 1997; Kamilaki, 2000; Pearce, 2004). The bacterial cells exhibited good ability in removing colour by degrading the chromophore of the dye. In qualifying the technique of using immobilised biological cells in decoloration of reactive dye solutions, free cells

standards were established (Section 2.2.3.3). It should be noted from "Sample 8", that the reduction of the black dye solution was rapidly achieved by the free biological cells. Actually, the colour removal started before the nitrogen degassing was complete, as indicated in Figure 3.2.5.

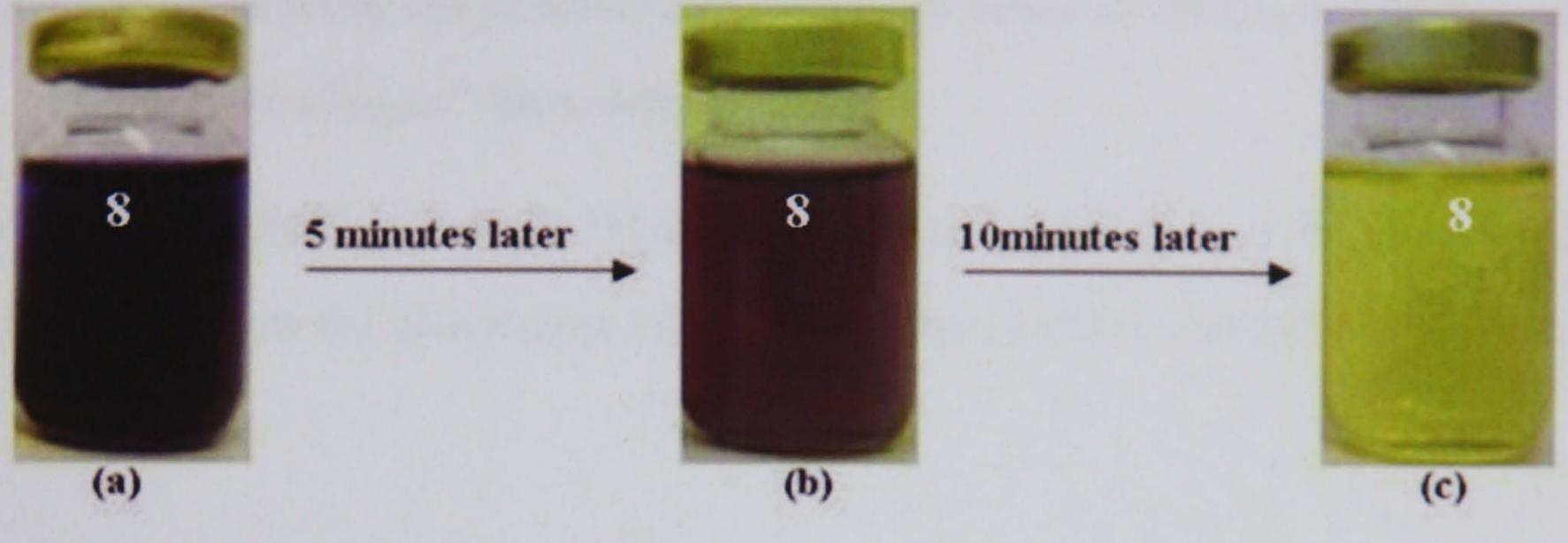


Figure 3.2.5 Decoloration Flow Profile for the Decoloration with Free *Shewanella* Cells (Sample 8). Images of the standards that were taken after different periods of incubation (a) 10 seconds after injection of bacterial cells; (b) 5 minutes later; (b) 10 minutes later still.

The decoloration of the dye solution with free cells was shown to be much faster than that which was achieved with the grafted cellulose immobilised cells. However, the "concentration" of the cells used for free cells standard was likely to be much greater than that used with the immobilised cells. The colour of the Remazol Black B dye solution was removed completely by the immobilised biological cells of *Shewanella* strain J18 143, within 24 hours of incubation. This result has shown much potential in using the chemical modified cellulose as an immobilisation substrate in the decoloration systems.

3.2.4 Measuring results from the decoloration of Remazol Black B solutions using immobilised cells

All of the experiments were carried out in triplicate and were repeated at least three times. The results below here are average values.

UV-visible spectophotometry (Section 2.5.3) was used as the main analysis support for evaluating the nature and the extent of decoloration of the dye solutions, as described in this section and also the following sections (Section 3.3 and Section 3.4).

3.2.4.1 Results from the decoloration of the of Remazol Black B solution using the "growing-in" immobilised cells

The numerical results shown in Figure 3.2.6, which correspond to the "Sample 1" in Figure 3.2.2, show the absorbance versus time behaviour for the respective systems.

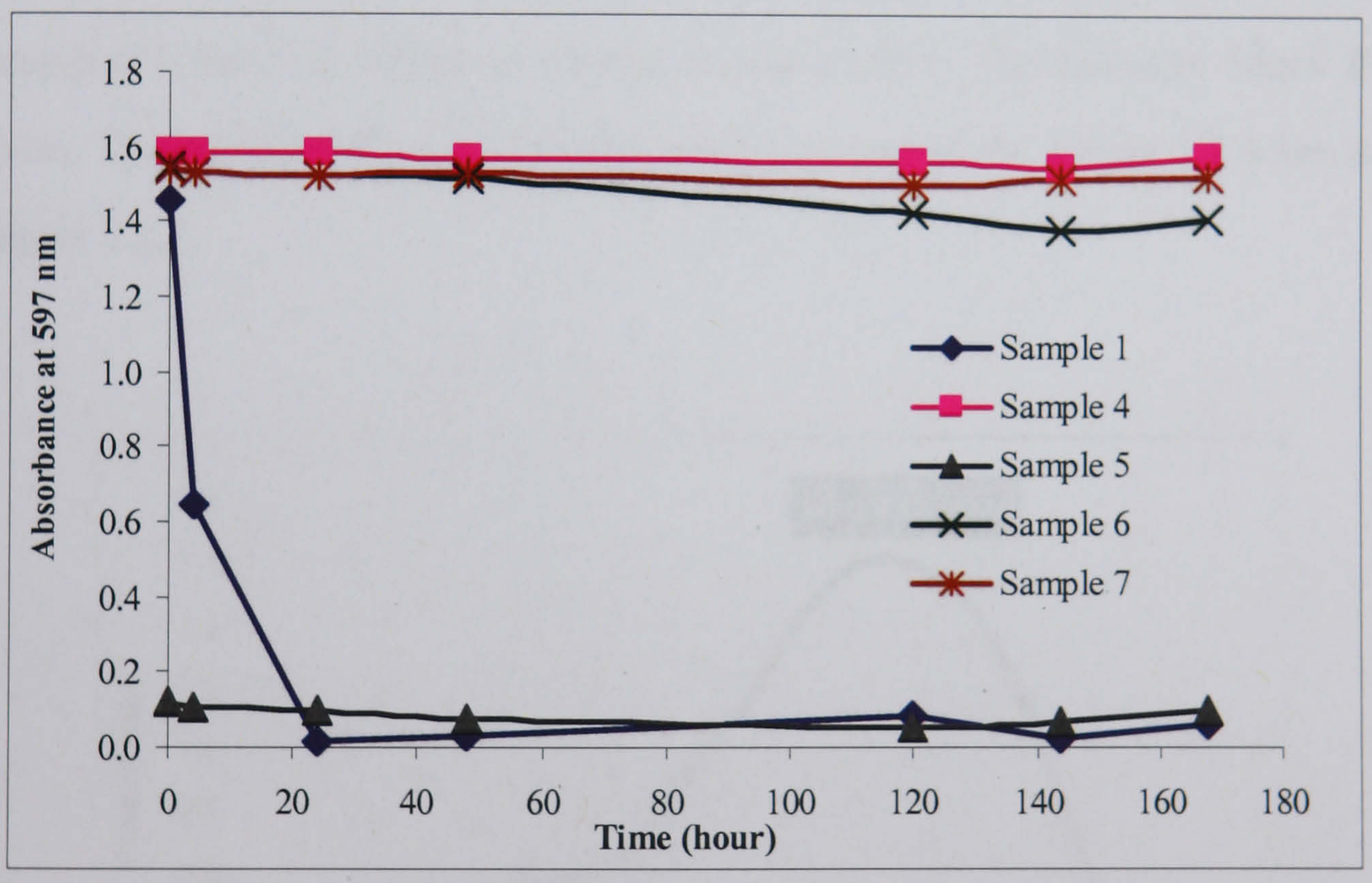


Figure 3.2.6 Results from the Decoloration of the Remazol Black B Dye Solution using Immobilised Biological Cells ("Sample 1" contained the dye solution that was treated with the "growing-in" immobilised cells; "Sample 4" is the dye solution standard; "Sample 5" is the standard solution that contained the immobilised cells; "Sample 6" contained the standard solution of the growth medium of the bacterial colony; "Sample 7" contained the control of the cellulosic graft copolymer)

All of the samples that contained the dye solution are at same concentration of the dye solution, i.e. the initial absorbance values of the samples ("Sample 1", "Sample

4", "Sample 6" and "Sample 7") are the same. However, "Sample 1", "Sample 7" and "Sample 6" exhibited relatively lower concentrations of the dye in the free solution than that of the "Sample 4". This is possibly because these three samples contained pieces of cellulosic graft copolymer that might bring a tiny amount of aqueous medium into the vials when being transferred from their washing containers to the vials. This point needs to be taken into account also when considering the results shown in Figure 3.2.8 and in Figure 3.2.10.

Figure 3.2.6 shows that the colour of the dye solution is removed completely within 24 hours by the "growing-in" immobilised cells. This result is also indicated in Figure 3.2.2. Compared with the standard of the dye solution ("Sample 4"), the cellulosic graft copolymer ("Sample 7") and the growth medium of the bacteria ("Sample 6") have no influence on the decoloration of the Remazol Black B dye solution. The corresponding UV-visible spectra to that of the Figure 3.2.6 are shown in Figure 3.2.7.

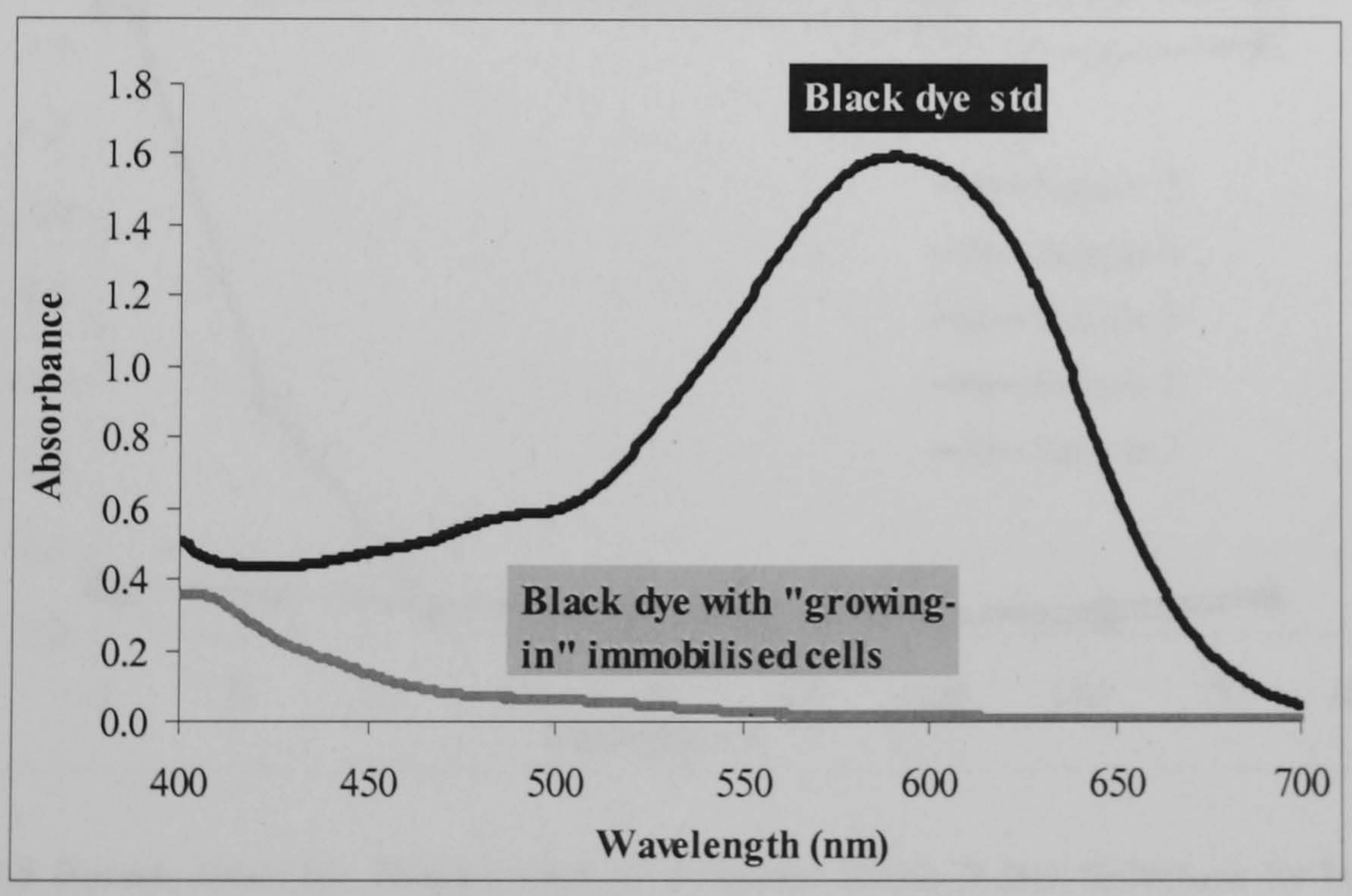


Figure 3.2.7 UV-Visible Spectra for Dye Solutions of Remazol Black B after 24 Hours Incubation without (Black dye std) and with (Black dye with "growing-in" immobilised cells) "Growing-in" Immobilised Cells of *Shewanella* Strain J18 143 at 30°C

It can be seen from the UV-visible spectra in Figure 3.2.7, that the visible peak at 597 nm was completely removed after 24 hours of incubation with the biological cells of *Shewanella* strain J18 143. This is no doubt an indication of complete removal of colour from the treated dye containing system. Figure 3.2.7 only provided the spectra in the visible region, 400 nm – 700 nm. The UV region was investigated by Pearce (2004). A significant increase in absorbance in the UV region was found that corresponds to the reduction of the azo chromophore and the formation of aromatic amines. The aromatic amines were formed by the reduction of the azo/ketohydrazone bonds in the dye molecules.

3.2.4.2 Results from the decoloration of the Remazol Black B solution using the chemically coupled immobilised cells

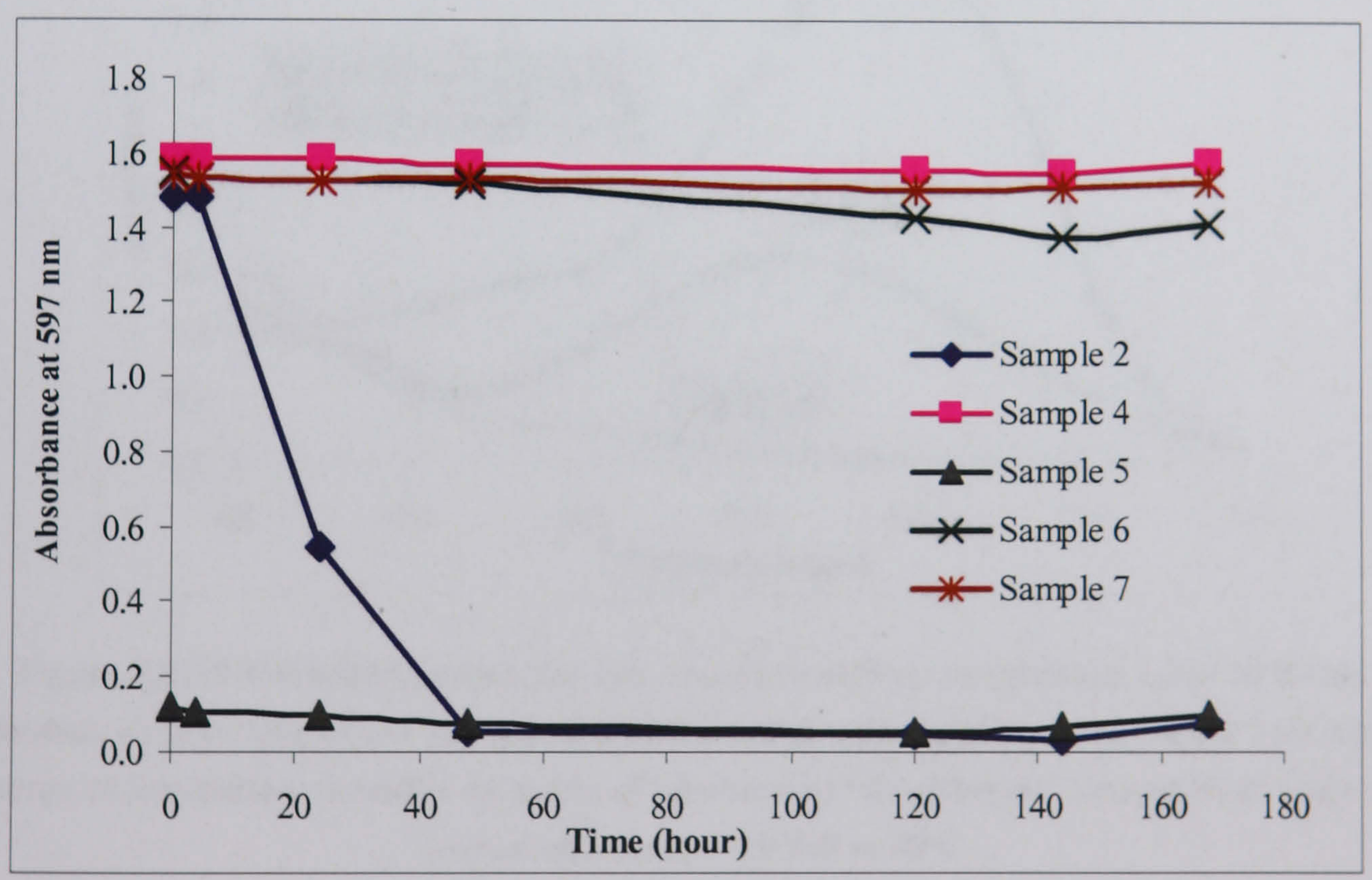


Figure 3.2.8 Results from the Decoloration of Remazol Black B Dye Solutions by Immobilised Biological Cells ("Sample 2" contained the dye solution treated with chemically coupled cells; "Sample 4" is the dye solution standard; "Sample 5" is the standard solution that contained the immobilised cells; "Sample 6" contained the standard solution of the growth medium of the bacterial cells; "Sample 7" contained the control of the cellulosic graft copolymer)

The results given in Figure 3.2.8, which correspond to the decoloration study of "Sample 2" in Figure 3.2.2, show the absorbance versus time behaviour for the respective systems. In "Sample 2", the biological cells were immobilised by the method of chemical coupling, as described in Section 2.1.3.3. It can be seen from the Figure 3.2.8 that the removal of the colour was complete compared with that of the standard of the immobilised cells. However, the decoloration that occurred was relative slow compared with that of the "growing-in" immobilised cells. The complete decoloration took about 48 hours of incubation. As stated above, this relative slowness in the decoloration process was more likely to be due to the lower concentration of the immobilised biological cells that existed in the system.

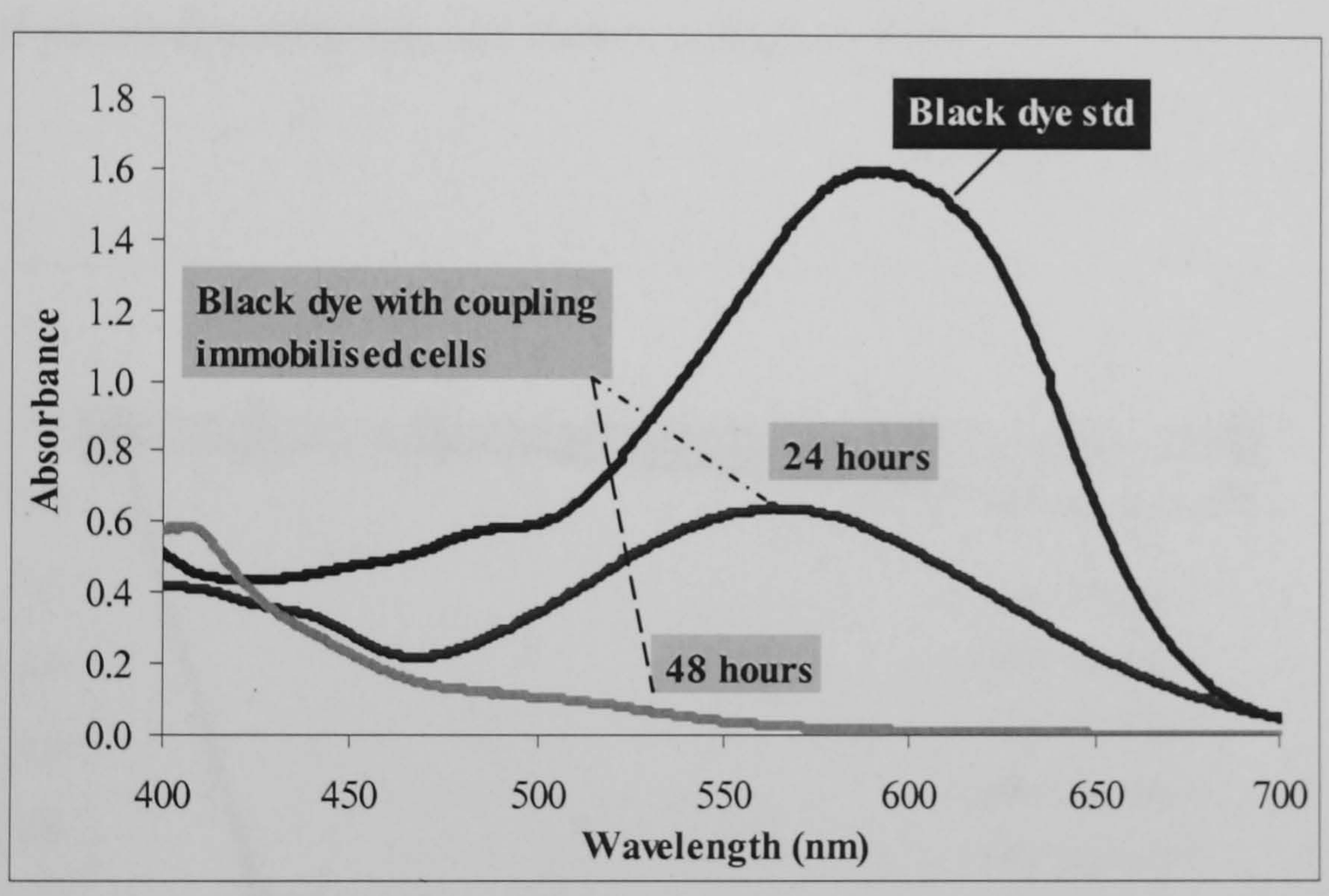


Figure 3.2.9 UV-Visible Spectra for Dye Solutions of Remazol Black B after 24 Hours Incubation without (Black dye std) and with (Black dye with coupling immobilised cells after 24 hours of incubation and after 48 hours of incubation) "Growing-in" Immobilised Cells of *Shewanella* Strain J18 143 at 30°C

The UV-visible spectra for the Remazol Black B solution, treated with immobilised *Shewanella* strain J18 143, by the immobilising method of chemical coupling, are shown in Figure 3.2.9. In the UV-visible spectrum for the dye solution containing chemically coupled cells after 24 hours of incubation (Figure 3.2.9), the absorbance

in the visible region at the λ_{max} for Remazol Black B (597 nm) had been reduced. However, there was still a significant peak in the visible region ("24 hours"). This peak represented a hypsochromic shift in the spectrum as a result of a structural change in the dye molecule. The colour of the dye solution was removed completely after 48 hours of incubation with the coupled cells as seen from the spectrum in Figure 3.2.9 ("48 hours").

3.2.4.3 Results from the decoloration of the Remazol Black B solution using the physically adsorbed immobilised cells

The results of the absorbance measurements associated with the decoloration of the Remazol Black B solution using immobilised biological cells, by the immobilising method of physical adsorption, are shown in Figure 3.2.10.

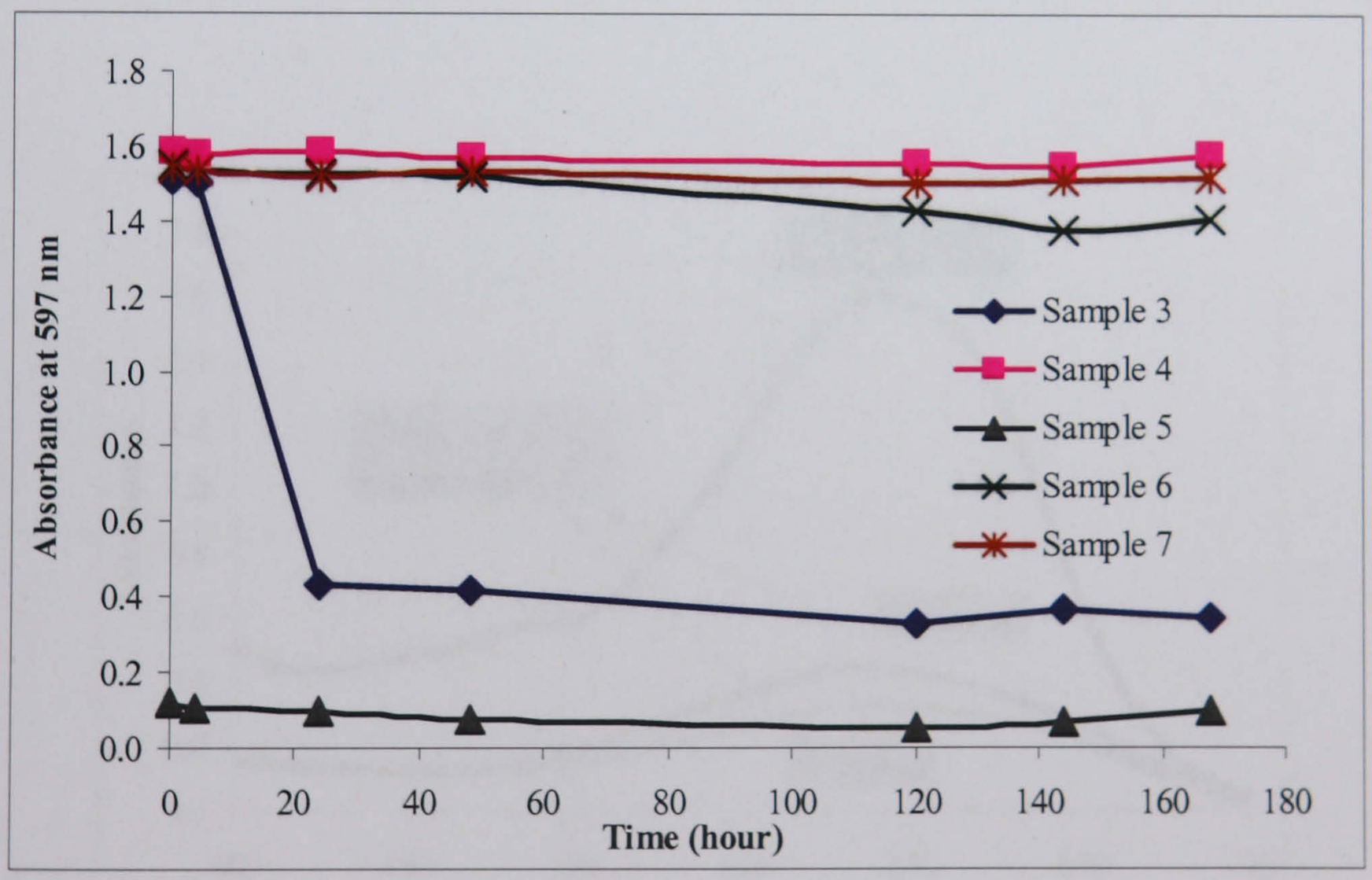


Figure 3.2.10 Results from the Decoloration of Remazol Black B Dye Solutions using the Immobilised Biological Cells ("Sample 3" contained the dye solution treated with the physically adsorbed cells; "Sample 4" is the dye solution standard; "Sample 5" is the standard solution containing the immobilised cells; "Sample 6" contained the standard solution of the growth medium of the bacterial cells; "Sample 7" contained the control of the cellulosic graft copolymer)

It can be seen from the curve of "Sample 3" that there were almost no change in colour within the first few hours. The colour was reduced at a relative slower rate than that of the "Sample 1" and that of "Sample 2". Complete decoloration was not achieved by "Sample 3".

The UV-visible spectra for the Remazol Black B dye solution treated with immobilised *Shewanella* strain J18 143, by the immobilising method of physical adsorption, are shown in Figure 3.2.11. In the UV-visible spectra for the dye solution containing chemically coupled cells after 24 hours of incubation and after 48 hours of incubation (Figure 3.2.11), the absorbance in the visible region at the λ_{max} for Remazol Black B (597 nm) had been reduced. There were still significant peaks that can be seen in the visible region ("24 hours" and "48 hours" in Figure 3.2.11). These peaks represented a hypsochromic shift in the spectrum as a result of a structural change in the dye molecule and also represent the fact that the colour of the dye solution was not completely removed.

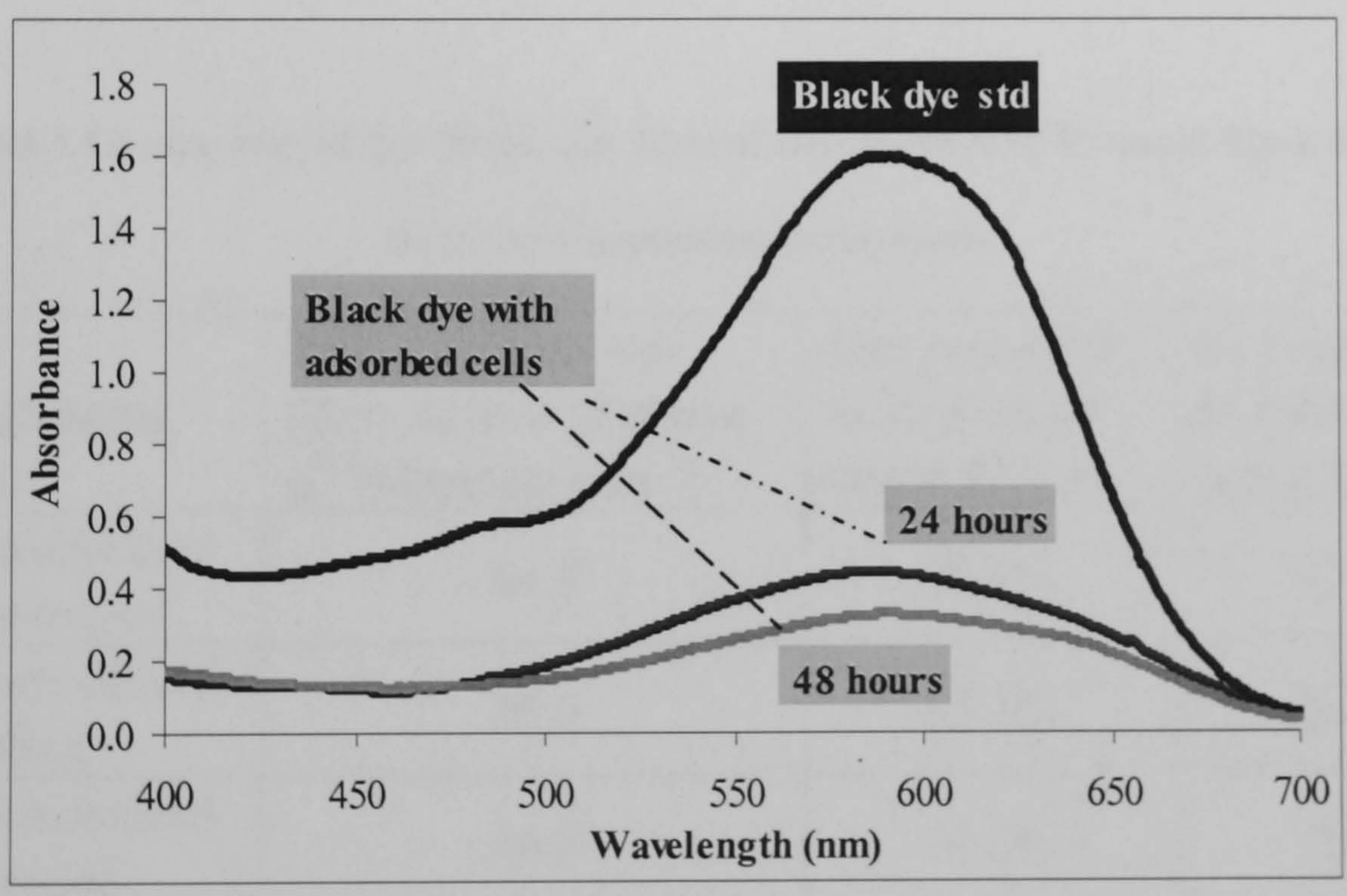


Figure 3.2.11 UV-Visible Spectra for Dye Solutions of Remazol Black B after 24 Hours Incubation without (Black dye std) and with (Black dye with adsorbed cells after 24 hours of incubation and after 48 hours of incubation) Physically Adsorbed Cells of *Shewanella* Strain J18 143 at 30°C

3.2.4.4 Comparison of decoloration effect of the immobilised cells between the three immobilisation methods

The results of this study have shown that the method of using grafted cellulose as a support for biological cells immobilisation is practicable and effective in the current context. Comparison of the decoloration results with those of the various samples with those of the two standards (shown in Figure 3.2.6, Figure 3.2.8 and Figure 3.2.10), shows that the optical densities of the dye solution in "Sample 1" (the bacterial cells were immobilised by the method of "growing-in") were reduced very rapidly. The dye solution in "Sample 2" (the bacterial cells were immobilised by the method of chemical coupling) became decolorised relatively slowly. However the extent of decoloration eventually reached the same level as that of "Sample 1". The absorbance of the dye solution in "Sample 3" (the bacterial cells were immobilised by the method of adsorption) gave a poorer result, as shown in Figure 3.2.10. Comparisons of the decoloration efficiency for "Sample 1", "Sample 2" and "Sample 3" are given in Table 3.2.1 and Figure 3.2.12 below.

Table 3.2.1 Comparison of Dye Reduction Rate of Decoloration of Remazol Black B for the Different Immobilisation Methods

Cell immobilisation method	**Dye reduction rate (first 24 hrs) [μM dye g^{-1} (biomass) min^{-1}]**	**Dye reduction in volumetric (after 24 hrs)**	**Dye reduction in volumetric (after 48 hrs)**
Cells immobilised by "growing-in"	66.0	99.0%	97.7%
Cells immobilised by coupling	26.0	63.3%	96.4%
Cells immobilised by adsorption	44.8	71.3%	72.1%

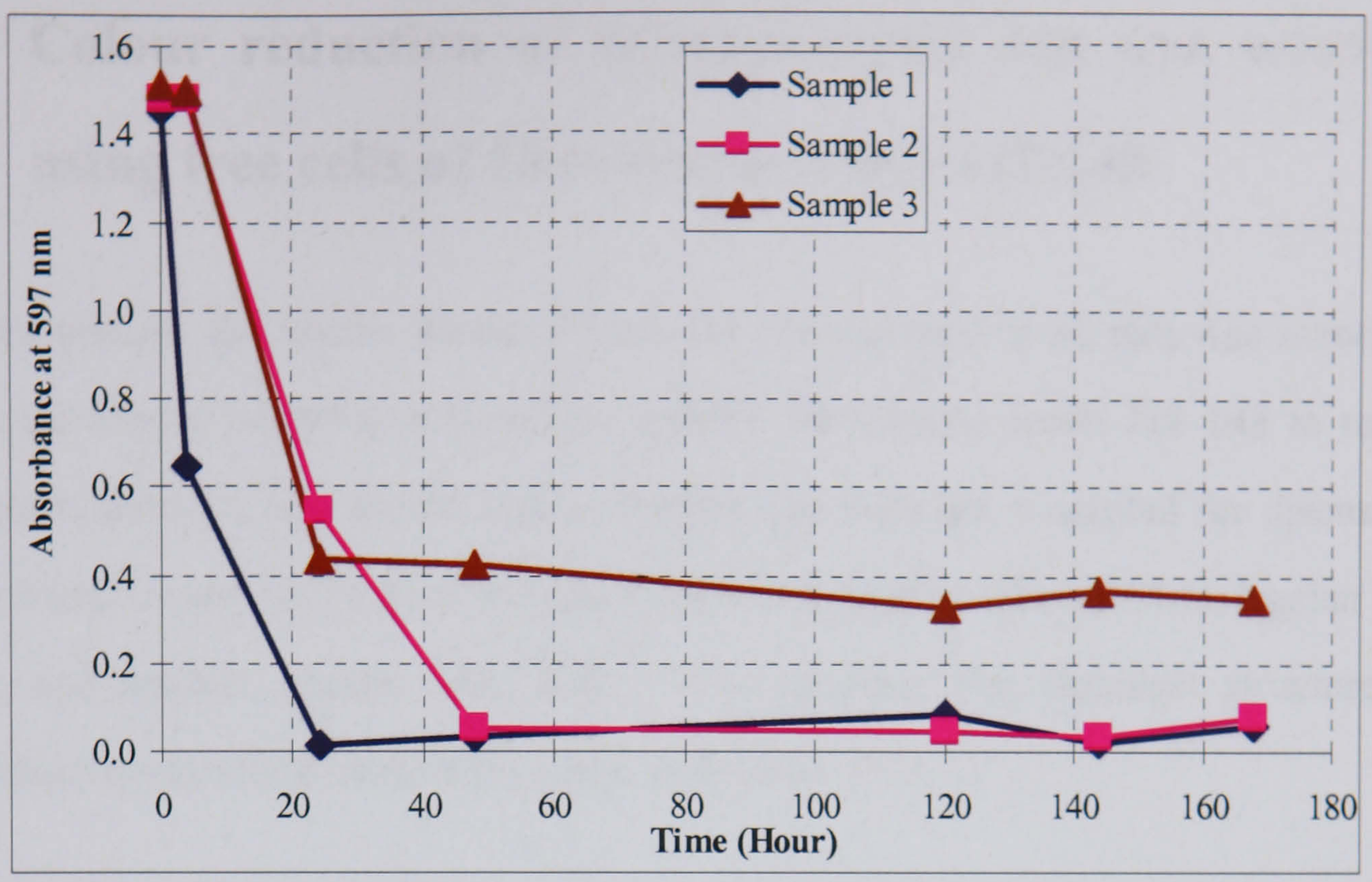


Figure 3.2.12 Comparison of the Effect of Colour Removal from Remazol Black B using Immobilised Cells based on the Three Different Immobilisation Methods ("Sample 1", the cells used in decoloration were immobilised by the method of "growing-in"; "Sample 2", the cells were immobilised by coupling; "Sample 3", the cells were immobilised by adsorption.)

To provide the data given in Table 3.2.1, the dye decoloration rate was calculated from the measuring results for the first 24 hours of incubation, based on the results from the protein assay of immobilised cells (Figure 3.1.6 and Table 3.1.1). The bacteria concentration used under each immobilisation method was an averaged value. It can be seen from Table 3.2.1 that the cells immobilised by the method of "growing-in" provided the most decoloration as evaluated from both the dye reduction rate as seen in the protein assay studies and in the dye reduction as assessed by volumetric means. The cells immobilised by the method of adsorption, in the first 24 hours of incubation, show a higher reduction rate than that of the coupling immobilised cells. However, after 48 hours of incubation, the Remazol Black B was almost completely removed from the system by the cells that were immobilised by coupling but not by the cells immobilised by adsorption. One can conclude that all of the three different methods involving the immobilised cells gave colour removal of the Remazol Black B from the investigated dye solution systems.

3.3 Colour reduction of metal-complex azo dye solutions using free cells of *Shewanella* strain J18 143

In this section, the results obtained from the experimental work that was associated with the use of bacterial cells of the species *Shewanella* strain J18 143 to reduce aqueous solutions of selected metal-complex azo dyes are presented for discussion. Four metal-complex azo dyes, Irgalan Grey GLN, Irgalan Black RBLN, Irgalan Blue 3GL and Irgalan Yellow 3RL KWL, were studied. The chemical structures of chromophores in these dyes were given in Section 2.3.1.1.

3.3.1 Colour reduction of solutions of Irgalan Grey GLN using the free cells of *Shewanella* strain J18 143

The Irgalan Grey GLN is a mixture of azo metal-complex dyes. It contains 70-80% of Irgalan Black RBLN and 5-10% of Irgalan Blue 3GL. The colour reduction of solutions of Irgalan Black RBLN and of Irgalan Blue 3GL was studied individually and ae considered in Section 3.3.2 and in Section 3.3.3.

3.3.1.1 Calibration curve for solutions of Irgalan Grey GLN metal-complex dye

To determine the dye reduction rate, calibration curves were prepared. This involved making dye solutions of the Irgalan Grey GLN metal-complex dye and then establishing the absorbance values at λ_{max} against known dye concentrations. The concentrations of the dye solutions were prepared at 0.0075, 0.015, 0.03, 0.06 and 0.12 g dm^{-3}. The calibration traces provided a basis for confidence in the subsequent analytical assessments.

The calibration curve shown in Figure 3.3.1 is one example of the various linear relationships that were established. The straight line plot indicates that the

Lambert-Beer law applies to the dye solutions over this range of concentrations. This calibration curve was used to ascertain the actual amount of the dye that was reduced by the *Shewanella* strain J18 143 cells.

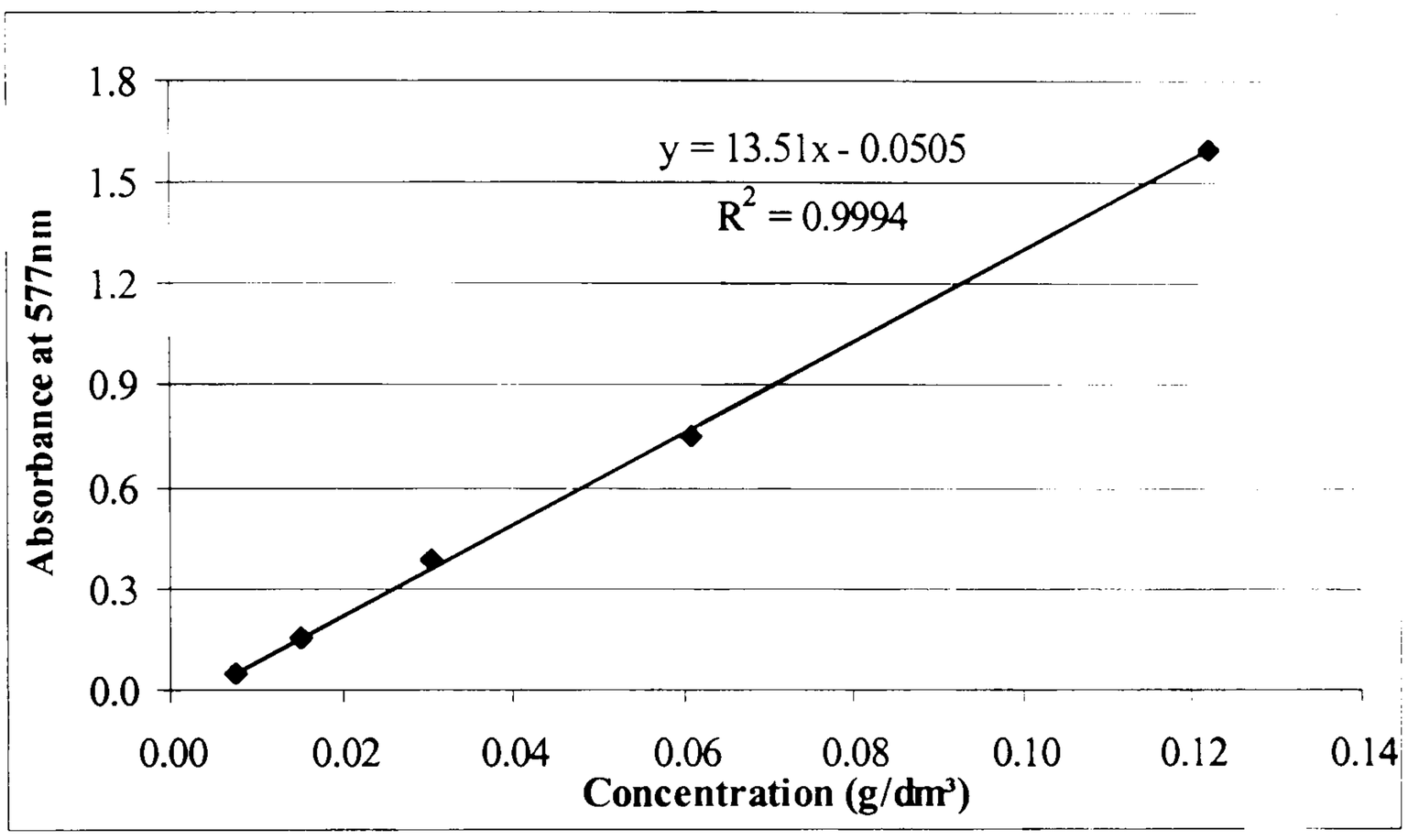


Figure 3.3.1 Calibration Plot for Solutions of Irgalan Grey GLN

3.3.1.2 Visual results from the reduction of solutions of Irgalan Grey GLN metal-complex dye using the free cells of *Shewanella* strain J18 143

The changes in colour of the samples were photographically recorded periodically as shown in Figure 3.3.2. Figure 3.3.2 consists of the images of the standard dye solution of the Irgalan Grey GLN metal-complex dye ("Sample 9", the final concentration of Irgalan Grey GLN in the sample was at 0.08 g dm^{-3}), the solution of the Irgalan Grey GLN metal-complex dye ("Sample 10", the final concentration was at 0.08 g dm^{-3}) being treated by the free cells of *Shewanella* stain J18 143 at different incubation periods and the standard suspension of the bacterial cells ("Sample 11").

It can be seen from Figure 3.3.2 that the colour of the dye solution of Irgalan Grey GLN was reduced slowly relative to the corresponding decoloration of the Remazol Black B dye solution when using the same loading of the resting cells of the

Shewanella strain J18 143 (Figure 3.2.4). However, most of the colour reduction was completed within 24 hours of incubation. A small amount of coloured settlement was observed in "Sample 10" at the bottom the vials after an incubation period of 18 hours. This is possible evidence for the fact that the dye molecule has been converted into less soluble particles. The colour of the dye solution was almost removed completely within 168 hours of incubation. It can be seen from Figure 3.3.2 that "Sample 9" was not a proper solution anymore after 72 hours of incubation. Some coloured precipitation was observed at the bottom of the vial. An investigation of these settlements was given and was discussed in Section 3.3.1.3.

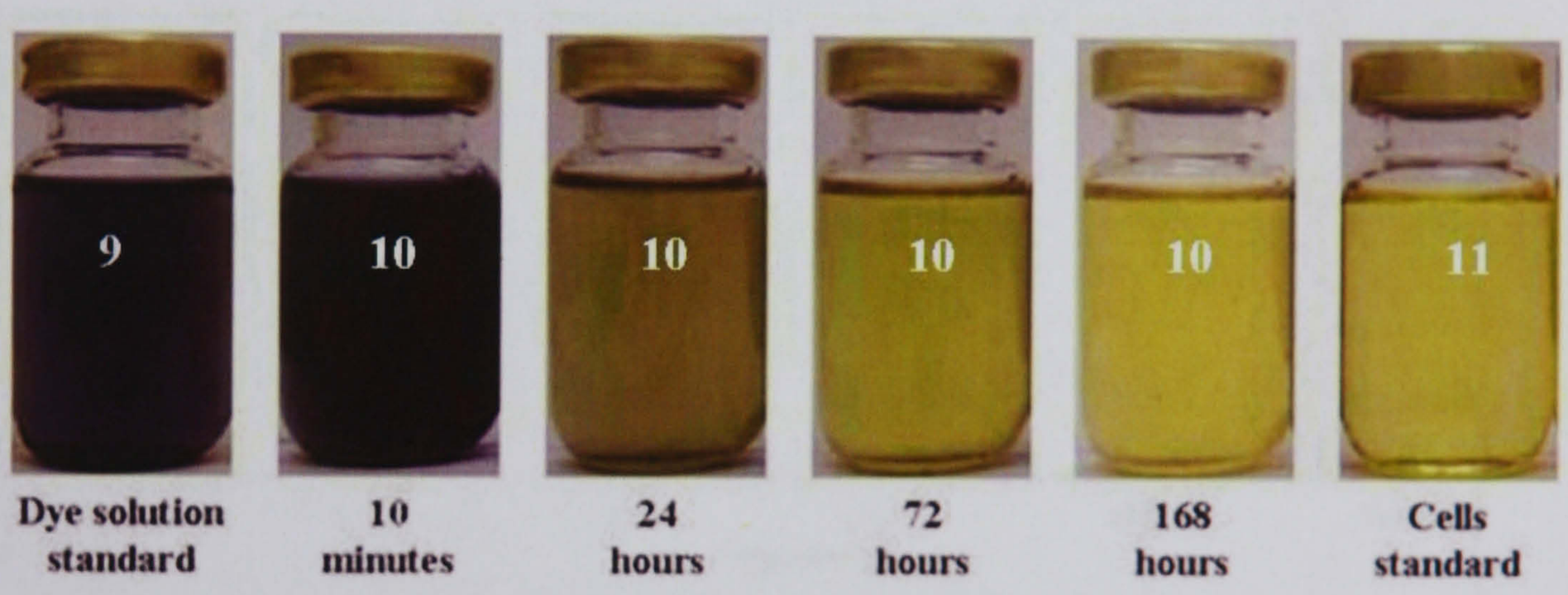


Figure 3.3.2 Flow File of Reduction of Dye Solution of Irgalan Grey GLN ("Sample 9" relates to the standard dye solution of Irgalan Grey GLN after 72 hours of incubation; "Sample 10" relates to the dye solution after 10 minutes of incubation, 18 hours of incubation, 72 hours of incubation and 168 hours of incubation as indicated with anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 11" presents the cell suspension standard)

3.3.1.3 Particle size investigation

As mentioned in Section 3.3.1.2, a small amount of coloured settlement was observed at the bottom of the vials containing the standard Irgalan Grey GLN metal-complex dye solution that had been incubated for 72 hours at 30°C ("Sample 9", Figure 3.3.2). The Irgalan Grey GLN contains sodium cations in its molecule. The dye could be precipitated out from the standard dye solution after a certain time

of incubation due to the interference by counter ions from the buffering solution (Broadbent, 2006, Personal communication). Thus, the settlement observed in the standard dye solution is from the reduced solubility of the by-products from the Irgalan Grey GLN. The particle size distribution from the particle analysis of the standard solution of Irgalan Grey GLN, after an incubation of 18 hours, is shown in Figure 3.3.3. The particles were detected across a wide range of size distributions, from 20 nm to 7000 nm. The volume content of the particles was up to 22%.

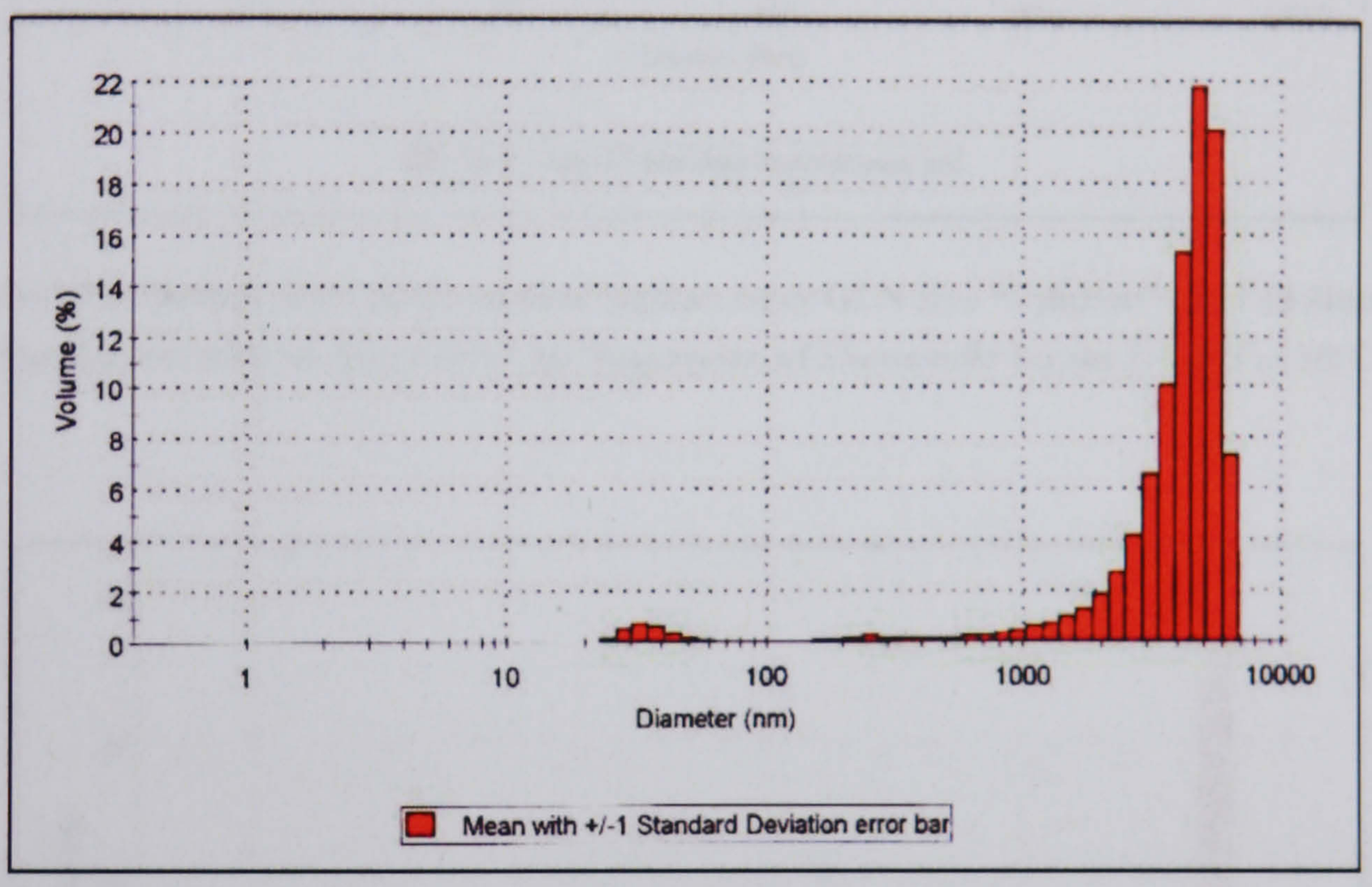


Figure 3.3.3 Particle Size Distribution of Standard Irgalan Grey GLN Dye "Solution" after 18 Hours of Incubation at 30°C

As also mentioned in Section 3.3.1.2, a small amount of a coloured, fluid "film" was observed at the bottom of the vials containing the dye solution that had been treated with cells after an incubation of 72 hours at 30°C ("Sample 10", Figure 3.3.2). The particle size analysis results are shown in Figure 3.3.4. The "particles" were distributed in size range of 35 nm to 65 nm (up to 5% in volume) and in size range of 4500 nm to 7000 nm (up to 50% in volume). This size range is much less than those of the by-products of the standard dye solution.

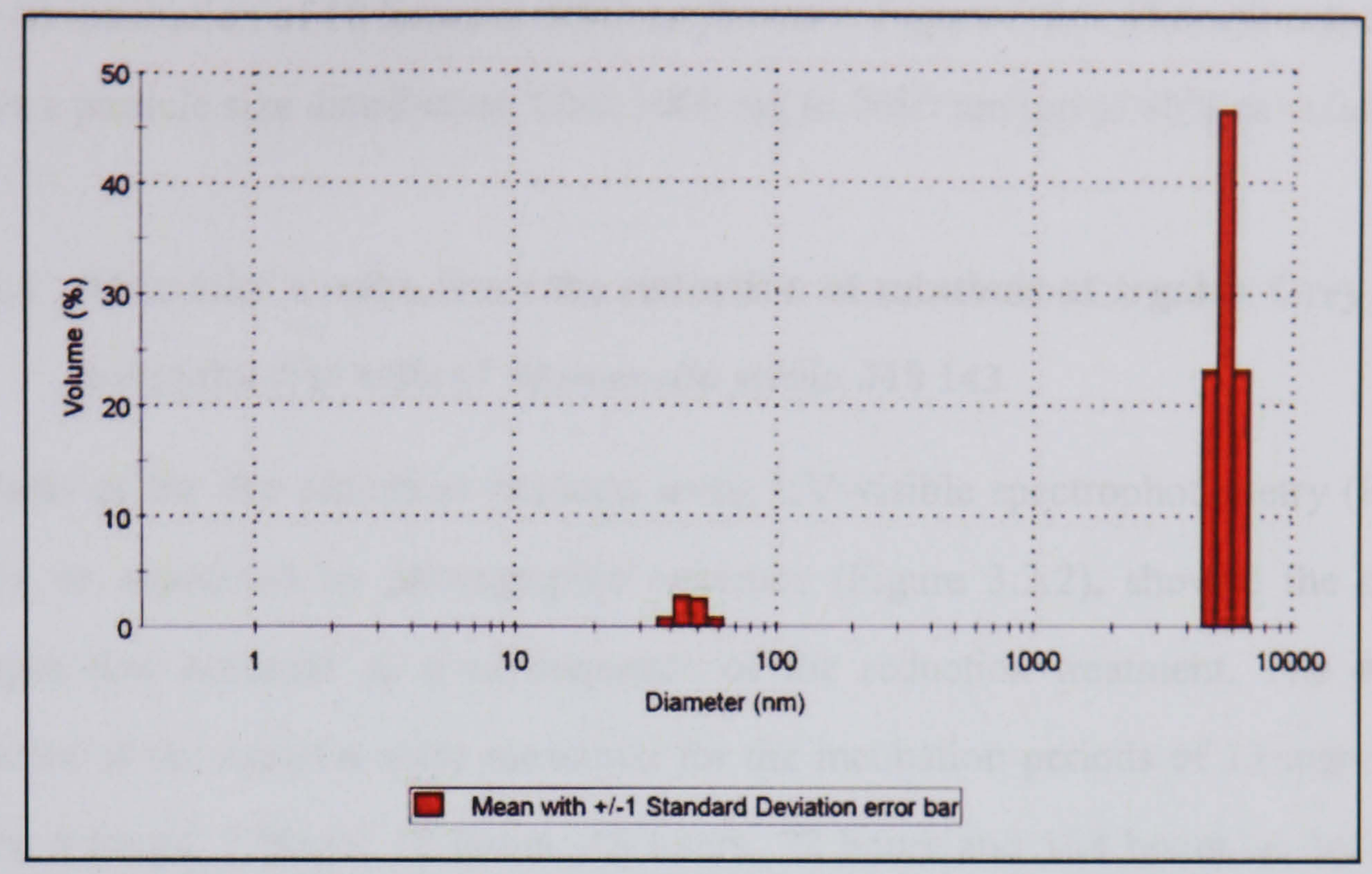


Figure 3.3.4 Particle Size Distribution of Irgalan Grey GLN Dye "Solution" after 18 Hours of Incubation with an Anaerobic Cell Suspension of *Shewanella* Strain J18 143 at 30°C

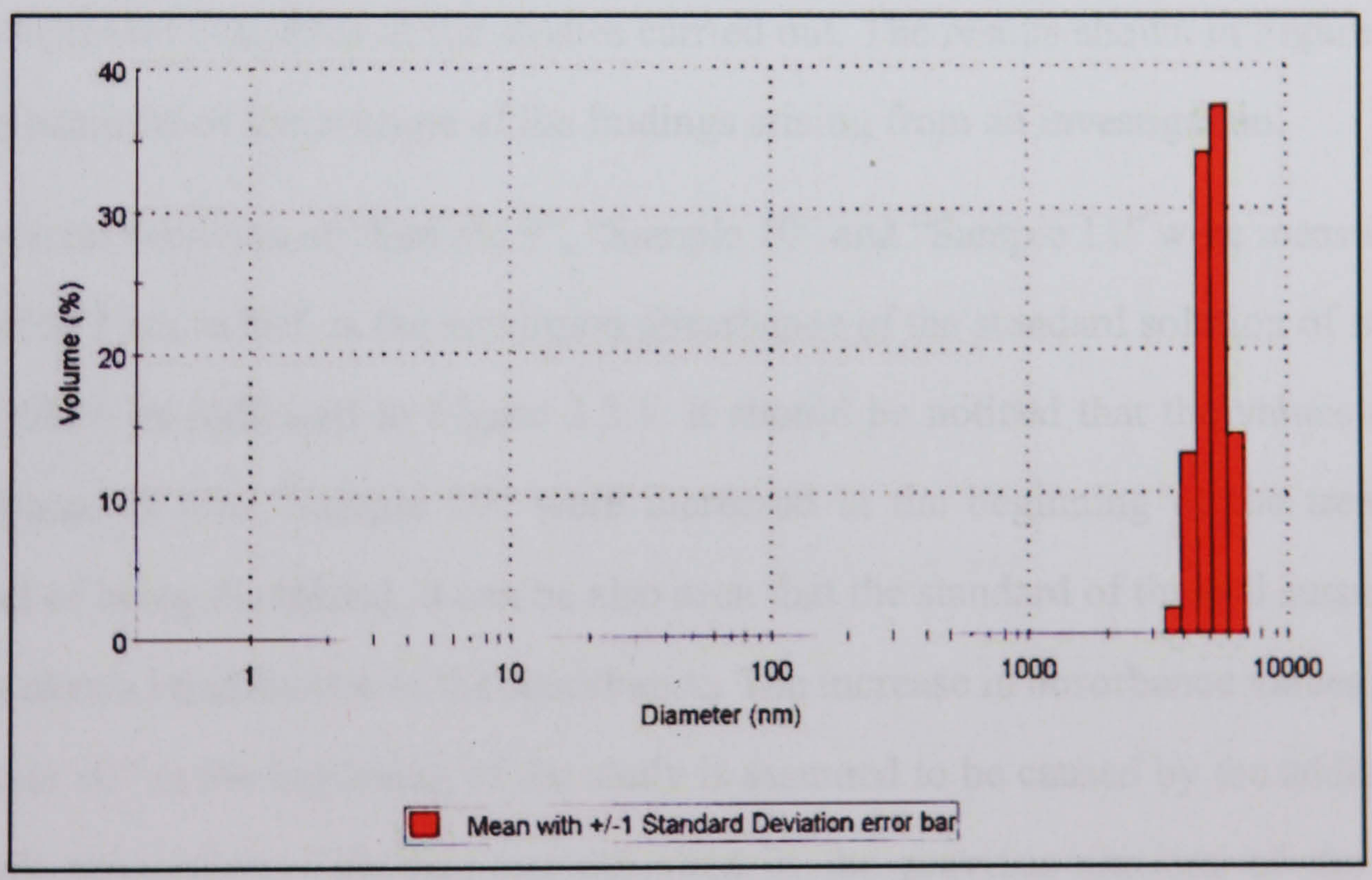


Figure 3.3.5 Particle Size Distribution of Standard Cell Suspension of *Shewanella* Strain J18 143 after 18 Hours of Incubation at 30°C

Based on the particle analysis results shown in Figure 3.3.3 and in Figure 3.3.4, it was realised that analysis of the particle size of the standard cell suspension would be

needed. The particle size distribution of the standard cell suspension (Sample 11), after an incubation of 18 hours at 30°C, is shown in Figure 3.3.5. The cell suspension shows a particle size distribution from 3000 nm to 7000 nm (up to 40% in volume).

3.3.1.4 Measured results from the reduction of solutions of Irgalan Grey GLN using the free cells of *Shewanella* strain J18 143

Analysis of the dye reduction products using UV-visible spectrophotometry (Figure 3.3.6), as supported by photographic evidence (Figure 3.3.2), showed the colour changes that occurred as a consequence of the reduction treatment. The optical densities of the samples were measured for the incubation periods of 13 minutes, 2 hours, 4 hours, 7 hours, 18 hours, 48 hours, 72 hours and 168 hours, as indicated (Section 2.3.3.2). The colour reduction of the solutions of Irgalan Grey GLN, at a concentration of 0.08 g dm^{-3}, using the bacterial cells was carried out in triplicate each time and was investigated at least three times. The images shown in Figure 3.3.2 represent examples of the studies carried out. The results shown in Figure 3.3.6 are an example of the average of the findings arising from an investigation.

The optical densities of "Sample 9", "Sample 10" and "Sample 11" were measured at λ_{max} of 577 nm, which is the maximum absorbance of the standard solution of Irgalan Grey GLN, as indicated in Figure 3.3.1. It should be noticed that the values of the absorbance of the "Sample 10" were increased at the beginning of the treatment instead of being decreased. It can be also seen that the standard of the cell suspension also makes a contribution to the absorbance. The increase in absorbance values of the "Sample 10" at the beginning of the study is assumed to be caused by the addition of the cell suspension. This was not observed in the previous sections of the study because, in those instances, the colour was removed too rapidly for one to be able to monitor any change caused by the addition of the cell suspension to the dye solution in the decoloration of the dye solution of the reactive azo dyes. Thus, a "corrected" relationship was established by subtracting the absorbance values of the standard of the cell suspension. This is presented as the "correction" line shown in Figure 3.3.7.

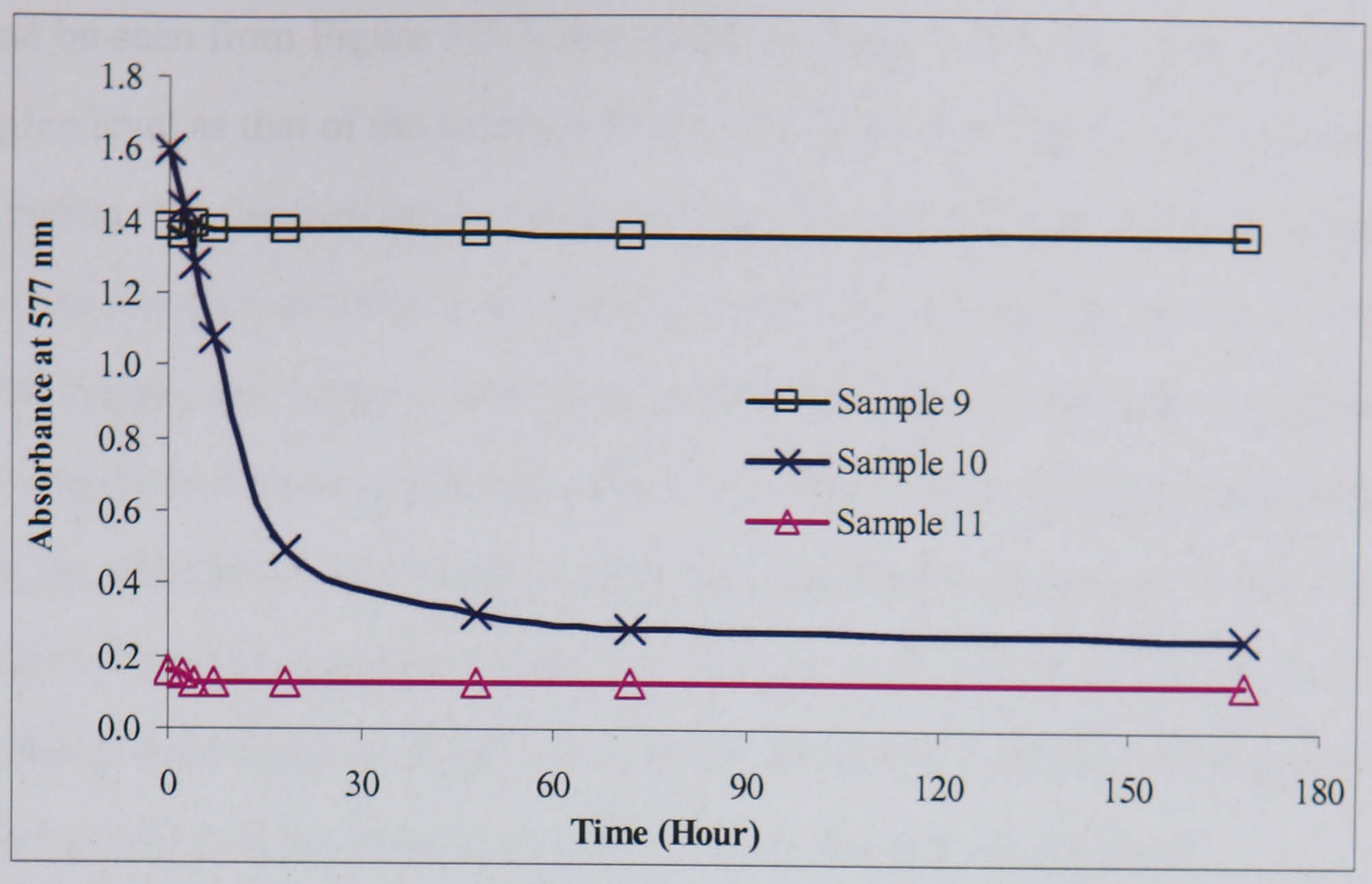


Figure 3.3.6 Decoloration of Solutions of Irgalan Grey GLN using Free Resting Biological Cells ("Sample 9" contained the standard dye solution of Irgalan Grey GLN; "Sample 10" contained the dye solution incubated with the anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 11" contained the cell suspension standard)

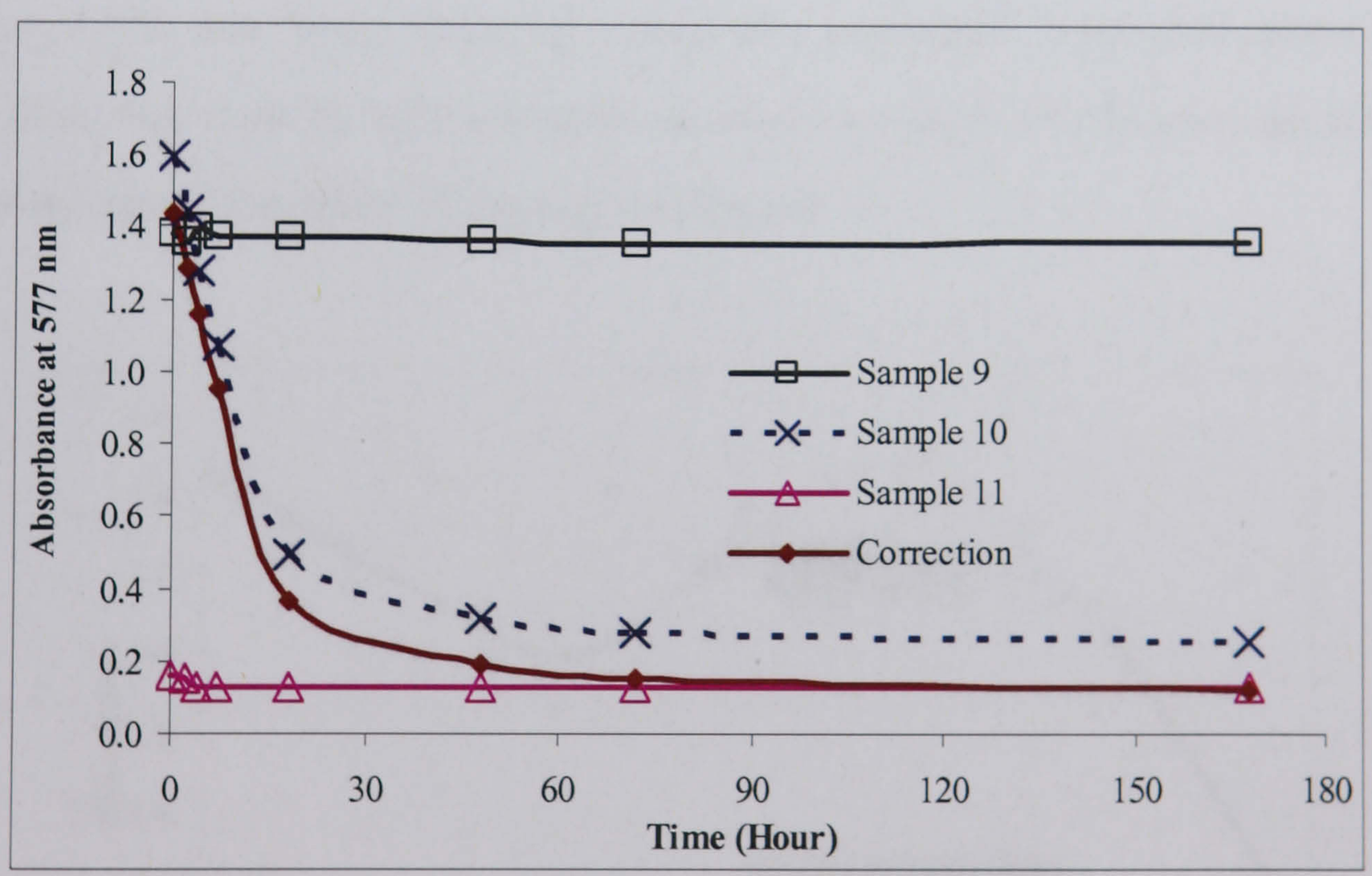


Figure 3.3.7 Corrected Results from the Decoloration of Solutions of Irgalan Grey GLN using Free Resting Biological Cells ("Sample 9" contained the standard dye solution of Irgalan Grey GLN; "Sample 10" contained the dye solution incubated with anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 11" contained the cell suspension standard)

As can be seen from Figure 3.3.7, the optical densities of the corrected values fell to the same level as that of the standard of the cell suspension. This finding supports the assumption that the increase in the absorbance, seen at the beginning, on adding the cells into the dye solution was caused by the cell suspension. In the UV-visible spectral region, the values of absorbance of the standard dye solution of Irgalan Grey GLN were measured, based on the reference of the corresponding buffering solution, while the absorbance of the cell-treated dye solutions was measured, based on the reference of the cell suspension standard, as mentioned in Section 2.2.3.1. As the cell suspension itself makes a contribution to the absorbance, the corrected curves give relative results so that comparisons of the colour changes can be made.

In the UV-visible spectra for the dye solutions containing cells, measured after 18 hours of incubation, the absorbance in the visible region at the λ_{max} for Irgalan Grey GLN (577 nm) was reduced (Figure 3.3.8). The lower line ("Grey dye with cells"), which represents the dye solution that was treated with the bacterial cells shown in Figure 3.3.8, has been corrected using the procedure mentioned above. The correction was done by subtracting the absorbance values arising from the standard cell suspension from those of the targeted sample.

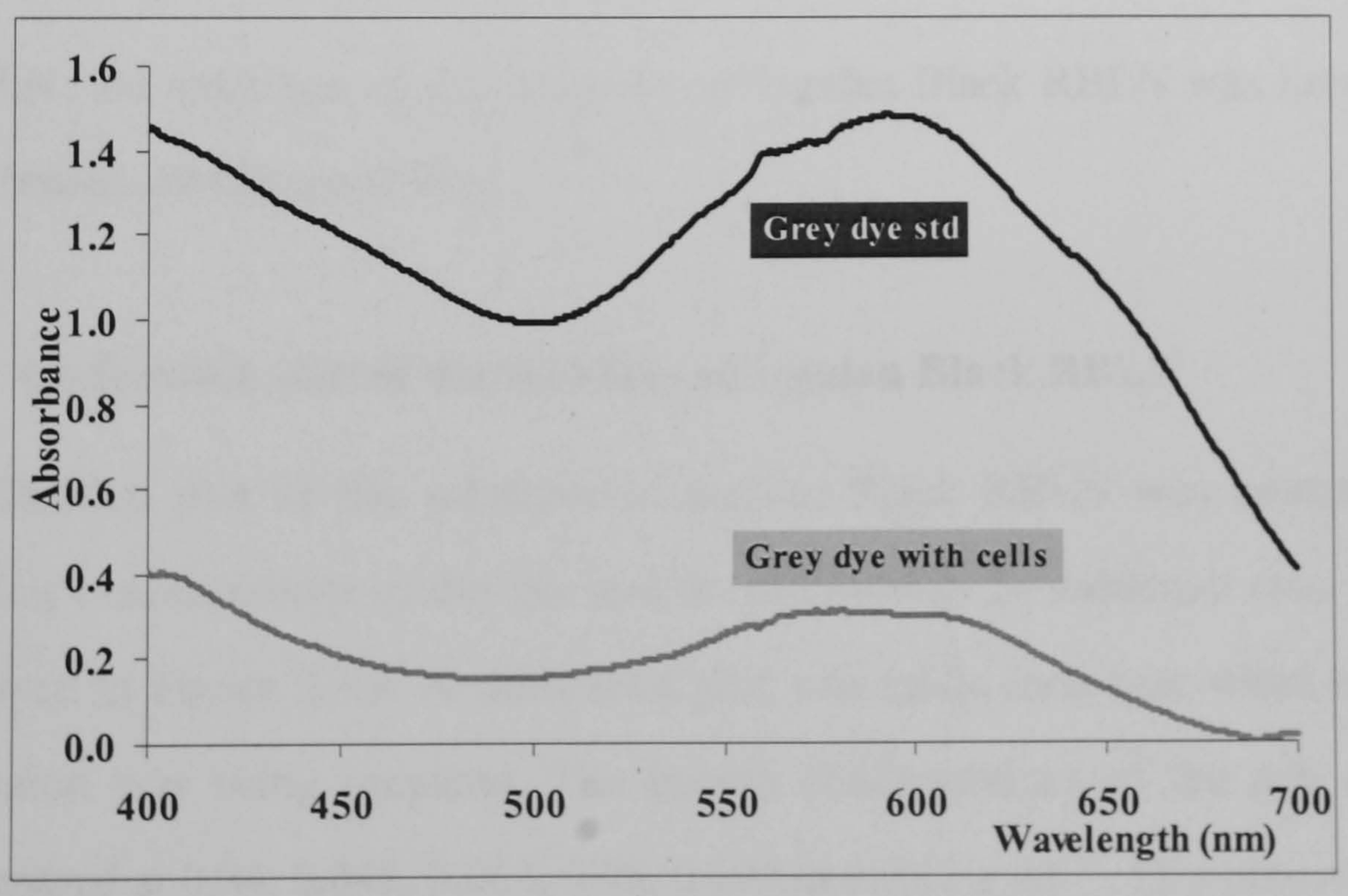


Figure 3.3.8 UV-Visible Spectra for Solutions of Irgalan Grey GLN after 18 Hours of Incubation, without and with Anaerobic Cell Suspension of *Shewanella* Strain J18 143, at 30°C

It is clearly shown in Figure 3.3.8 that there was a reduction in absorbance by the dye solution. That is an indication of reduction in colour in conformity with the information mentioned in the Section 3.2.4.1. All of the results from the visual observation and from the data evaluation have shown that the colour of the dye solution of the selected metal-complex azo dye, Irgalan Grey GLN, can be reduced by the resting cells of *Shewanella* strain J18 143. This finding gives insight into the potential use of the bacterial cells of *Shewanella* strain J18 143 in the reduction of selected metal-complex azo dyes.

The information provided by the manufacturer (Ciba Specialty Chemicals) of the Irgalan Grey GLN dye, states that the dye is a mixture of the chromophore of Irgalan Black RBLN and the chromophore of Irgalan Blue 3GL. For this reason, it was decided that further studies of the biological reduction of these two "parent" metal-complex dyes were needed.

3.3.2 Colour reduction of solutions of Irgalan Black RBLN using the free cells of *Shewanella* strain J18 143

Based on the results obtained from the colour reduction of dye solutions of Irgalan Grey GLN, the reduction of dye solutions of Irgalan Black RBLN was investigated and the results are discussed here.

3.3.2.1 Calibration plot of the solutions of Irgalan Black RBLN

The calibration plot of the solutions of Irgalan Black RBLN was assembled for calculating concentrations of the dye and for monitoring the reduction rate. The plot is displayed in Figure 3.3.9. A calibration plot was made each time when each new dye solution was being prepared. The known concentrations of the dye solutions were prepared at 0.04, 0.048, 0.064, 0.08, 0.104 and 0.12 g dm^{-3}. This precaution was taken to ensure that any changes in dye would be recognised.

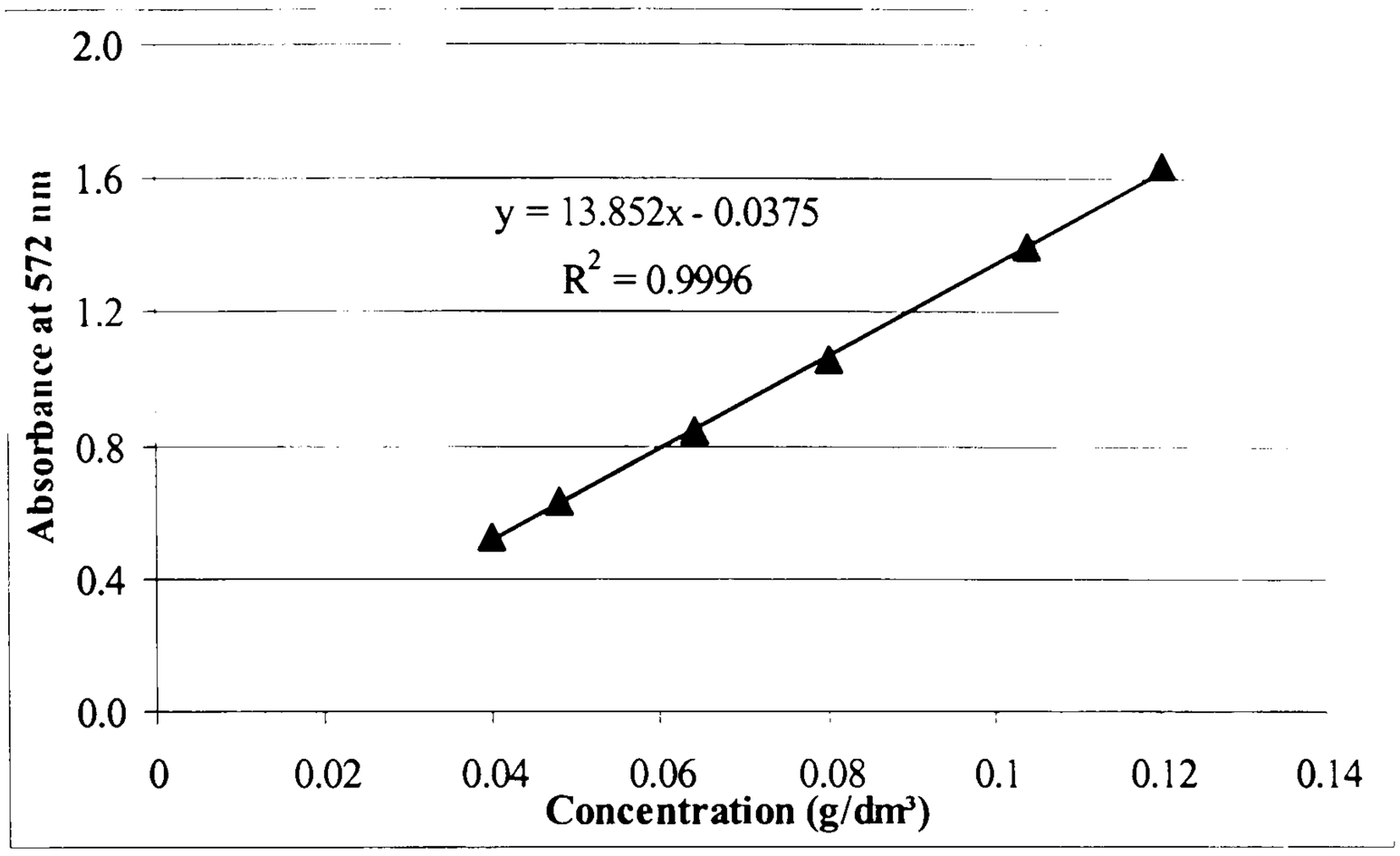


Figure 3.3.9 Calibration Plot for Solutions of Irgalan Black RBLN

It can be seen in Figure 3.3.9 that the Lambert-Beer law applies to the calibration plot of the Irgalan Black RBLN metal-complex azo dye solution, over the prepared range of dye concentrations, because of the linear relationship between the absorbance and the dye concentration.

3.3.2.2 Visual results from the colour reduction of Irgalan Black RBLN dye solutions using the free cells of *Shewanella* strain J18 143

The reduction of Irgalan Black RBLN, using free resting cells of *Shewanella* strain J18 143, was recorded photographically. Figure 3.3.10 displays the images of the standard dye solution of Irgalan Black RBLN (Sample 11), the dye solution having been treated with the bacterial cells (Sample 13) at different periods of incubation (10 minutes, 18 hours, 28 hours and 48 hours) and the standard suspension of free resting cells of *Shewanella* strain J18 143 (Sample 14).

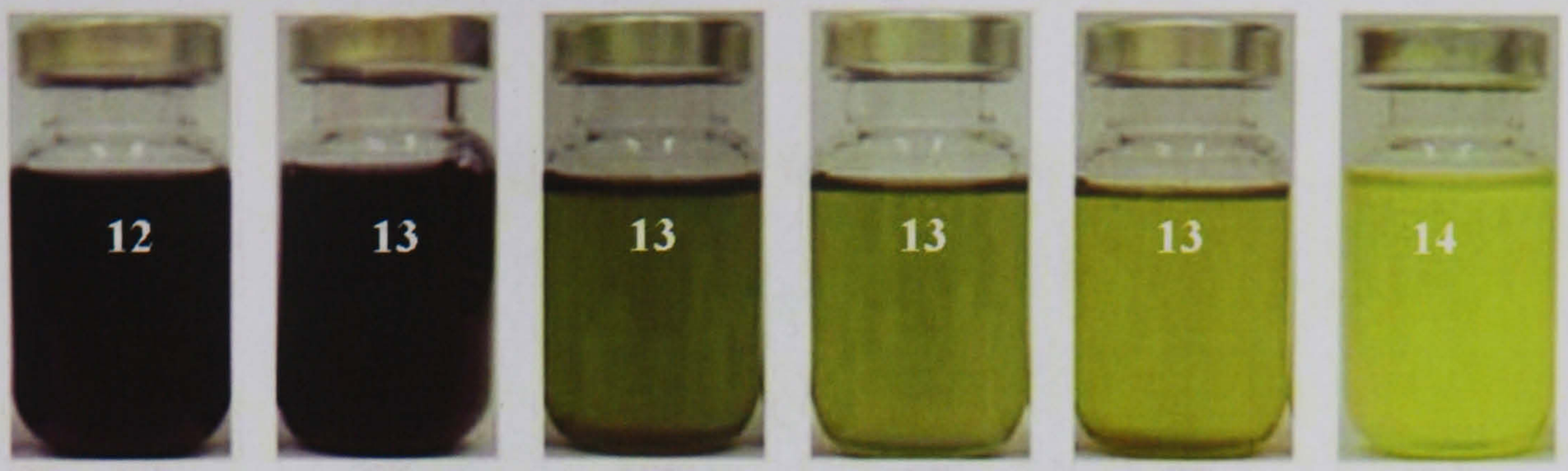


Figure 3.3.10 Flow File of Reduction of Solutions of Irgalan Black RBLN ("Sample 12" relates to the standard dye solution of Irgalan Black RBLN; "Sample 13" relates to the dye solution after 10 minutes incubation, 18 hours incubation, 28 hours incubation and 48 hours incubation respectively with anaerobic resting cell suspension of *Shewanella* strain J18 143, at 30°C; "Sample 14" presents the cell suspension standard)

It can be observed from the images that the colour is reduced mostly within 28 hours. The colour was not removed completely in comparison with the standard of the cell suspension. Some coloured particles became suspended over the top of the evaluated solution sample surface. Settlement was found at the bottom of certain samples. For other reductions of the solutions of Irgalan Black RBLN, it was decided that anaerobic cuvettes would be used to provide the evaluated system with a less disturbed environment, both with respect to physical disturbance and with respect to the anaerobic conditions. To measure the absorbance of the samples (Section 2.3.4.2), ~1 cm^{-3} of sample was transferred into disposable cuvettes using a sterile syringe with a sterile needle, for the measurement of the optical densities. This amount of sample was injected into its parent vial after measurement. There is no doubt that some oxygen could be transferred into the vial during such processes. Strict anaerobic conditions could then not be guaranteed. Furthermore, when the sample was transferred out of or injected into the system, the solution was disturbed. To reduce these possible factors, parallel experiments were prepared in sealed, anaerobic, quartz precision cuvettes (10 mm) as described in Section 2.3.4.2. The dye concentration in both of the evaluating systems was 0.08 g dm^{-3}. The visual results are displayed in Figure 3.3.11.

Figure 3.3.11 Photographs of Standard Dye Solutions of Irgalan Black RBLN and Reduction of the Dye Solutions using *Shewanella* Strain J18 143 after 18 Hours of Incubation, at 30°C

In Figure 3.3.11, the left two images represent the standard solutions of Irgalan Black RBLN (0.08 g dm^{-3}) after 18 hours' incubation in an incubator at 30°C. The right two images contained the solutions (0.08 g dm^{-3}) that had been treated with free resting cells of *Shewanella* strain J18 143, for 18 hours under the same conditions. Some coloured material can be observed on the surface of the aqueous solution of the right two samples.

3.3.2.3 Measured results from the reduction of solutions of Irgalan Black RBLN using the free cells of *Shewanella* strain J18 143

The corresponding measured results relevant to the images shown in Figure 3.3.10 ("Sample 12", "Sample 13" and "Sample 14") that were analysed by UV-Visible spectroscopy are shown in Figure 3.3.12. The measurements were taken after the samples had been incubated, at 30°C, for 10 minutes, 28 minutes, 96 minutes, 186 minutes, 248 minutes and 1493 minutes respectively. It is important to notice that the optical densities of "Sample 12" and of "Sample 14" were measured, based on references of the phosphate buffer saline solution (P.B.S.). The optical densities of

"Sample 13" were measured on the basis of the reference of the standard of the cell suspension (Sample 14). This point is also raised in Section 3.3.3.2 in that the cell suspension itself makes a contribution to the absorbance values.

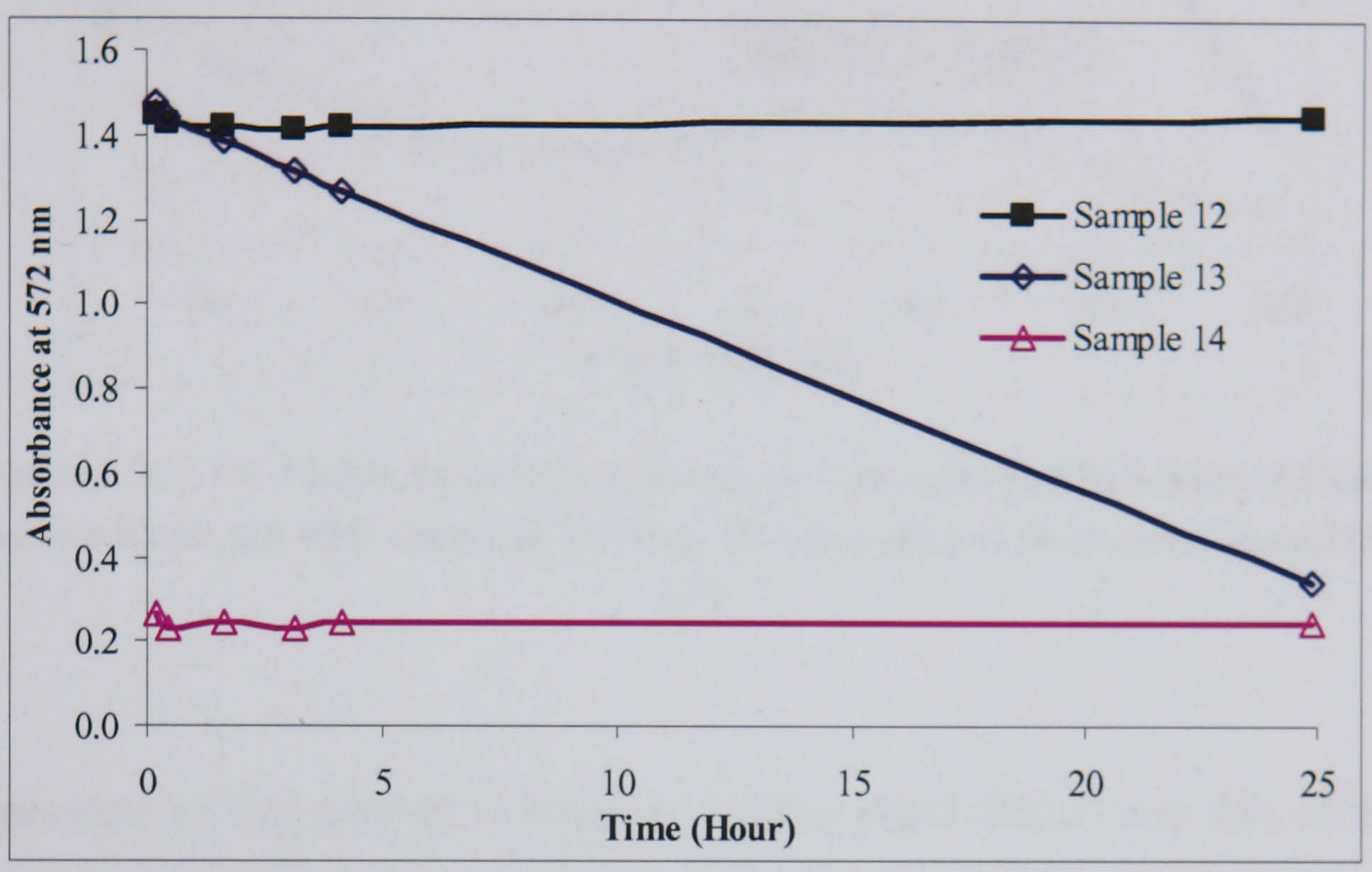


Figure 3.3.12 Measured Results from the Colour Reduction of Solutions of Irgalan Black RBLN using Free Resting Biological Cells ("Sample 12" contained the standard dye solution of Irgalan Black RBLN; "Sample 13" contained the dye solution that was incubated using the anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 14" contained the cell suspension standard)

Figure 3.3.12 shows that the optical densities of the standard solution of Irgalan Black RBLN (Sample 12) and those of the standard cell suspension (Sample 14) at same level. However, the optical densities of the bacterial cells-treated dye solution decrease linearly. The optical densities were measured at 248 minutes after the addition of the bacterial cells and 1493 minutes after such addition. There was a gap of about 20 hours due to limitations in accessing to the experimental area. The trend of the curves of "Sample 13" in Figure 3.3.12 will not fully represent the full tendency in colour reduction. The value of each point in the figure is the real measuring result.

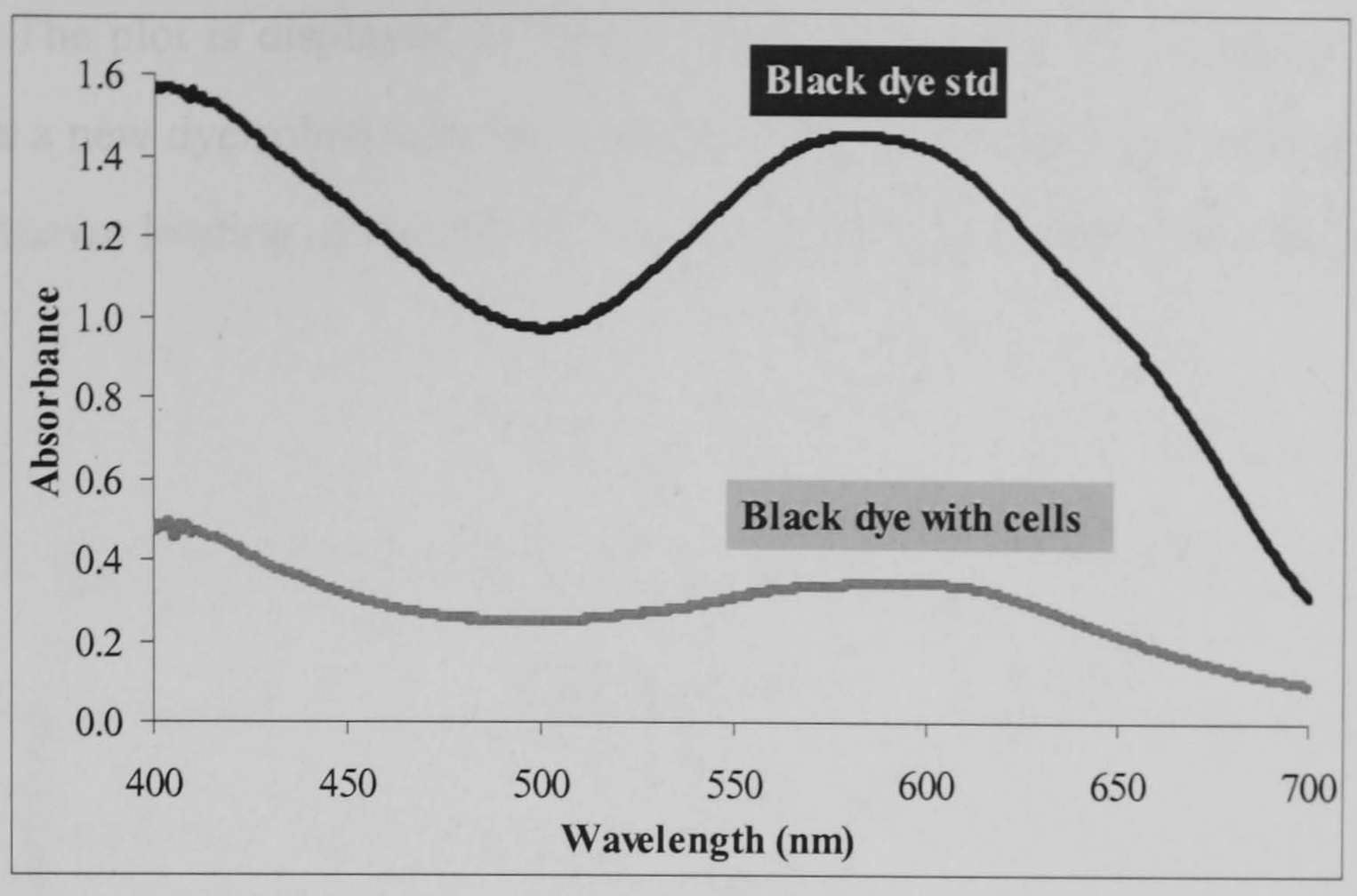


Figure 3.3.13 UV-Visible Spectra for Solutions of Irgalan Black RBLN after 24 Hours Incubation without and with Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 at 30°C

The spectrum of the standard solution of Irgalan Black RBLN and that of the cell treated dye solution after an incubation of 24 hours are displayed in Figure 3.3.13. It can be seen that the absorbance at the λ_{max} of the dye solution of Irgalan Black RBLN (the curve of the dye solution standard) dropped to a lower level (the curve of the dye solution with cells). This is no doubt strong support for the reduction of the colour intensity.

3.3.3 Colour reduction of solutions of Irgalan Blue 3GL using the free cells of *Shewanella* strain J18 143

The results from the biological reduction of solutions of Irgalan Blue 3GL are given and discussed in this section.

3.3.3.1 Calibration plot of the solutions of Irgalan Blue 3GL

The calibration plot of solutions of Irgalan Blue 3GL was assembled to be used in calculating the concentration of dye and the reduction rates, as described in later

sections. The plot is displayed in Figure 3.3.14. A new calibration curve was made each time a new dye solution being prepared. The optical densities were measured at λ_{max} for known loading of the dye solutions of 0.02, 0.03, 0.05, 0.07, 0.09 and 0.12 g dm^{-3}.

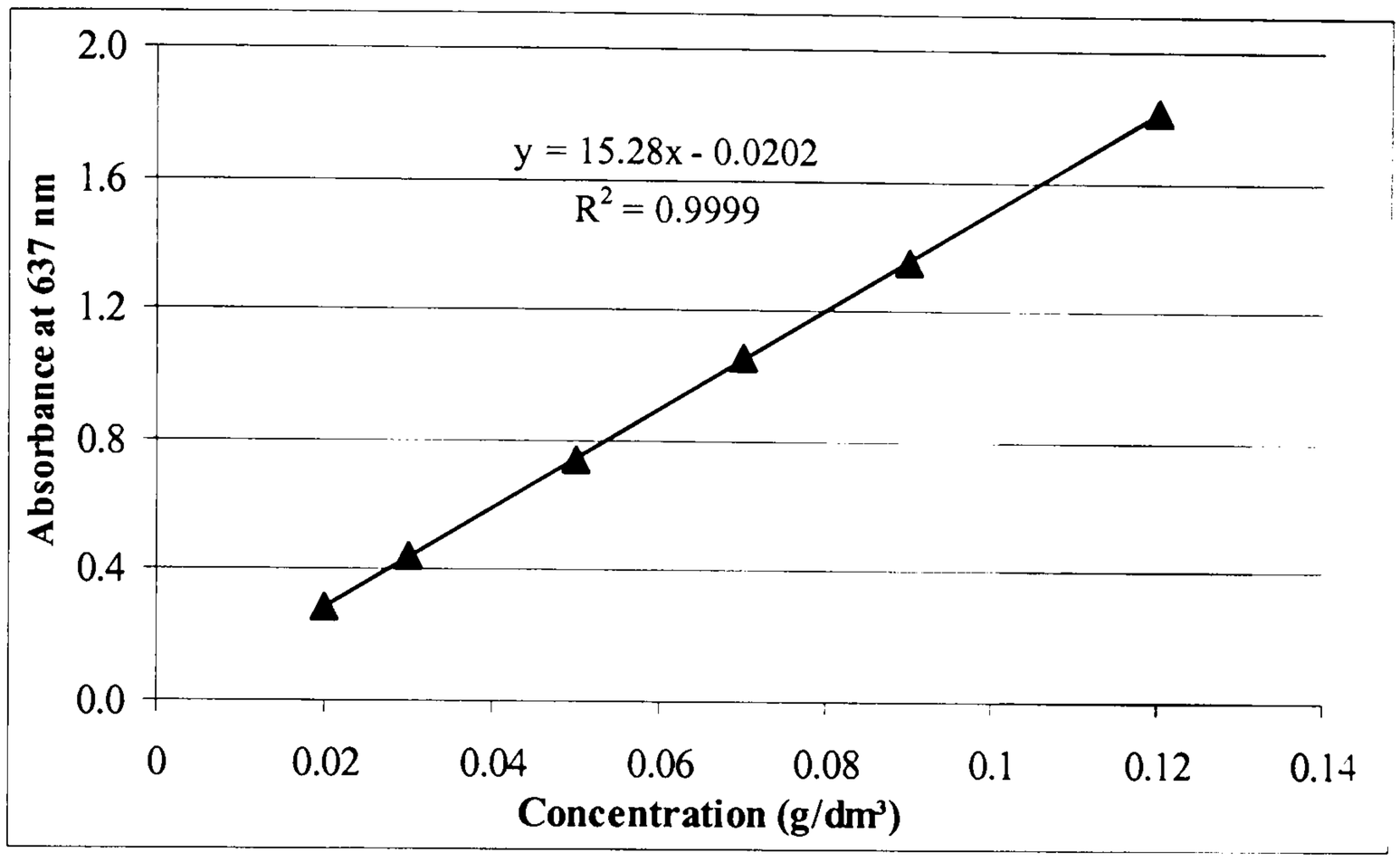


Figure 3.3.14 Calibration Curve for Solutions of Irgalan Blue 3GL (Absorbance measured at λ_{max} for the dye solution of Irgalan Blue 3GL)

A straight line was formed between the measured absorbance values and the values of the dye concentration (Figure 3.3.14). This is an indication that the Lambert-Beer law was applied over the prepared range of the dye concentration.

3.3.3.2 Visual results from the reduction of solutions of Irgalan Blue 3GL using the free cells of *Shewanella* strain J18 143

The reduction of Irgalan Blue 3GL using free resting cells of *Shewanella* strain J18 143 was recorded photographically. Figure 3.3.15 displays the images of the standard solutions of Irgalan Blue 3GL. This is the dye solution that was treated with the bacterial cells for different periods of incubation time (10 minutes, 18 hours, 28 hours and 48 hours) and with the standard suspension of free resting cells of *Shewanella* strain J18 143.

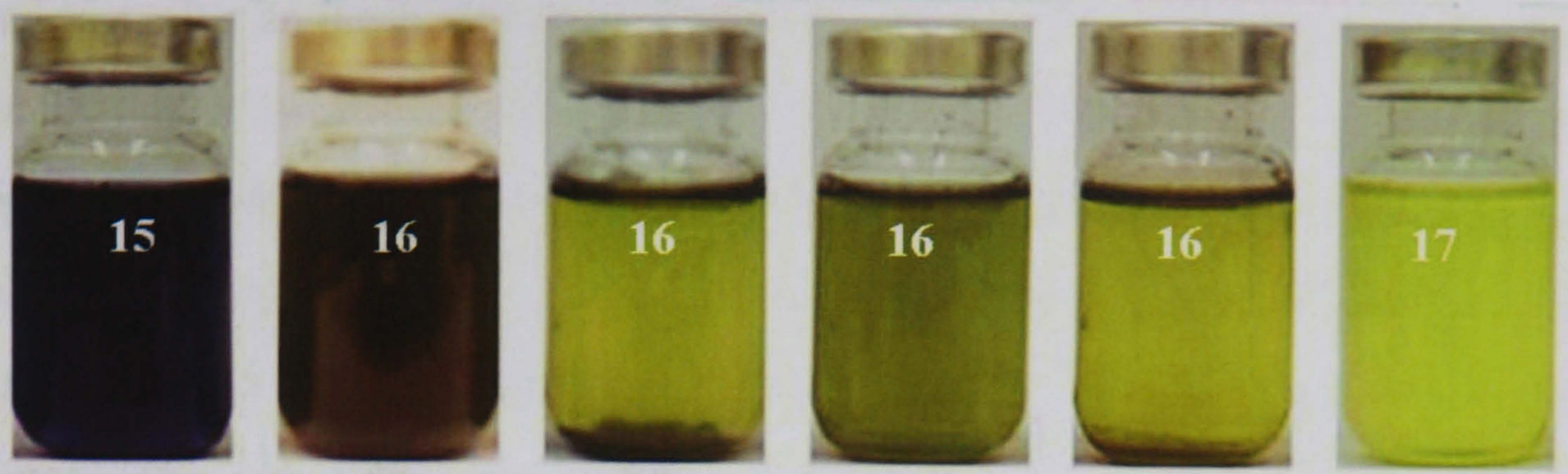


Figure 3.3.15 Sequence in the Reduction of Solutions of Irgalan Blue 3GL ("Sample 15" relates to the standard dye solution of Irgalan Black RBLN; "Sample 16" relates to the dye solution after 10 minutes of incubation, 18 hours of incubation, 28 hours of incubation and 48 hours of incubation respectively, with anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 17" presents the cell suspension standard)

It can be seen from the Figure 3.3.15 that the colour of the dye solution changed to green within ten minutes, after the addition of cell suspension (image taken of "10 minutes later"). The colour of the dye solution was then further reduced to a reduced intensity. The image of "18 hours later" shows a settlement of coloured particles at the bottom of the vial and some coloured material over the top of the liquid system. A coloured film-like "biomass" also developed at the bottom of the vial. The image corresponding to the dye solution that had been treated for 28 hours did not show a clear settlement in the vial. This may be due to the shaking of the sample before taking the photograph.

As employed in the experiments used in the reduction of solutions of Irgalan Black RBLN, stricter, anaerobic and more stable conditions were used for the reduction of Irgalan Blue 3GL (Figure 3.3.16). The samples were prepared at the same concentrations as that of the samples shown in Figure 3.3.15.

Four cuvettes were prepared for the investigation. Two of the cuvettes contained the standard solution of Irgalan Blue 3GL (0.08 g dm^{-3}). Two others contained the same concentration of the dye solution with the addition of the bacterial cell suspension. In

Figure 3.3.16, the left hand side two cuvettes represent the image of the standard dye solution after an incubation of 18 hours. The difference between these two samples in each set of two (left hand side and right hand side) is that the second left sample was shook before the photograph was taken while the first left sample was not shaken. The right hand side two cuvettes contained the dye solution and the bacterial cells. The left one was shook before being taken the photograph while the right sample was not. There is no difference in the pairs of images displayed in Figure 3.3.15. The measured samples were taken from the vials using a syringe/needle combination for the measurement using UV-Visible spectro- photometry. All of the samples were shaken well before taking such measurements. The image of the dye solution standard is shown in Figure 3.3.15, taken 10 minutes after the preparation. Here, no settlement can be seen.

Figure 3.3.16 Photographs of Standard Solutions of Irgalan Blue 3GL and Reduction of the Dye Solutions using *Shewanella* Strain J18 143 after 18 Hours' Incubation, at 30°C pH 7

3.3.3.3 Measured results from the reduction of solutions of Irgalan Blue 3GL using the free cells of *Shewanella* strain J18 143

The measured absorbance results corresponding to the samples, prepared as indicated in Figure 3.3.15, are shown in Figure 3.3.17. The measurements were taken by

UV-Visible spectrophotometry, after the samples had been incubated at 30°C, for 10 minutes, 28 minutes, 96 minutes, 186 minutes, 248 minutes and 1493 minutes respectively. The optical densities of "Sample 15" and of "Sample 17" were measured, based on references of the phosphate buffer saline solution (P.B.S.). The optical densities of solutions relating to "Sample 16" were measured based with respect to the reference of the standard of the cell suspension (Sample 17). All of the absorbance values were measured after shaking the vials.

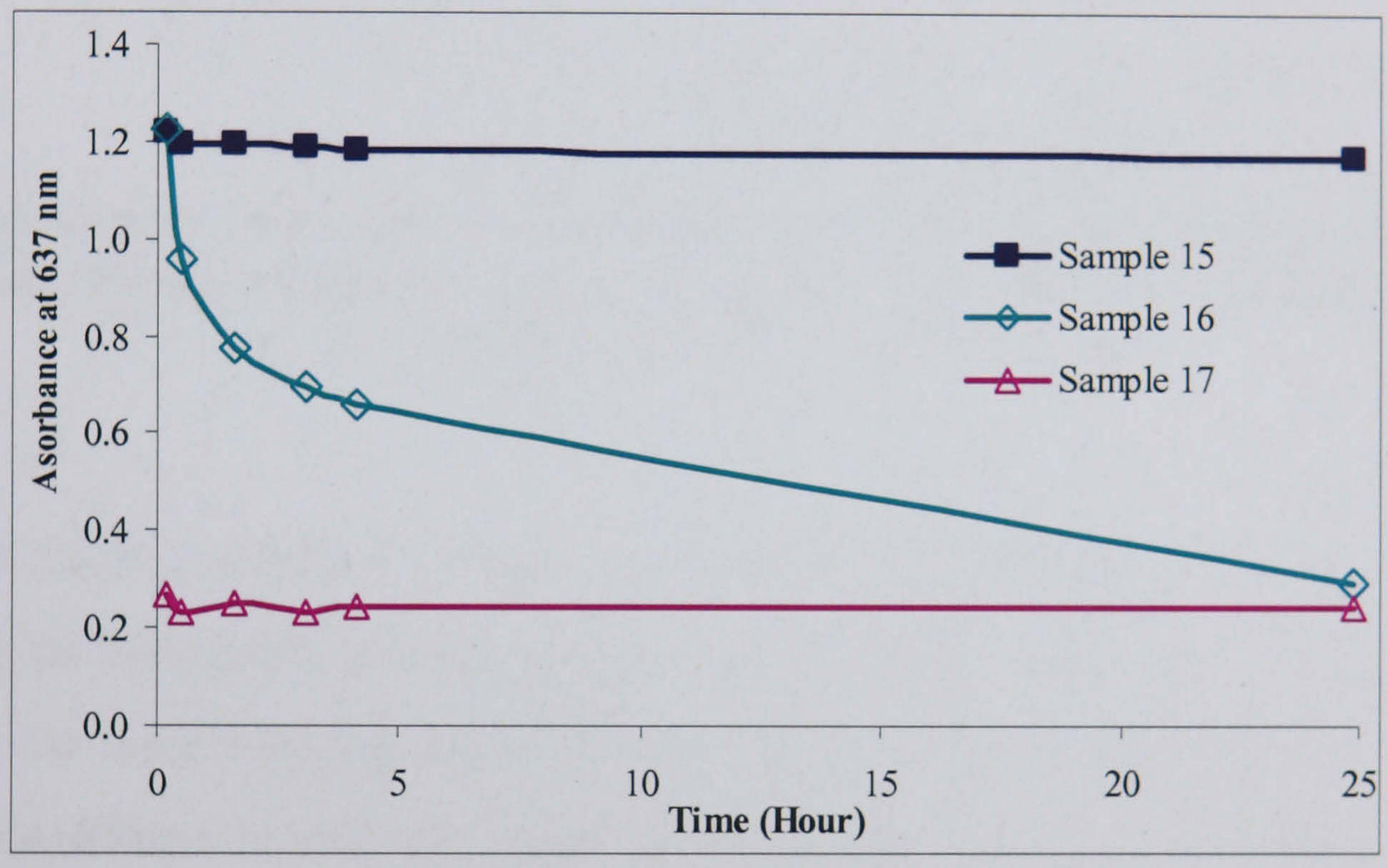


Figure 3.3.17 Results from the Colour Reduction of Solutions of Irgalan Blue 3GL using Free Resting Biological Cells ("Sample 15" contained the standard solution of Irgalan Blue 3GL; "Sample 16" contained the dye solution that was incubated with anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 17" contained the cell suspension standard)

As shown in Figure 3.3.17, the optical densities of the standard dye solution and the cell suspension standard did not change with time. The optical densities of the biologically treated dye solution gradually reduced with time (25 hours) to a level that was slightly greater than that of the cell suspension standard. The colour intensity of the dye solution was successfully reduced at the λ_{max} of the Irgalan Blue 3GL.

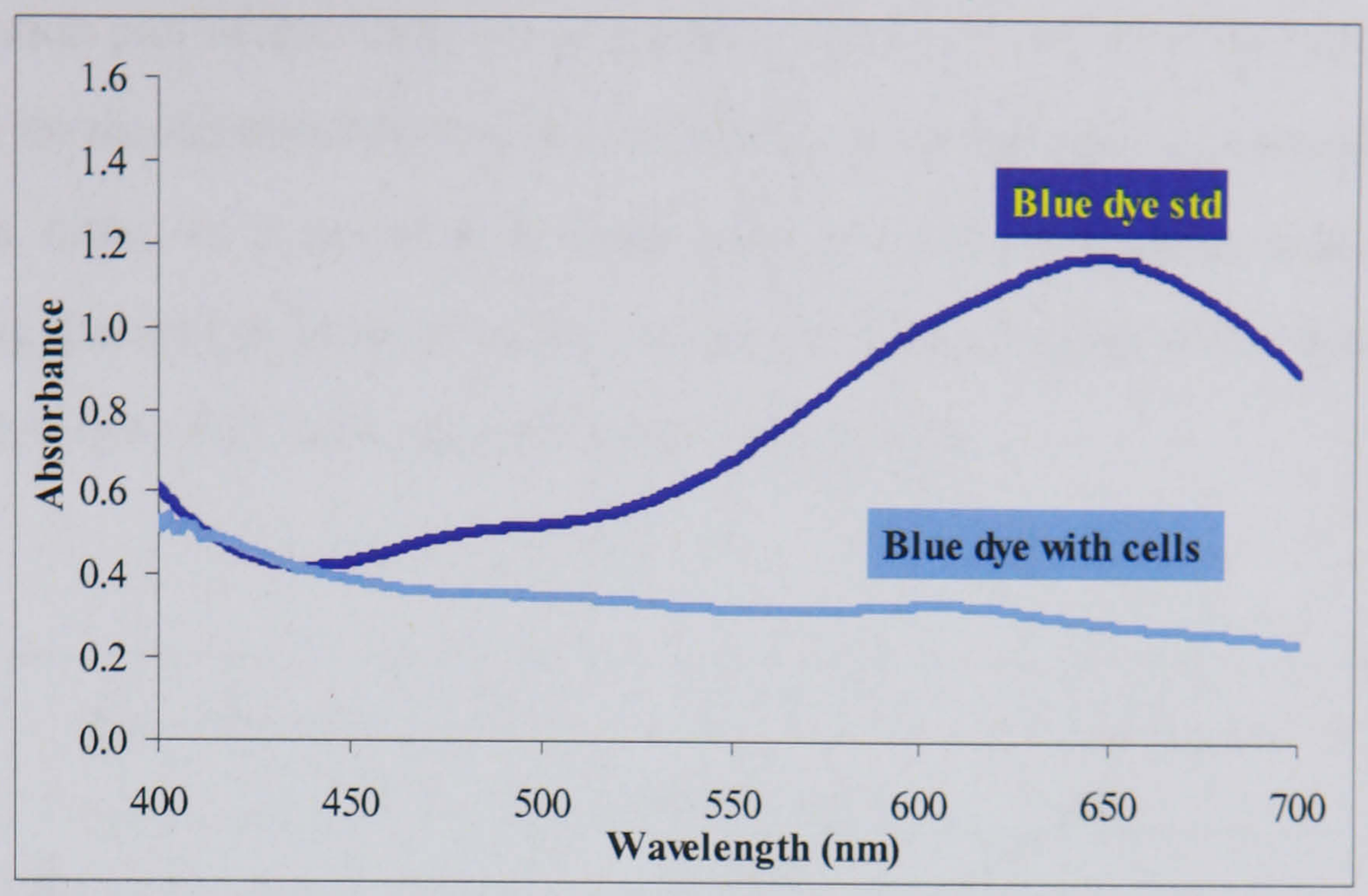


Figure 3.3.18 UV-Visible Spectra for Solutions of Irgalan Blue 3GL after 24 Hours Incubation without (Blue dye std) and with Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 (Blue dye with cells) at 30°C

Figure 3.3.18 shows the UV-visible spectrum of the standard of the dye solution and that of the biologically treated dye solution, after 24 hours of incubation with cells, at 30°C. The much lower maximum adsorption of the "blue dye with cells" was shifted to lower different wavelength, indicating that a change in colour had happened. The absorbance of the dye solution with cells fell to such extent that the evidence for reduction of colour of the treated solution is clearly seen.

3.3.4 Colour reduction of solutions of Irgalan Yellow 3RL KWL using the free cells of *Shewanella* strain J18 143

Irgalan Yellow 3RL KWL is a metal-complex azo dye that was studied, for any colour reduction that might occur, using the *Shewanella* strain J18 143. The chemical structure of this yellow dye was given in Section 2.3.1.1 (Figure 2.3.3). The experimental results from the reduction of solutions of Irgalan Yellow 3RL KWL are given here and are discussed in this section.

3.3.4.1 Calibration plot of the solutions of Irgalan Yellow 3RL KWL

A calibration plot of the solutions of Irgalan Yellow 3RL KWL (Figure 3.3.19) was prepared for the determination of concentrations of the dye and for the calculation of reduction rates, as considered in later sections. This calibration was made by measuring the optical densities at λ_{max} of known concentrations of the dye solutions at 0.0075, 0.015, 0.03, 0.06 and 0.09 g dm^{-3} respectively.

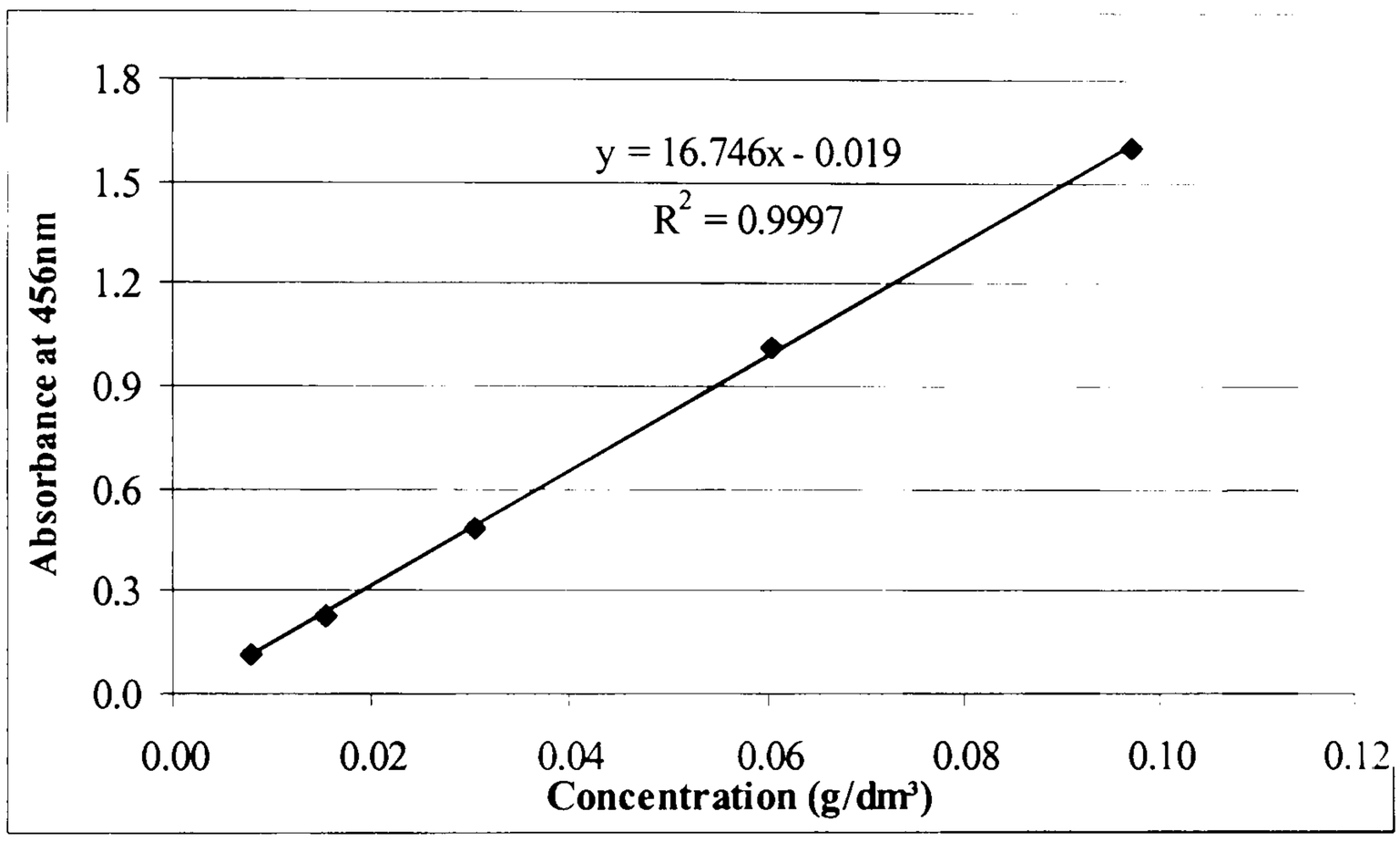


Figure 3.3.19 Calibration Plot for Solutions of Irgalan Yellow 3RL KWL (Absorbance measured at λ_{max} for the dye solution of Irgalan Yellow 3RL KWL)

The straight line in Figure 3.3.19 indicates that the Lambert-Beer law applies to the dye solutions over this range of concentrations.

3.3.4.2 Visual results from the reduction of solutions of Irgalan Yellow 3RL KWL using the free cells of *Shewanella* strain J18 143

The reduction of solutions of Irgalan Yellow 3RL KWL, using the free cells of *Shewanella* strain J18 143, was recorded photographically. These images are shown in Figure 3.3.20.

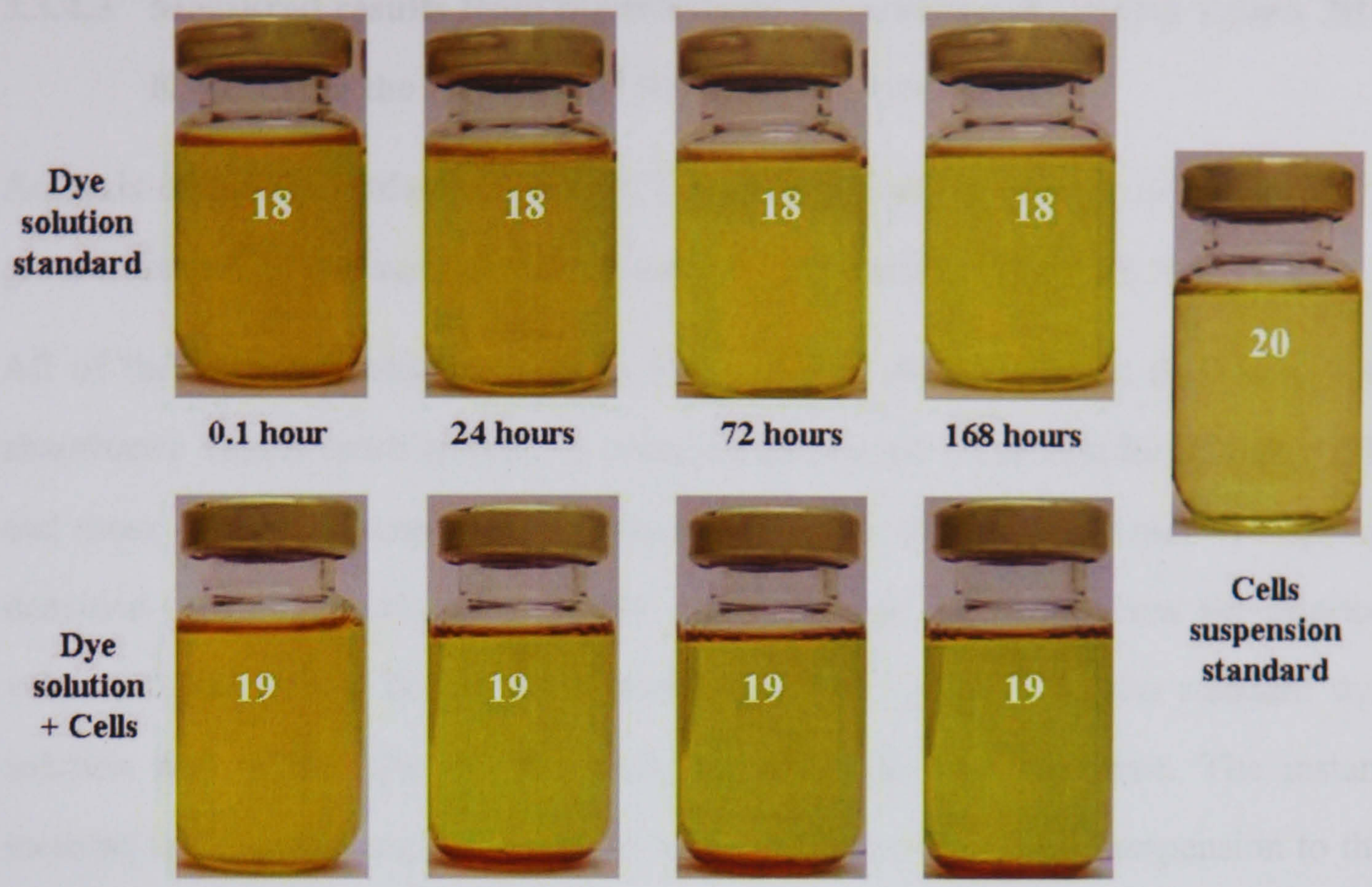


Figure 3.3.20 Flow File of Reduction of Solutions of Irgalan Yellow 3RL KWL (Standard dye solutions at different incubation periods; Dye solution after 0.1hour, 24 hours, 72 hours and 168 hours incubation respectively, with anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; and the cell suspension standard)

Figure 3.3.20 displays the images of the standard of the dye solution (Sample 18), of the dye solution with existing of the bacterial cells (Sample 19) and those of the standard cell suspension (Sample 20). All of the images shown in Figure 3.3.20 were taken without any prior shaking of the samples. As shown in Figure 3.3.20, some particles arising from what was the standard yellow dye solution precipitated to the bottom of the evaluating system. The images of the standard dye solution were taken at different incubation periods and are shown in Figure 3.3.20. Only a slight change can be seen in the images of the dye solutions that were treated with the biological cells. One difference that can be recognised from these images is that for the standard dye solutions no precipitation was produced during the incubation period.

3.3.4.3 Measured results from the reduction of solutions of Irgalan Yellow 3RL KWL using the free cells of *Shewanella* strain J18 143

Analysis of the dye reduction using UV-visible spectrophotometry (Figure 3.3.21), gives information that supports the photographic evidence (Figure 3.3.20).

All of the measurements were taken after shaking the samples in their vial. The absorbance values (with respect to time) of the standard dye solution (Sample 18) and those of the cell suspension (Sample 20) did not change with time. The optical densities of the dye solution with the bacterial cells, decreased from the original value (at time zero). The initial concentration (0.12 g dm^{-3}) in the standard dye solution and in the dye solution with bacterial cells was the same. The instant increase in the absorbance was caused by the addition of the cells suspension to the dye solution.

A correction plot was assembled, as shown in Figure 3.3.22, taking into account the effect of the biological cells.

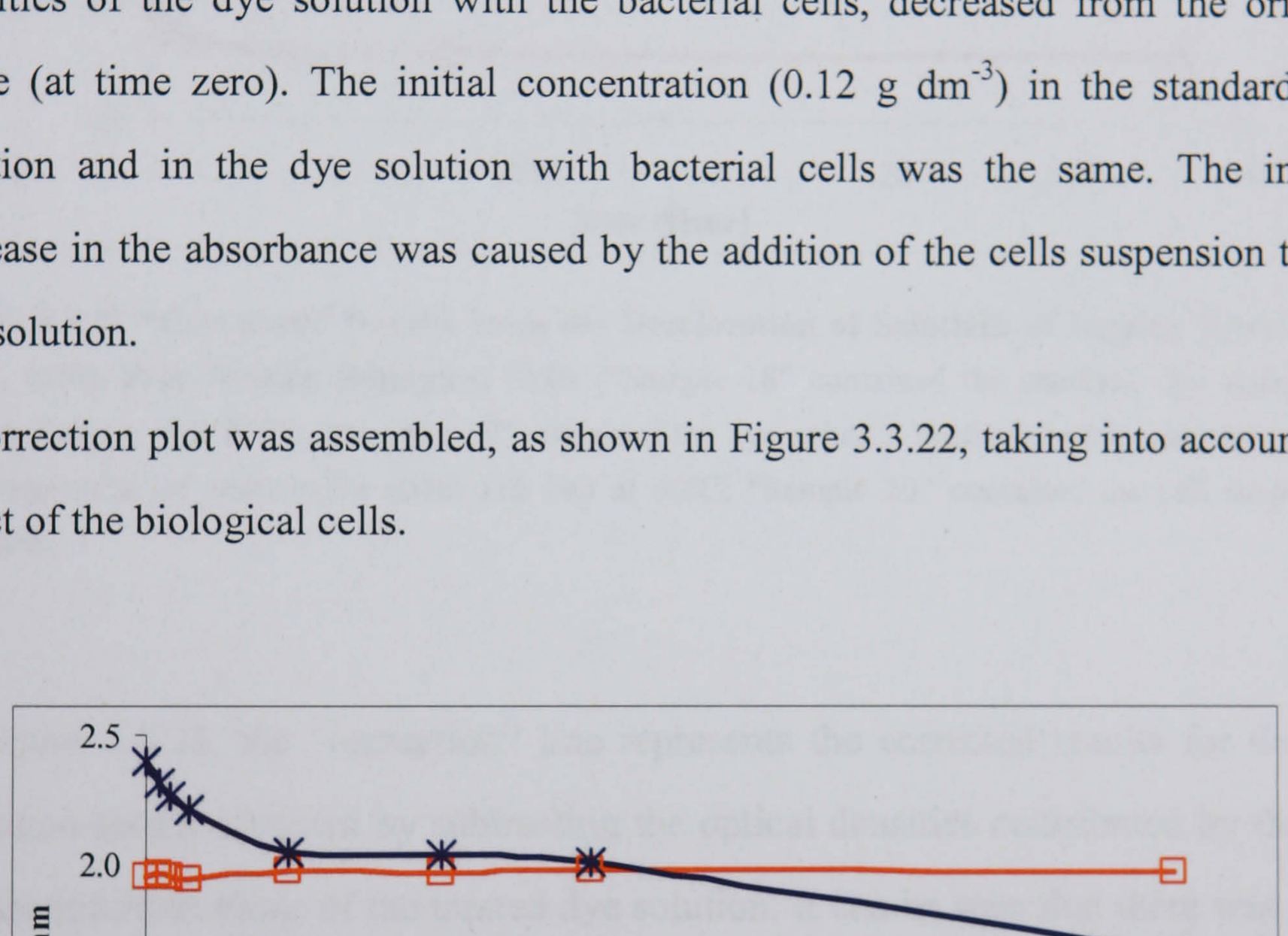


Figure 3.3.21 Results from the Colour Reduction of Solutions of Irgalan Yellow 3RL KWL using Free Resting Biological Cells ("Sample 18" contained the standard dye solution of Irgalan Yellow 3RL KWL; "Sample 19" contained the solution that was incubated with anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 20" contained the cell suspension standard)

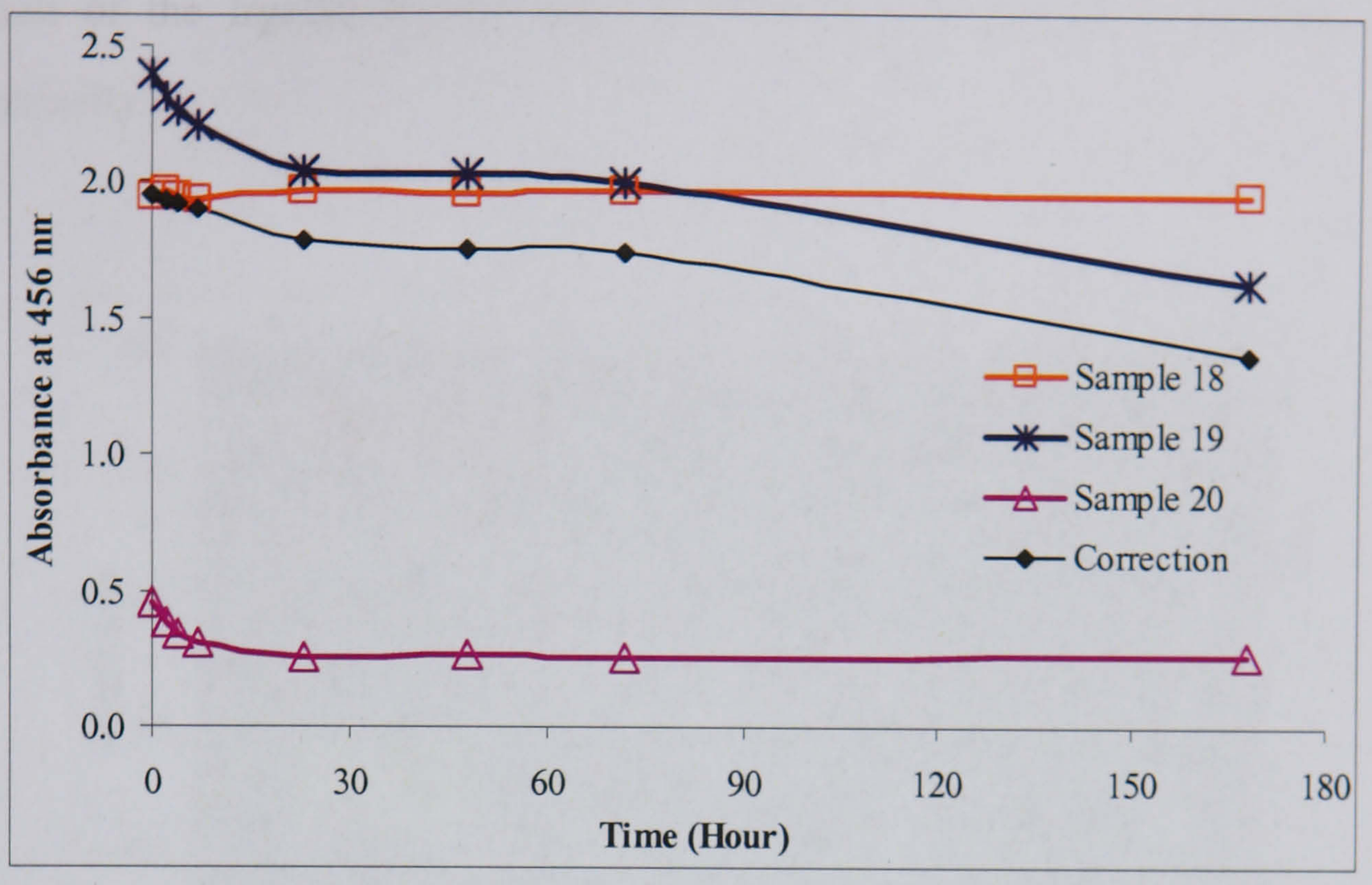


Figure 3.3.22 "Corrected" Results from the Decoloration of Solutions of Irgalan Yellow 3RL KWL using Free Resting Biological Cells ("Sample 18" contained the standard dye solution of Irgalan Yellow 3RL KWL; "Sample 19" contained the dye solution incubated with anaerobic resting cell suspension of *Shewanella* strain J18 143 at 30°C; "Sample 20" contained the cell suspension standard)

In Figure 3.3.22, the "correction" line represents the corrected results for the dye reduction study, obtained by subtracting the optical densities contributed by the cell suspension from those of the treated dye solution. It can be seen that there was some reduction in optical densities of the biological treated dye solutions, but this was not as significant as the reductions previously mentioned for the solutions of the other three Irgalan dyes.

In the UV-visible spectral region, the absorbance of the Irgalan Yellow 3RL KWL dye solution standards was measured based on the reference of corresponding buffering solution. Also, the absorbance of the cell-treated dye solutions was measured, based on the reference of the cell suspension standard, as mentioned in Section 2.2.3.1. As the cell suspension itself makes a contribution to the absorbance, the corrected curve gives relative results for comparisons of the colour changes. In the UV-visible spectra of the dye solutions that contained cells, after 18 hours of

incubation (Figure 3.3.23), the absorbance in the visible region at the λ_{max} for the dye solution of the Irgalan Yellow 3RL KWL (456 nm) is clearly reduced, if not dramatically.

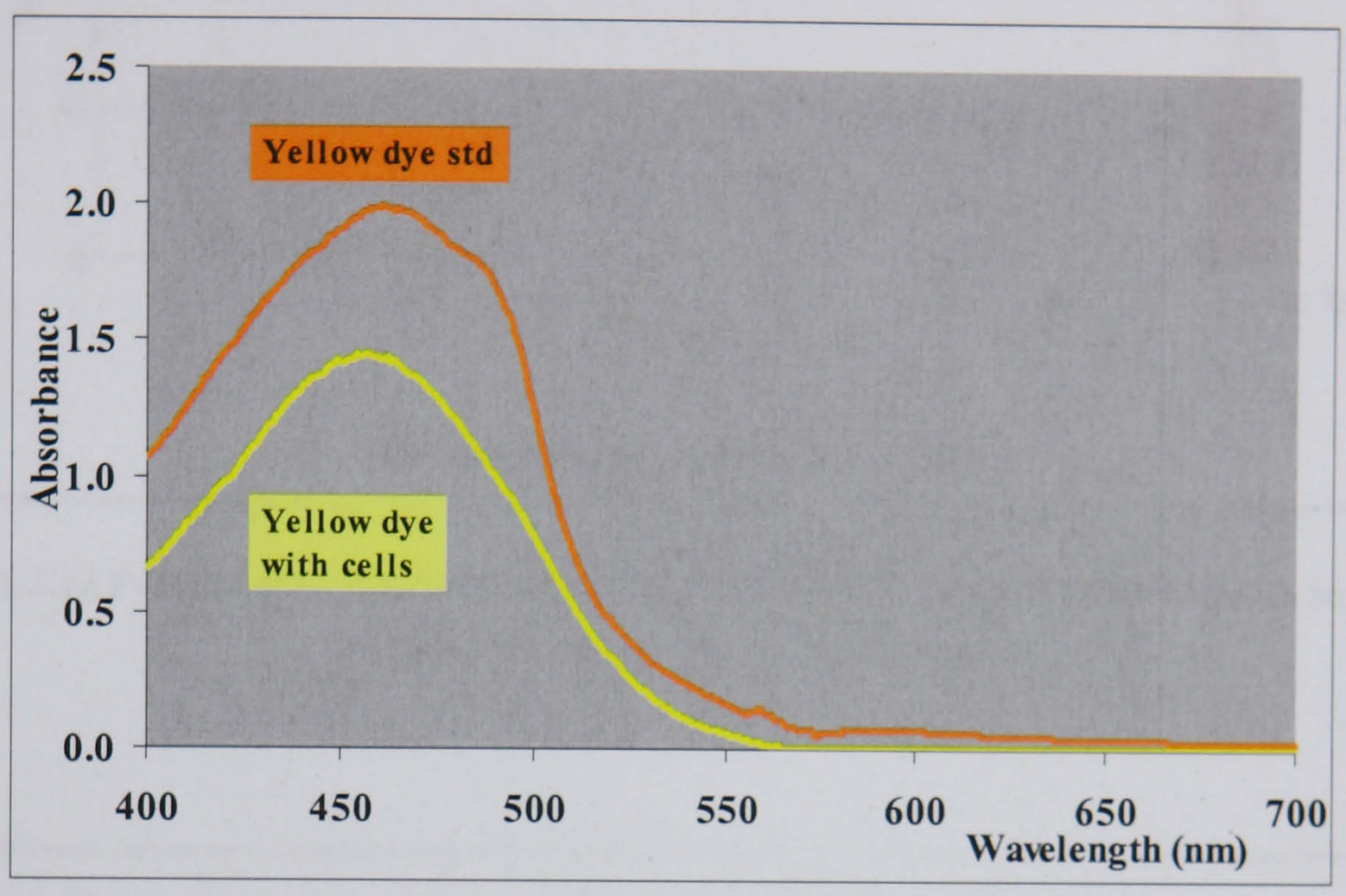


Figure 3.3.23 UV-Visible Spectra for the Solution of Irgalan Yellow 3RL KWL and for the Dye Solution that was Treated with Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 after 18 Hours Incubation, at 30°C

3.3.4.4 Particle size investigation

As can be seen from Figure 3.3.20, a small amount of a coloured "film" was observed at the bottom of the vials of the Irgalan Yellow 3RL KWL solution which contained the cell suspension of *Shewanella* strain J18 143. Particle size analysis was carried out to evaluate the reduction systems containing Irgalan Yellow 3RL KWL dye solutions. No evidence of "coloured film" formation was seen in vials containing only a solution of the dye. Particle size analysis was carried out on the solutions of Irgalan Yellow 3RL KWL and its biological treated form.

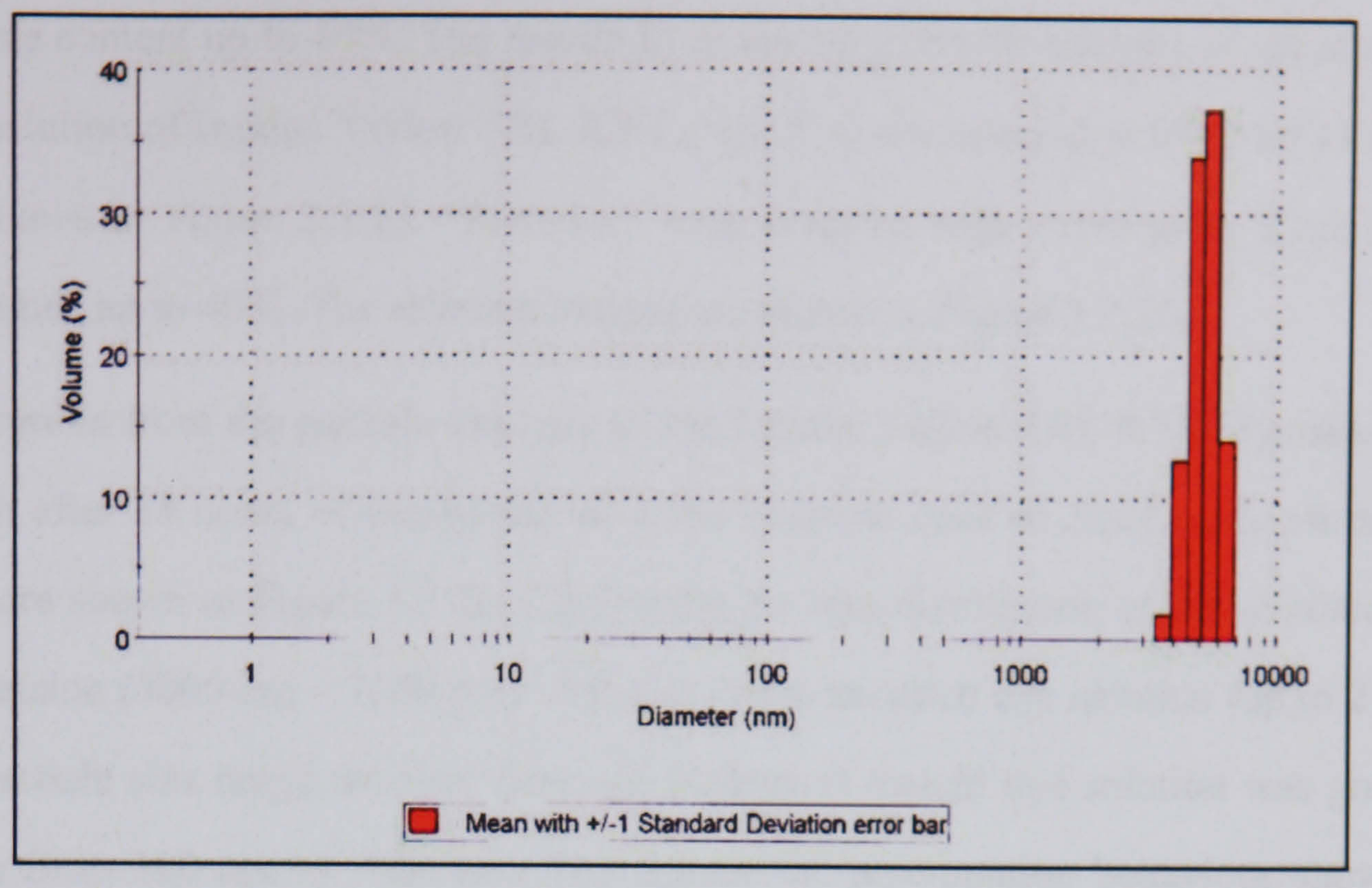


Figure 3.3.24 Particle Size Distribution of Standard Cell Suspension of *Shewanella* Strain J18 143 after 18 Hours of Incubation at 30°C

Figure 3.3.25 Particle Size Distribution of Standard Irgalan Yellow 3RL KWL Dye "Solution" after 18 Hours of Incubation at 30°C

Figure 3.3.24 displays the particle size distribution of the standard cell suspension, after an incubation period of 18 hours at 30°C. It can be seen that the cells that were suspended in the systems gave a size distribution from 3000 nm to 7000 nm with

volume content up to 40%. The results from the particle size analysis of the standard dye solution of Irgalan Yellow 3RL KWL, which was incubated at 30°C for 18 hours, are shown in Figure 3.3.25. "Particles" were detected with a size up to 2 nm and a "volume" up to 40%. The relevant images are shown in Figure 3.3.20.

The results from the particle analysis of the Irgalan Yellow 3RL KWL dye solution, which after 18 hours of incubation with the bacterial cells of *Shewanella* strain J18 143, are shown in Figure 3.3.26. Comparing the size distribution of the standard cell suspension (3000 nm – 7000 nm) with that of the standard dye solution (up to 2 nm), the particle size range detected from the biological treated dye solution was greater, range from 500 nm to 7000 nm. This agrees the precipitation behaviour shown in Figure 3.3.20. Further more, the volume content of the detected particles was reduced in the biologically treated dye solutions. It is possible that the dye molecules became aggregated among the cells. Thus, the dye molecules became adsorbed onto the biomass.

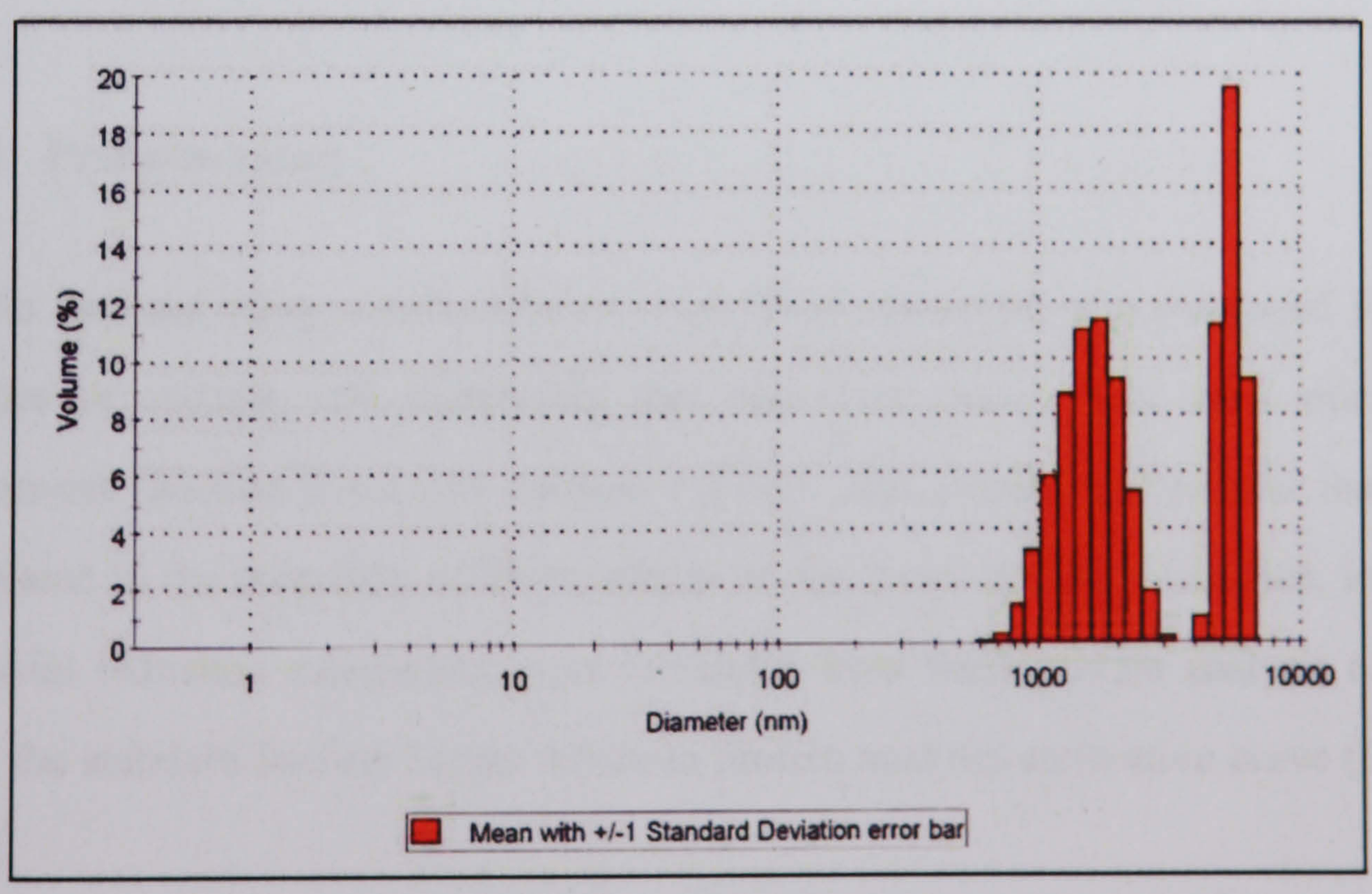


Figure 3.3.26 Particle Size Distribution of Irgalan Yellow 3RL KWL "Solution" after 18 Hours of Incubation with Anaerobic Cell Suspension of *Shewanella* Strain J18 143 at 30°C

3.4 Factors affecting the reduction of solutions of metal-complex azo dyes using free resting cells of *Shewanella* strain J18 143

The factors that may affect the decoloration of the solutions of reactive azo dyes using the whole cells of *Shewanella* strain J18 143 have been documented by Pearce (2004). These factors include oxygen, temperature, pH, the colorant concentration, the colorant structure, any colorant hydrolysis, the role of electron donors, the role of the electron shuttles, and the presence of alternative electron acceptors. The experimental conditions set up for the current study was the same as those used by Pearce (2004). A more limited study of the effects of the colorant concentration, temperature and pH on the reduction of the solutions of metal-complex azo dyes was carried out (Section 2.4). The other parameters used were those specified by Pearce (2004). The results from the experimental work undertaken are given and are discussed in this section.

3.4.1 Protein assay

Protein analysis using a bicinchoninic acid (BCA) assay kit was employed for the samples of resting cell suspension that had been taken from each microbial experiment (Section 2.4.2 and Section 2.5.5.2). The amount of biomass that was implicated in the reduction of the solutions of the metal-complex azo dyes, in each microbial reduction experiment, was calculated from these protein analysis results, using the standard Bovine Serum Albumin protein analysis calibration curve (Figure 3.4.1).

Figure 3.4.1 gives one example of the calibration curves that were assembled. The calibration curve was made each time before the dye reduction treatment was commenced. The reduction of the dye solutions by *Shewanella* strain J18 143 cells

was then expressed as a rate, this being the concentration of dye solution reduced per gram of biomass per minute.

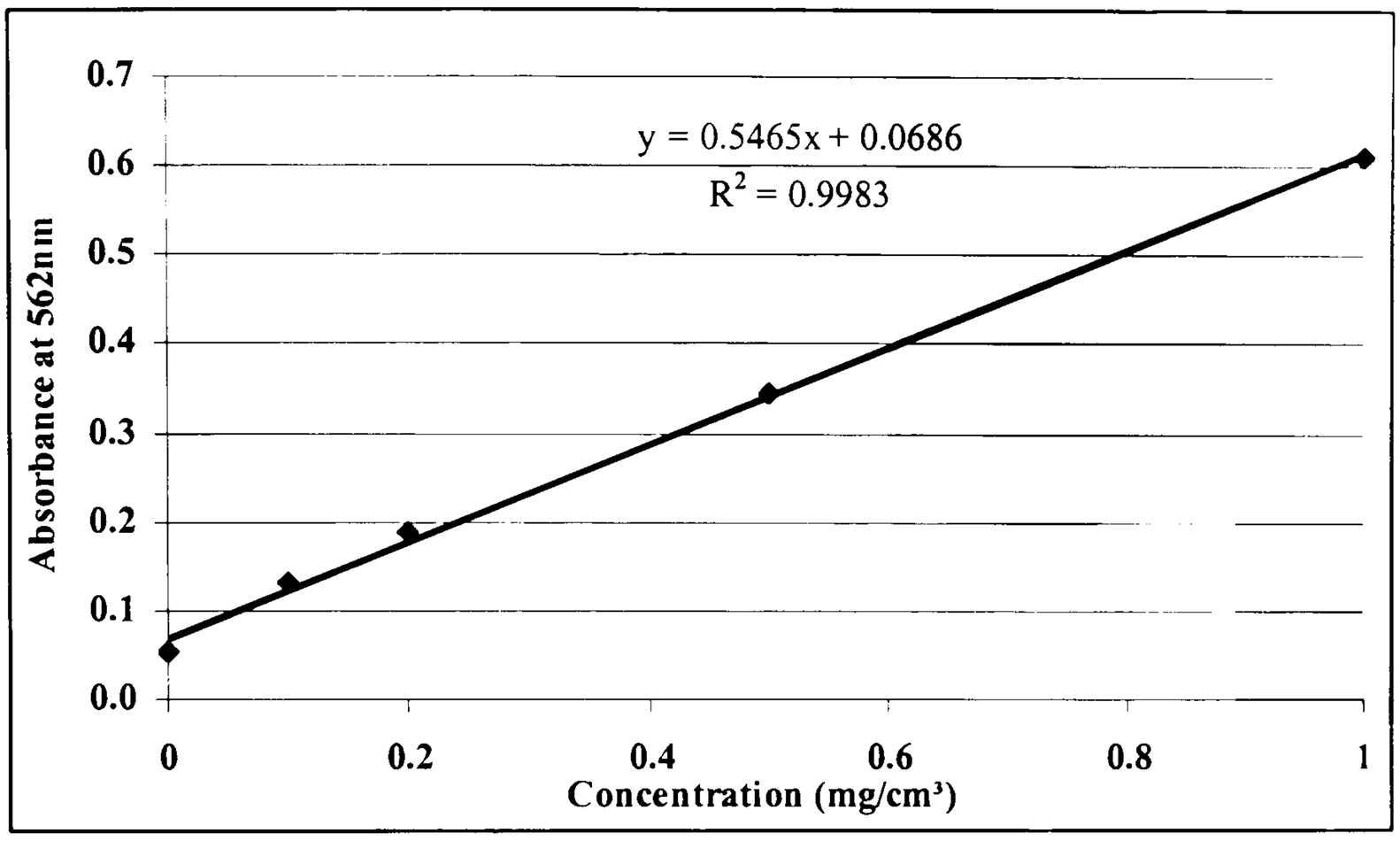


Figure 3.4.1 Calibration Curve of Standard Bovine Serum Albumin Protein Assay

3.4.2 Factors affecting the reduction of Irgalan Grey GLN solutions using free resting cells of *Shewanella* strain J18 143

The factors of the dye concentration, the temperature and the pH are considered in this section on the study of the colour reduction of Irgalan Grey GLN solutions.

3.4.2.1 Effect of dye concentration on the dye reduction

The concentration of dye in the system can influence the efficiency of the biologically induced reduction through a combination of factors, including the possible toxicity of the dye at higher concentrations and the ability of enzymic system to recognise the dye substrate efficiently at very low concentrations (Pearce, 2004). The effect of solutions of the Irgalan Grey GLN dye concentration on the dye reduction efficiency is clearly shown in Figure 3.4.2.

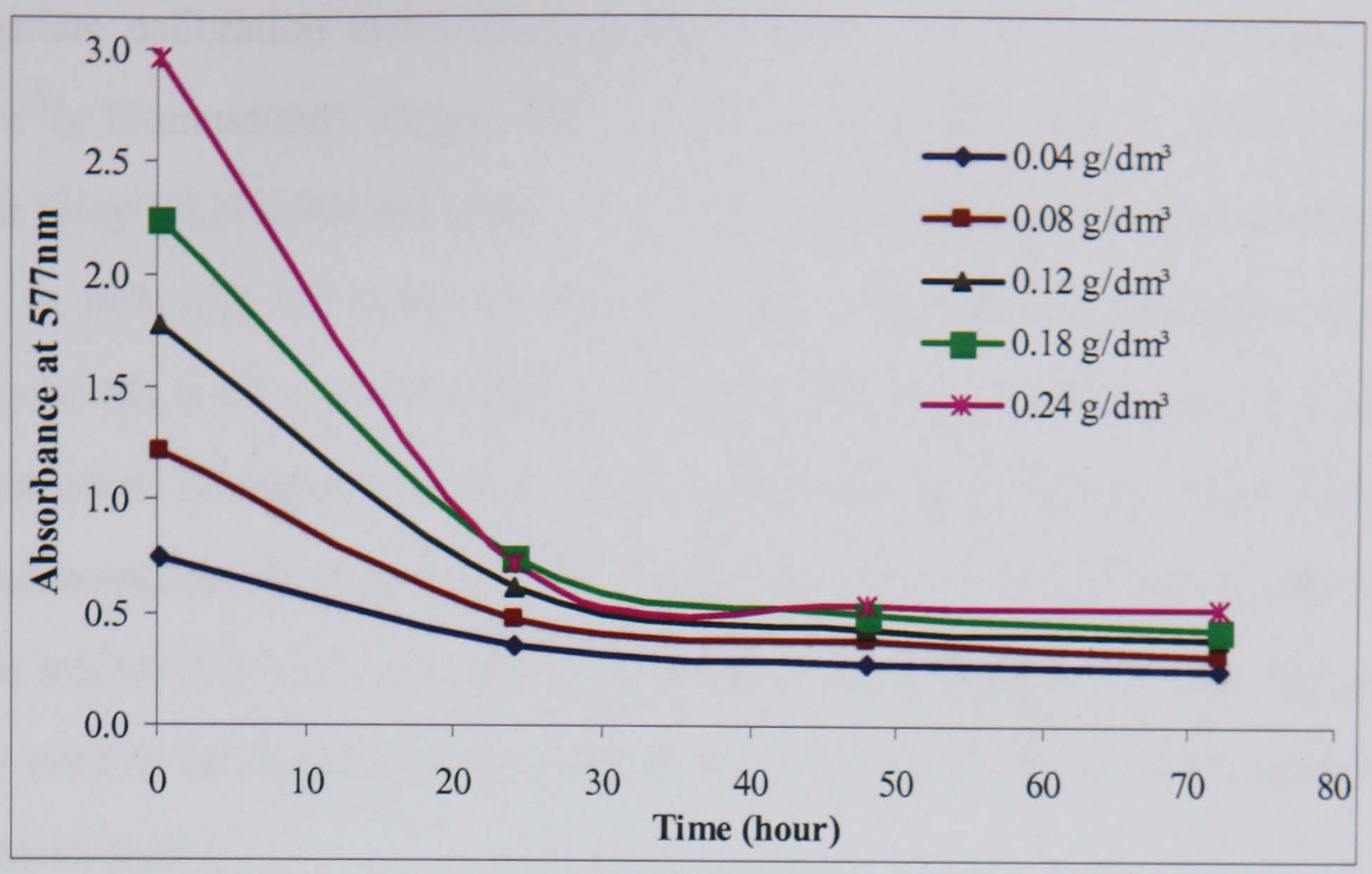


Figure 3.4.2 Reduction of Irgalan Grey GLN Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C

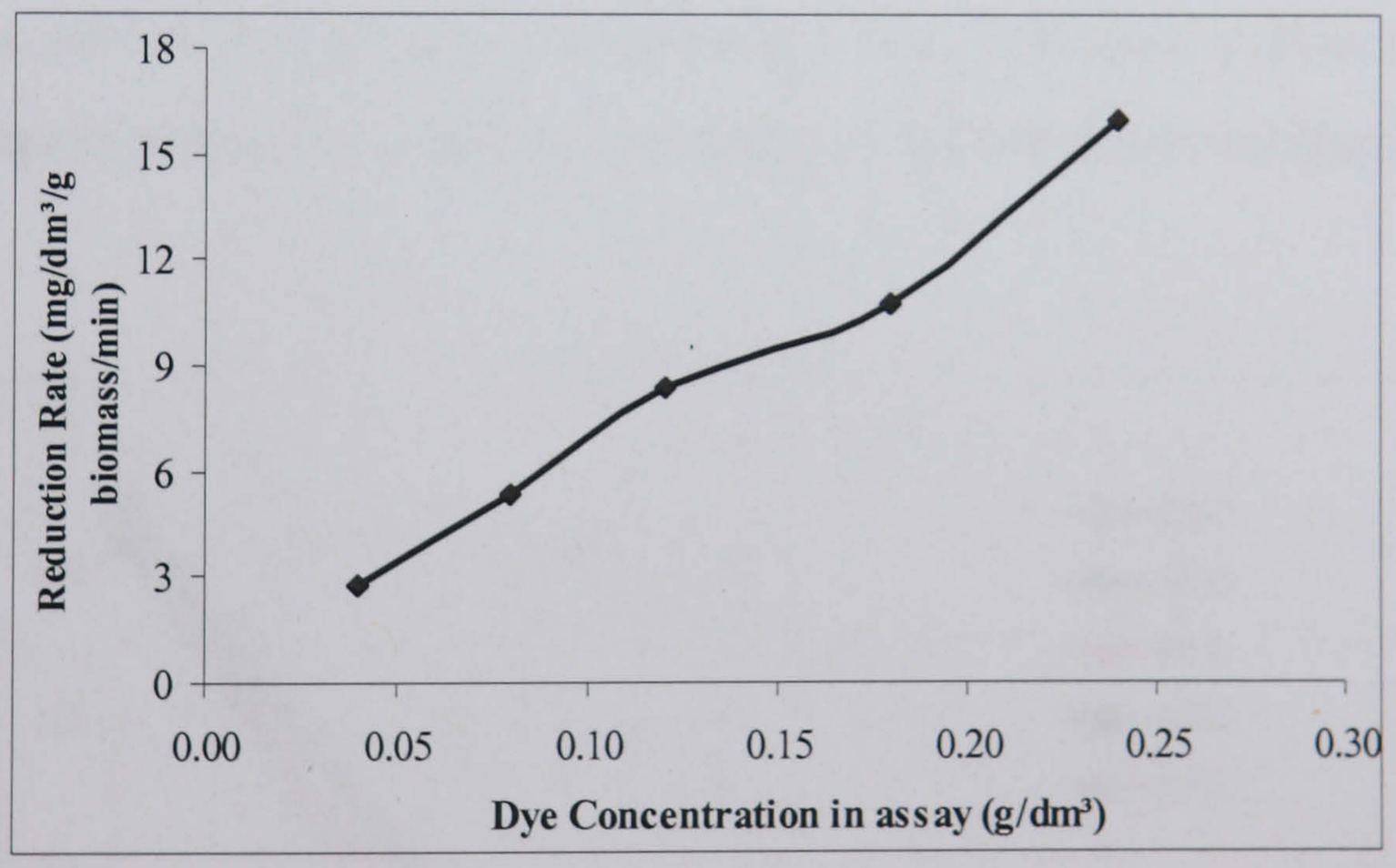


Figure 3.4.3 Effect of Concentration on the Rate of the Irgalan Grey GLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

Figure 3.4.2 shows that the bacterial cells of *Shewanella* strain J18 143 performed effective dye reduction at all of the dye concentrations considered. The rate of the reduction of the dye solutions at each concentration was calculated using the

appropriate calibration curve (Figure 3.4.1). The reduction rate was expressed as mg/dm^{-3}/g biomass/min. Figure 3.4.3 gives the reduction rate for the reduction of Irgalan Grey GLN solutions against the dye concentration of the evaluated systems. It can be seen that the reduction rate increases as the dye concentration increases. This provides a clear relationship of between the dye reduction rate and the dye concentration. However, one should not assume that this relation would apply over all concentrations. The implication for industrial dye effluents is that, unless specific further calibrations are undertaken, for higher concentration of dye, the effluent would need to be "balanced" to bring its concentration with the limits specified by the current study.

3.4.2.2 Effect of temperature on dye reduction

To investigate the effect of temperature on dye reduction, washed anaerobic cells of *Shewanella* strain J18 143 were challenged with solutions of Irgalan Grey GLN (0.12 g dm^{-3}) at pH 6.8, over a range of temperatures from 22°C to 60°C (Section 2.4.4). The change in absorbance at the λ_{max} of the dye solutions is shown in Figure 3.4.4.

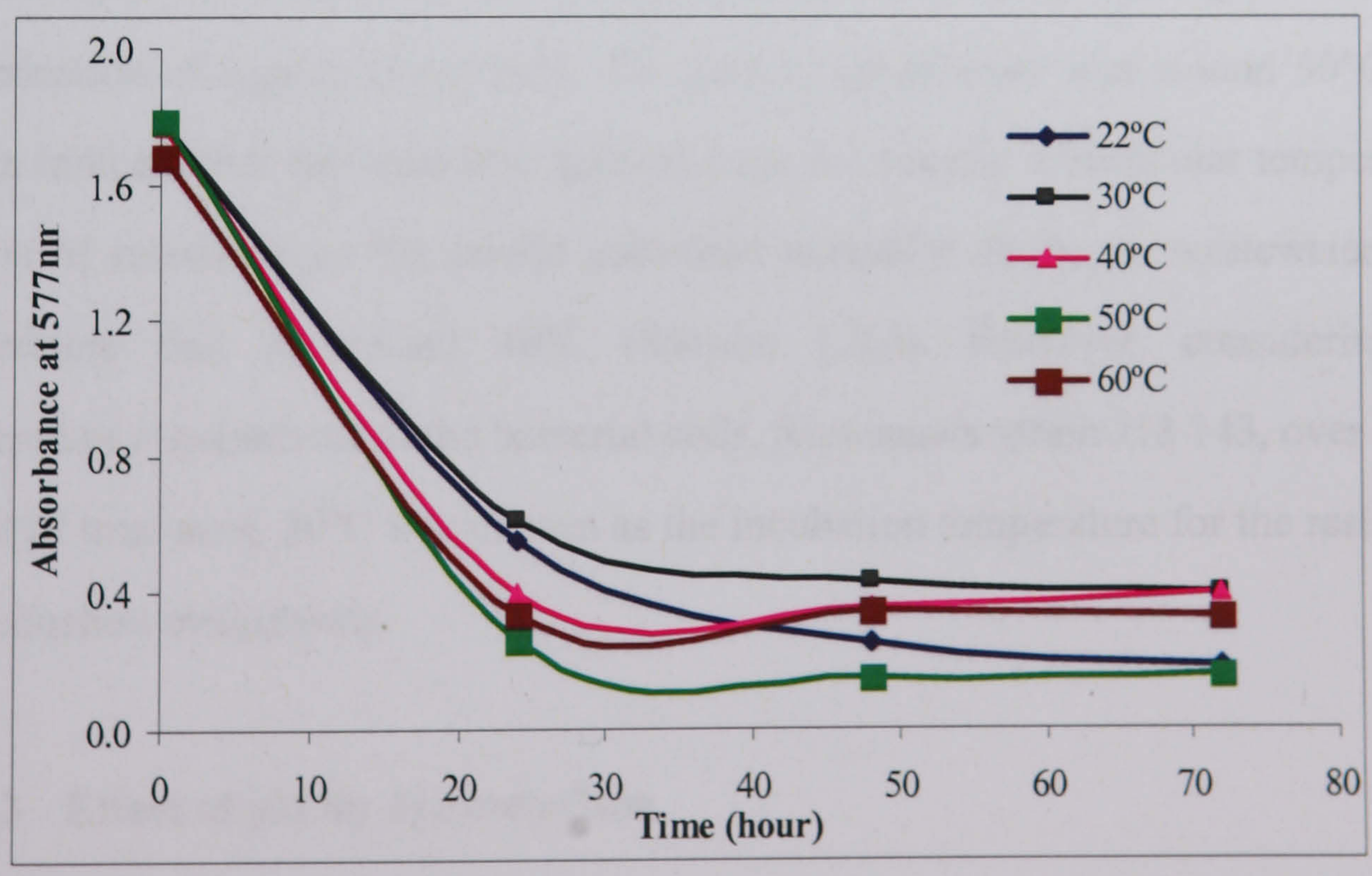


Figure 3.4.4 Reduction of Irgalan Grey GLN Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8)

It can be seen in Figure 3.4.4 that the bacterial cells of *Shewanella* strain J18 143 are capable of reducing the colour of the dye solutions over quite a wide range of temperatures. This effect is, perhaps, more clearly demonstrated in Figure 3.4.5.

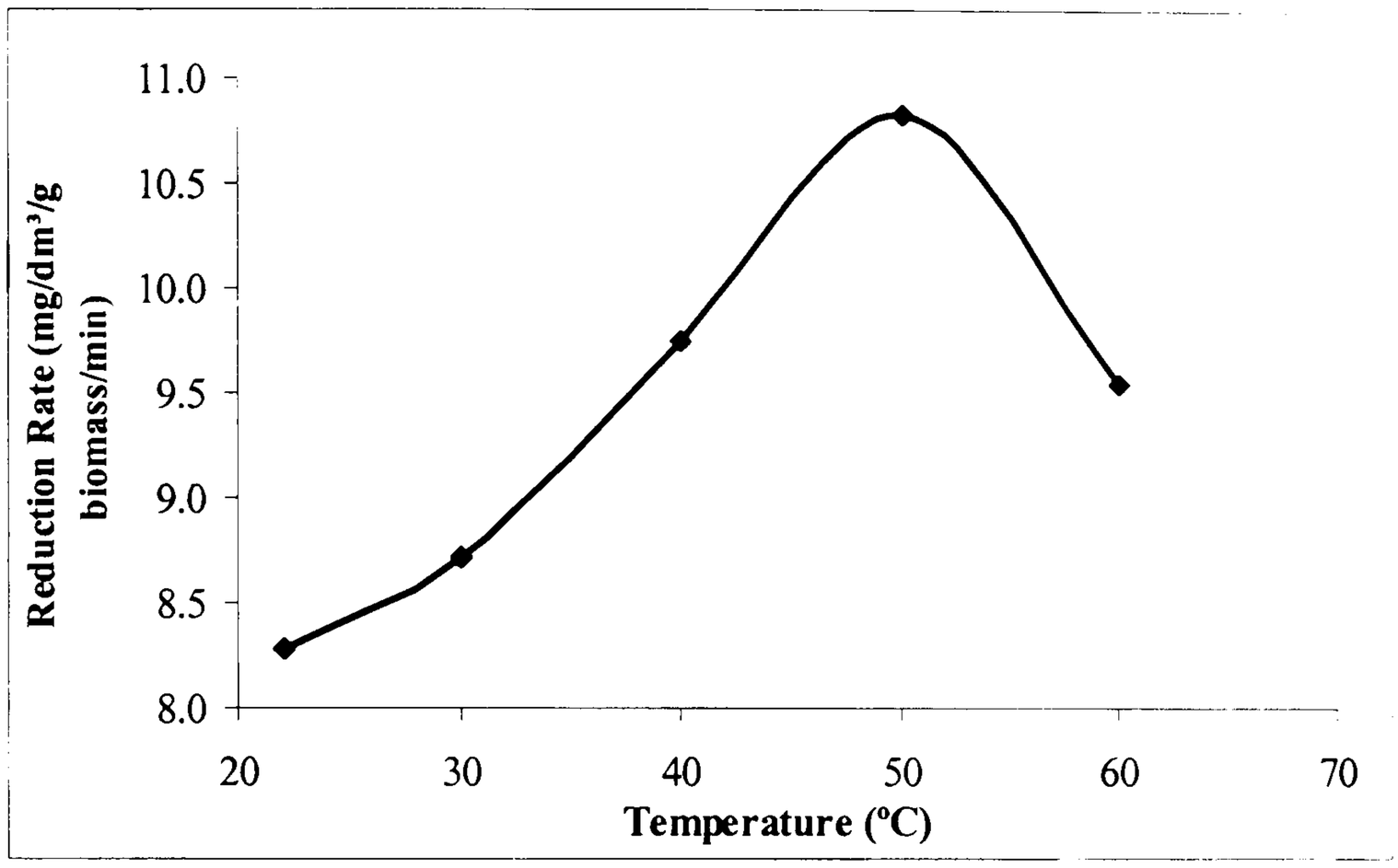


Figure 3.4.5 Effect of Temperature on the Rate of Irgalan Grey GLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

The rate of reduction of the dye solutions by the biomass was calculated. Data concerning of dye reduction rate versus temperature are shown in Figure 3.4.5. For the reduction of Irgalan Grey GLN, the optimal temperature was around 50°C. The results indicate that the reduction process does not require a particular temperature. This is of relevance as the textile industries normally discharge wastewaters at a temperature that is around 40°C (Section 1.2.4). However, considering the temperature requirement of the bacterial cells, *Shewanella* strain J18 143, over a long period of treatment, 30°C was chosen as the incubation temperature for the rest of the dye reduction evaluations.

3.4.2.3 Effect of pH on dye reduction

To determine the optimum pH for dye reduction, washed anaerobic cells of *Shewanella* strain J18 143 were challenged with solutions of Irgalan Grey GLN at

30°C, using a range of different buffers to produce a pH range from 4.4 to 9.2 (Figure 3.4.6).

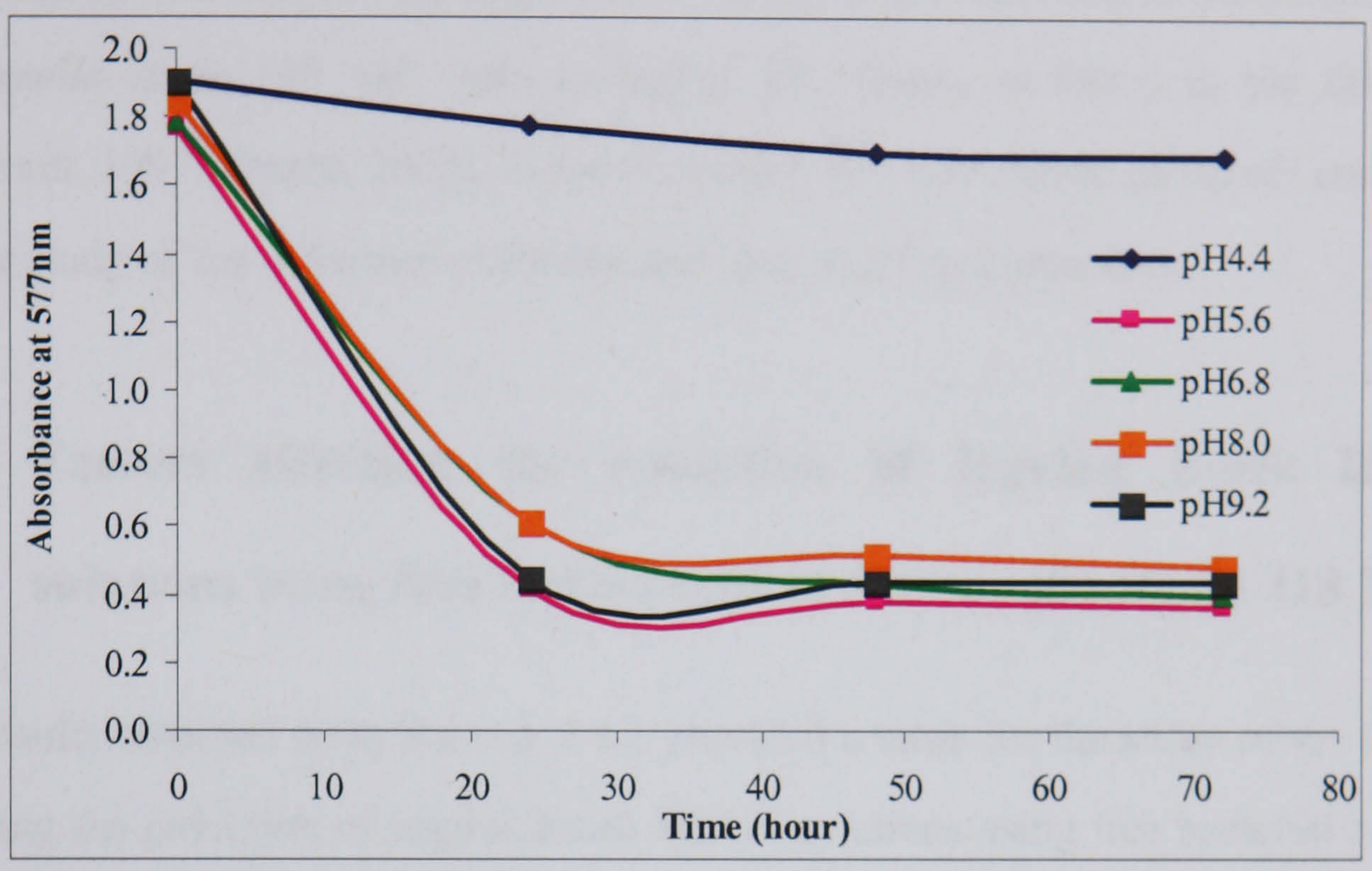


Figure 3.4.6 Reduction of Irgalan Grey GLN Solutions under Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C)

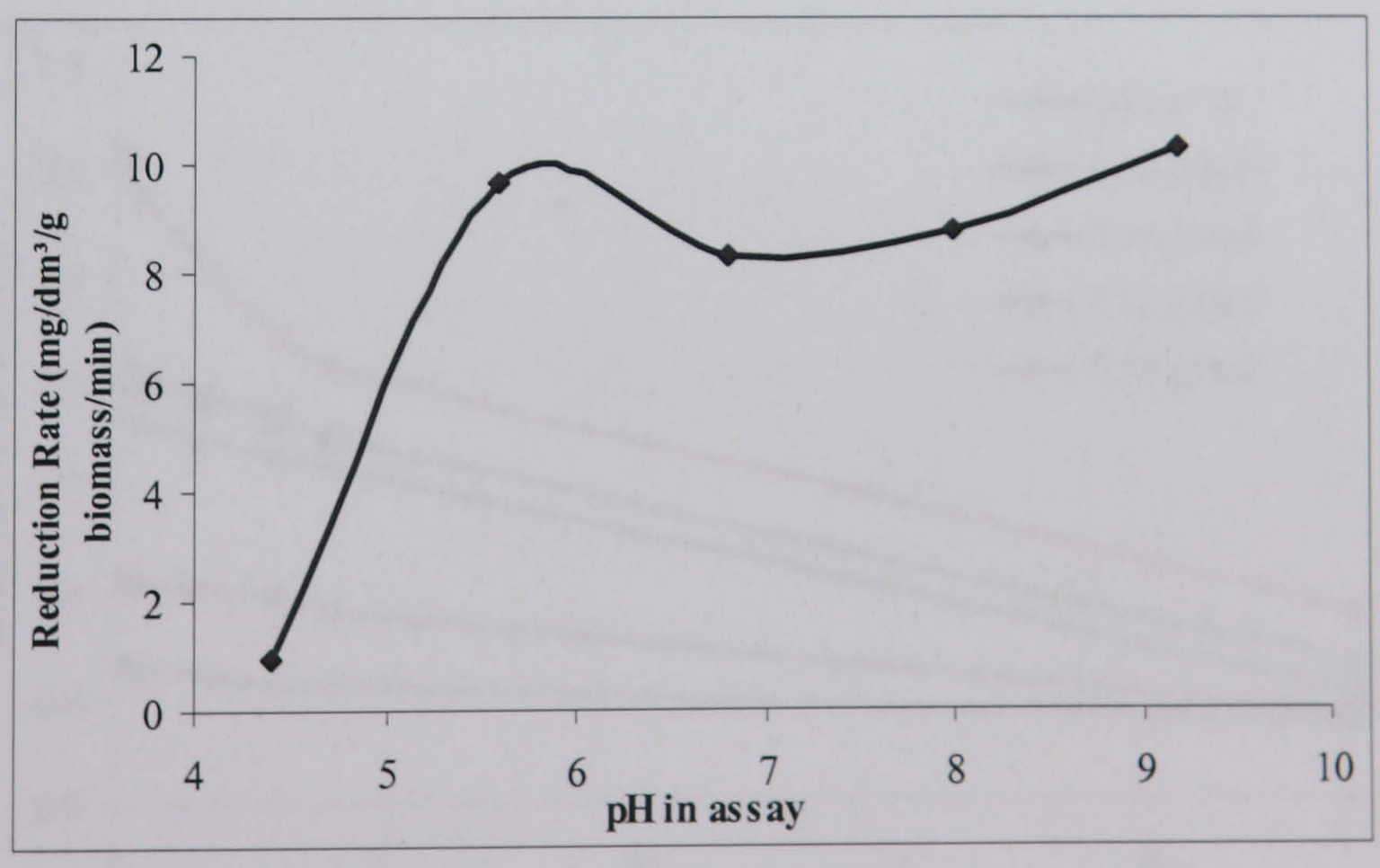


Figure 3.4.7 Effect of pH Conditions on the Rate of Irgalan Grey GLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

As can be seen from Figure 3.4.6, almost no dye reduction occurred at pH 4.4. A significant dye reduction rate occurs at pH 5.6. The results in Figure 3.4.7 show that the bacterial cells of *Shewanella* strain J18 143 can decolorise the dye solutions of Irgalan Grey GLN at pH values up to 9.2. The optimal conditions for reduction using *Shewanella* strain J18 143 were at higher pH values, as found in the literature (Willmott, 1997; Pearce, 2004). In the research, pH 7 was chosen as the pH condition for the study of the reduction of the Irgalan Grey GLN dye solutions.

3.4.3 Factors affecting the reduction of Irgalan Black RBLN solutions using free resting cells of *Shewanella* strain J18 143

The results obtained from Section 3.4.2 provided a basis for the study of the factors affecting the reduction of Irgalan Black RBLN solutions using free bacterial cells of *Shewanella* strain J18 143.

3.4.3.1 Effect of dye concentration on dye reduction

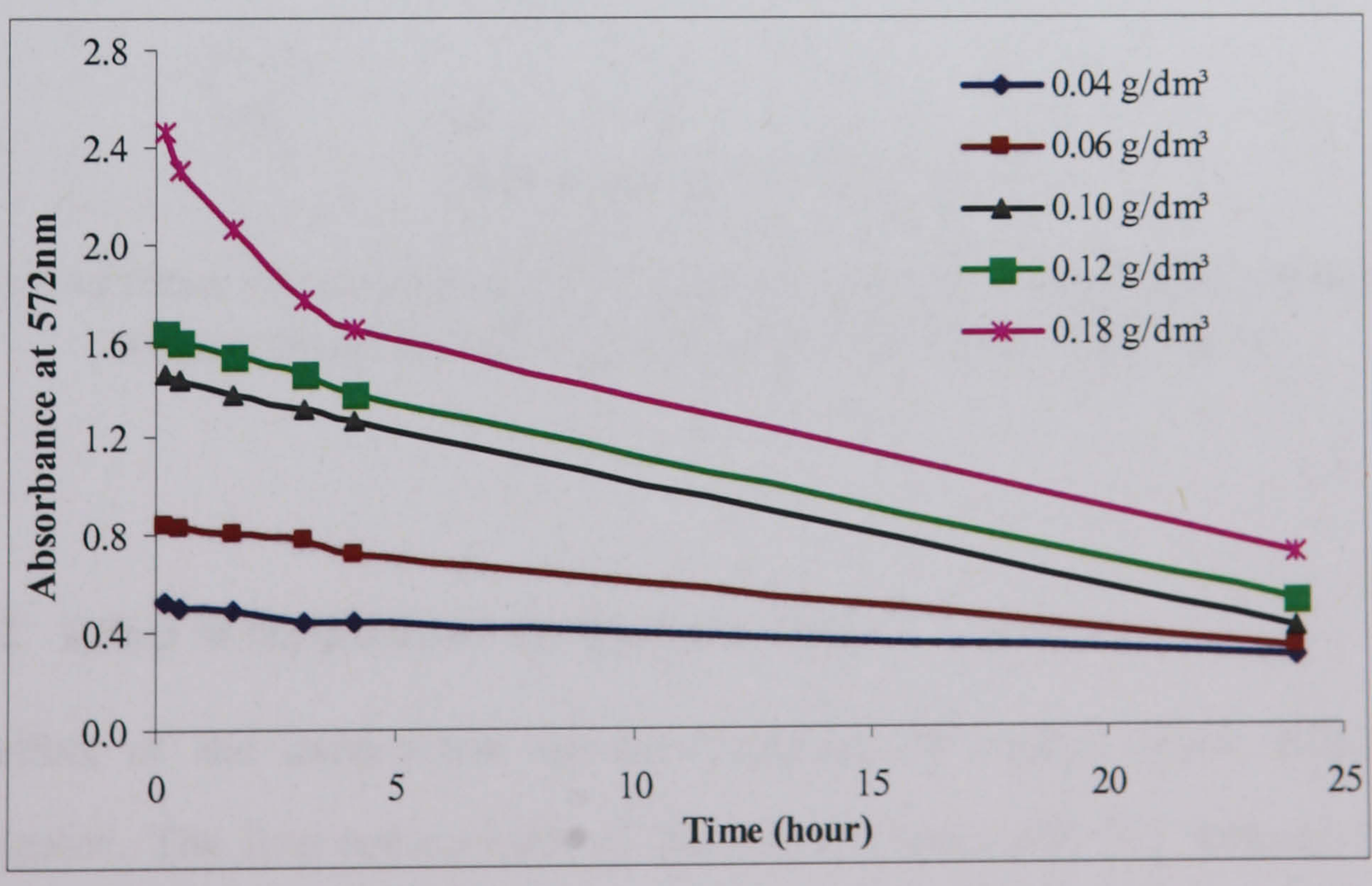


Figure 3.4.8 Reduction of Irgalan Black RBLN Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C

The effect of the concentration of the Irgalan Black RBLN solution on the dye reduction was investigated. The results are shown in Figure 3.4.8.

It can be seen from Figure 3.4.8 that the biological cells of *Shewanella* strain J18 143 are capable of reducing the colour of the dye solution over the considered concentration range. The reduction rate was calculated based on the standard protein assay and the calibration plot of the Irgalan Black RBLN solutions. The reduction rate was expressed as $mg/dm^{-3}/g$ biomass/min. Figure 3.4.9 gives the reduction rate for the reduction of Irgalan Black RBLN solutions against the dye concentration of the evaluated systems. The reduction rate increases as the dye concentration increases.

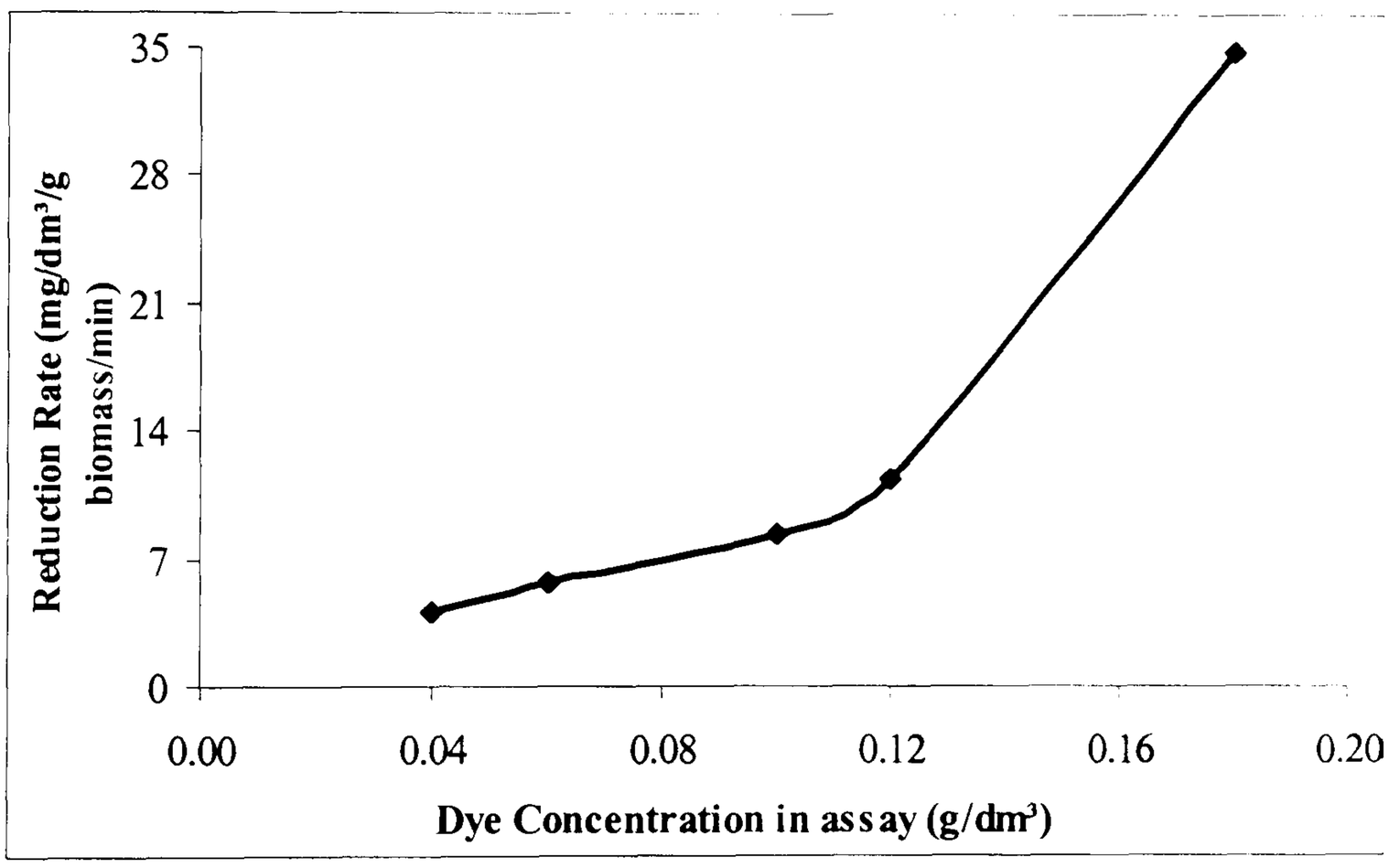


Figure 3.4.9 Effect of Concentration on the Rate of the Irgalan Black RBLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

3.4.3.2 Effect of temperature on dye reduction

The effect of the temperature on the reduction of Irgalan Black RBLN was investigated. The free resting cells of *Shewanella* strain J18 143 were challenged with solutions of Irgalan Black RBLN (0.08 g dm^{-3}) at a pH 6.8, over a range of

temperatures from 22°C to 60°C (Section 2.4.4). The results are shown in Figure 3.4.10.

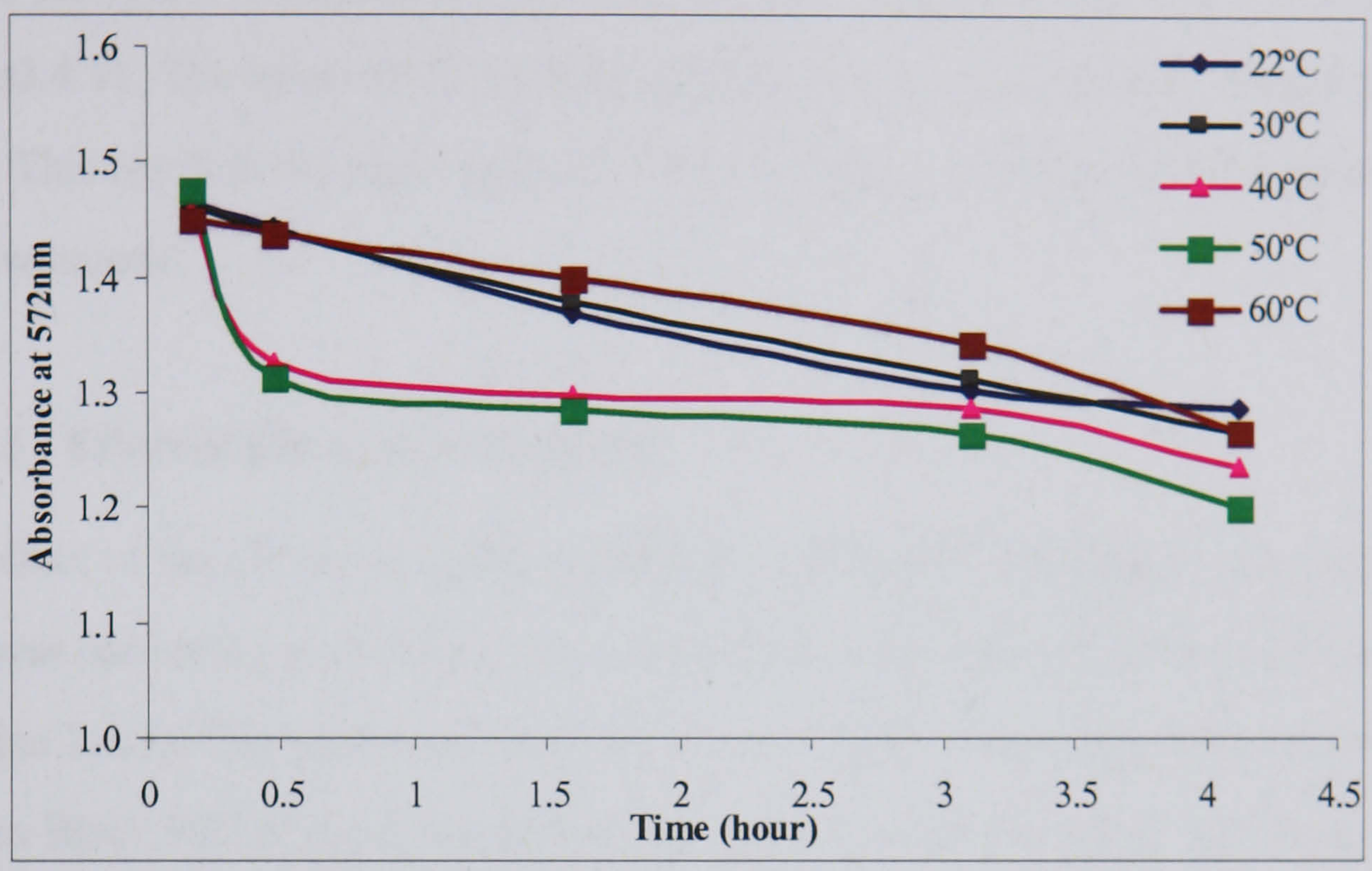


Figure 3.4.10 Reduction of Irgalan Black RBLN Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8)

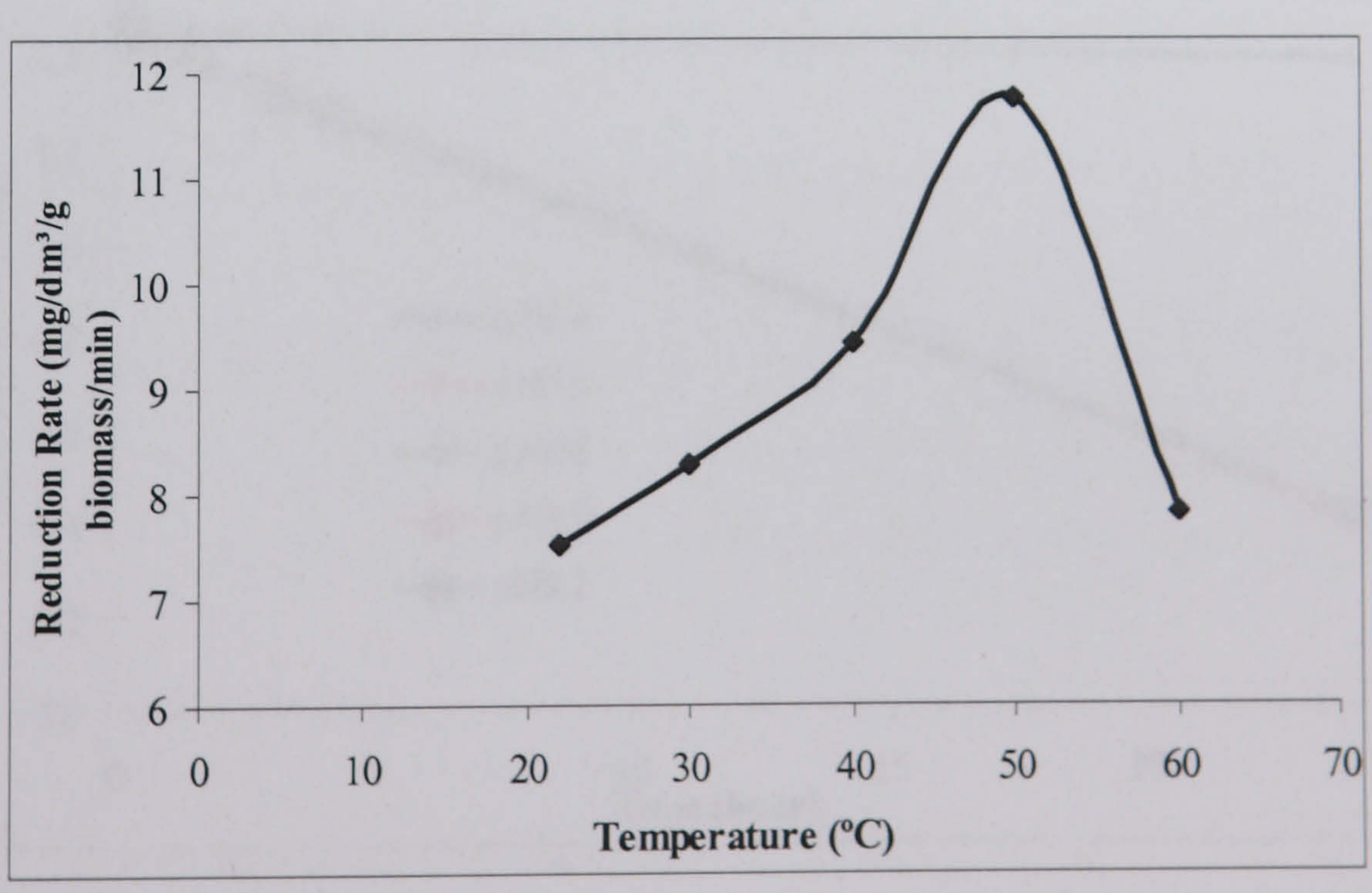


Figure 3.4.11 Effect of Temperature on the Rate of Irgalan Black RBLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

Figure 3.4.10 shows that the free resting cells of *Shewanella* strain J18 143 are capable of reducing the colour of solutions of Irgalan Black RBLN over a wide range of temperatures. The optimisation of the effect of the temperature on the reduction rate of the colour reduction of the dye solutions of Irgalan Black RBLN is shown in Figure3.4.11. The most effective colour reduction was found below a temperature of 50°C. This result is the same as found from the colour reduction of the Irgalan Grey GLN solutions.

3.4.3.3 Effect of pH on dye reduction

The effect of the pH on the colour reduction of Irgalan Black RBLN was evaluated. This was carried by preparing a range of buffers to produce a pH range of 4.4 to 9.2 (Section 2.4.1). The results are shown in Figure 3.4.12. The colour of the solutions of Irgalan Black RBLN could not be reduced at pH 4.4. The bacterial cells have shown the ability of reducing the colour from the solutions of Irgalan Black RBLN, from pH 5.6 to pH 9.2.

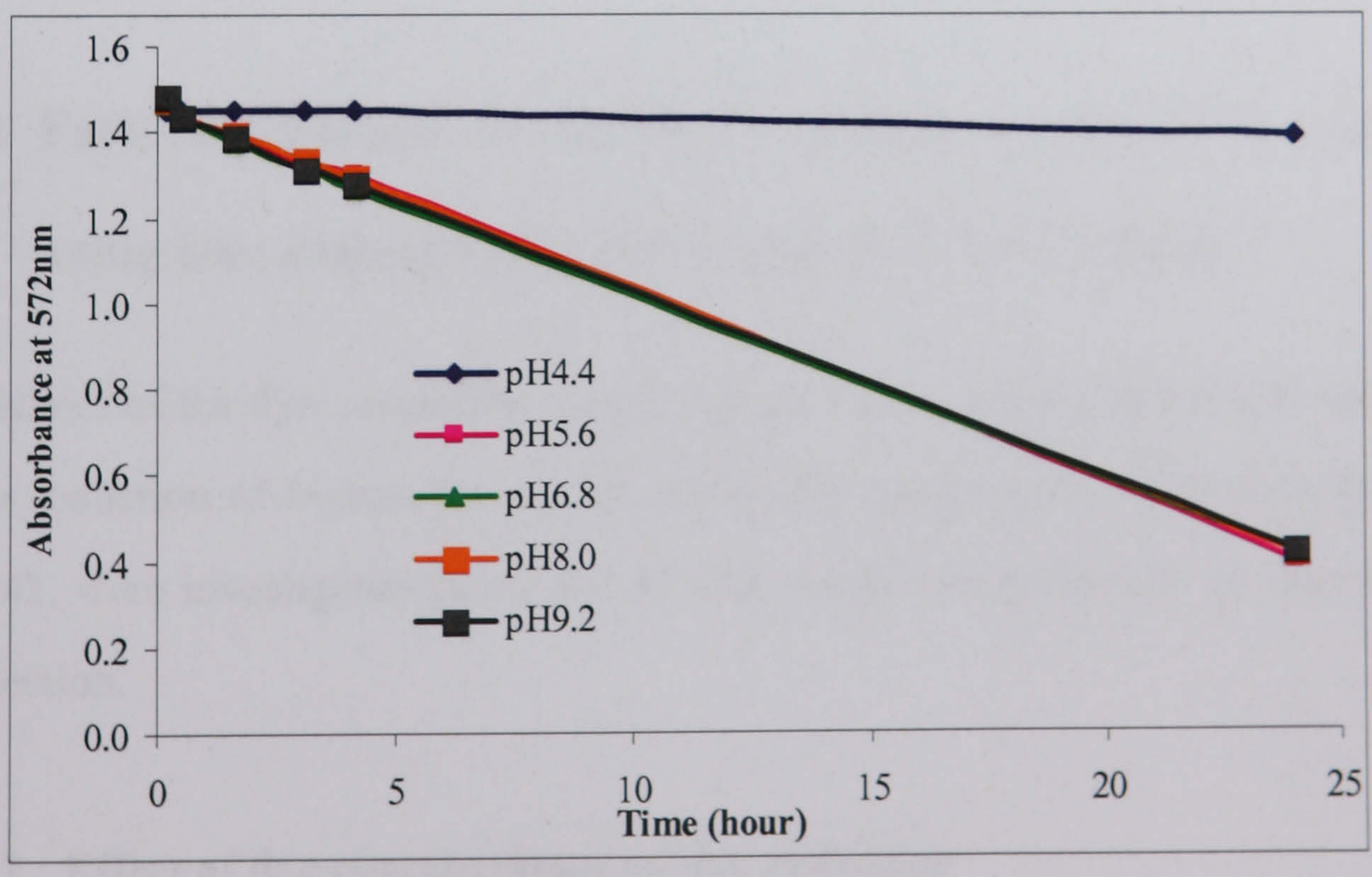


Figure 3.4.12 Reduction of Irgalan Black RBLN Solutions under Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C)

The calculated reduction rates are shown in Figure 3.4.13. The free resting cells of *Shewanella* strain J18 143 show more effective reduction behaviour at higher pH ranges, from pH 6 to pH 9.2.

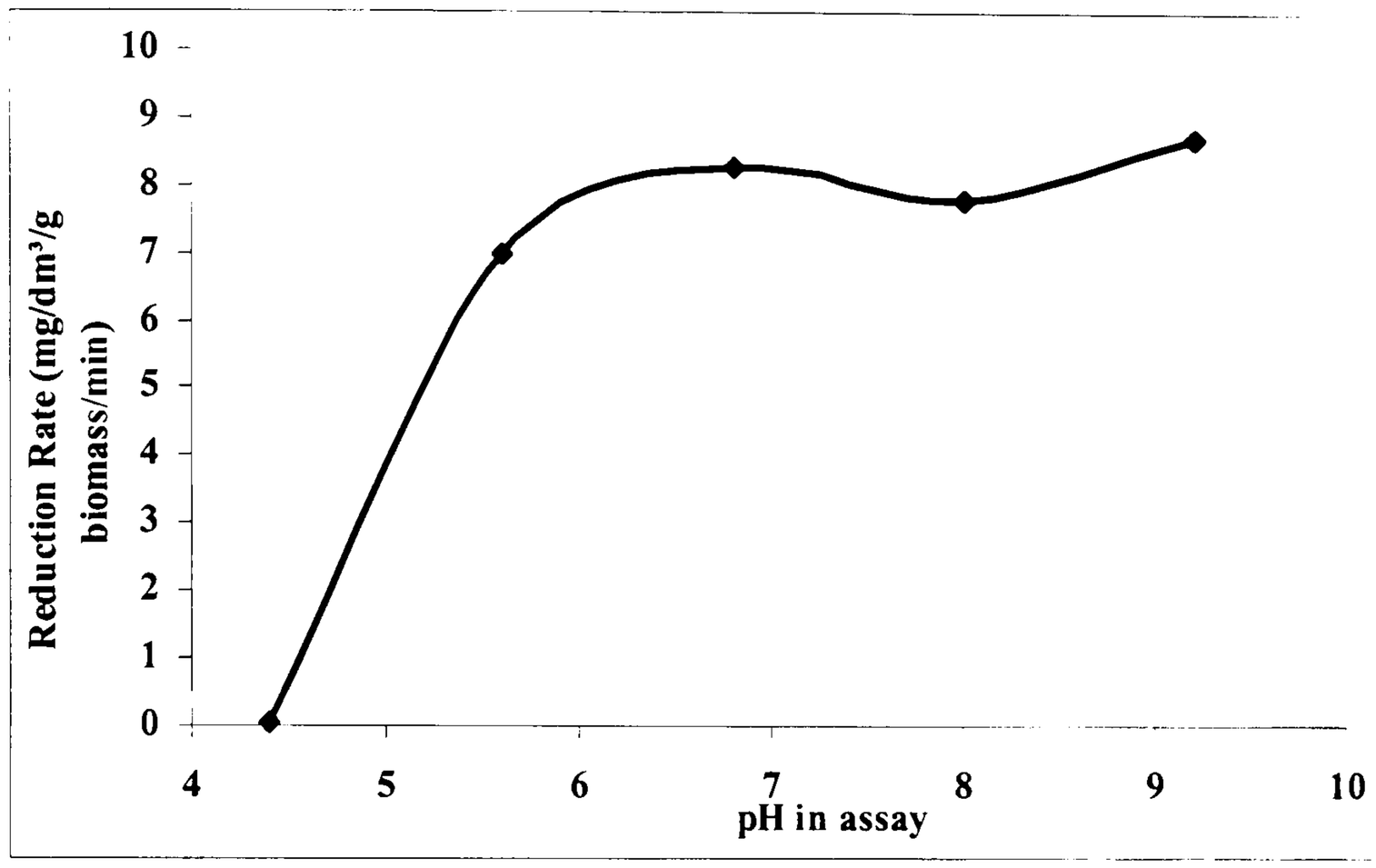


Figure 3.4.13 Effect of pH Conditions on the Rate of Irgalan Black RBLN Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

3.4.4 Factors affecting the reduction of Irgalan Blue 3GL solutions using free resting cells of *Shewanella* strain J18 143

The factors of the dye concentration, the temperature and the pH that may affect the colour reduction of Irgalan Blue 3GL, using free resting cells of *Shewanella* strain J18 143, were investigated (Section 2.4). The results are given and are discussed in this Section.

3.4.4.1 Effect of dye concentration on dye reduction

The effect of the dye concentration on the colour reduction of Irgalan Grey GLN and Irgalan Black RBLN was discussed in Section 3.4.2 and in Section 3.4.3 respectively.

The same evaluation routine was carried out on the Irgalan Blue 3GL. The results are shown in Figure 3.4.14.

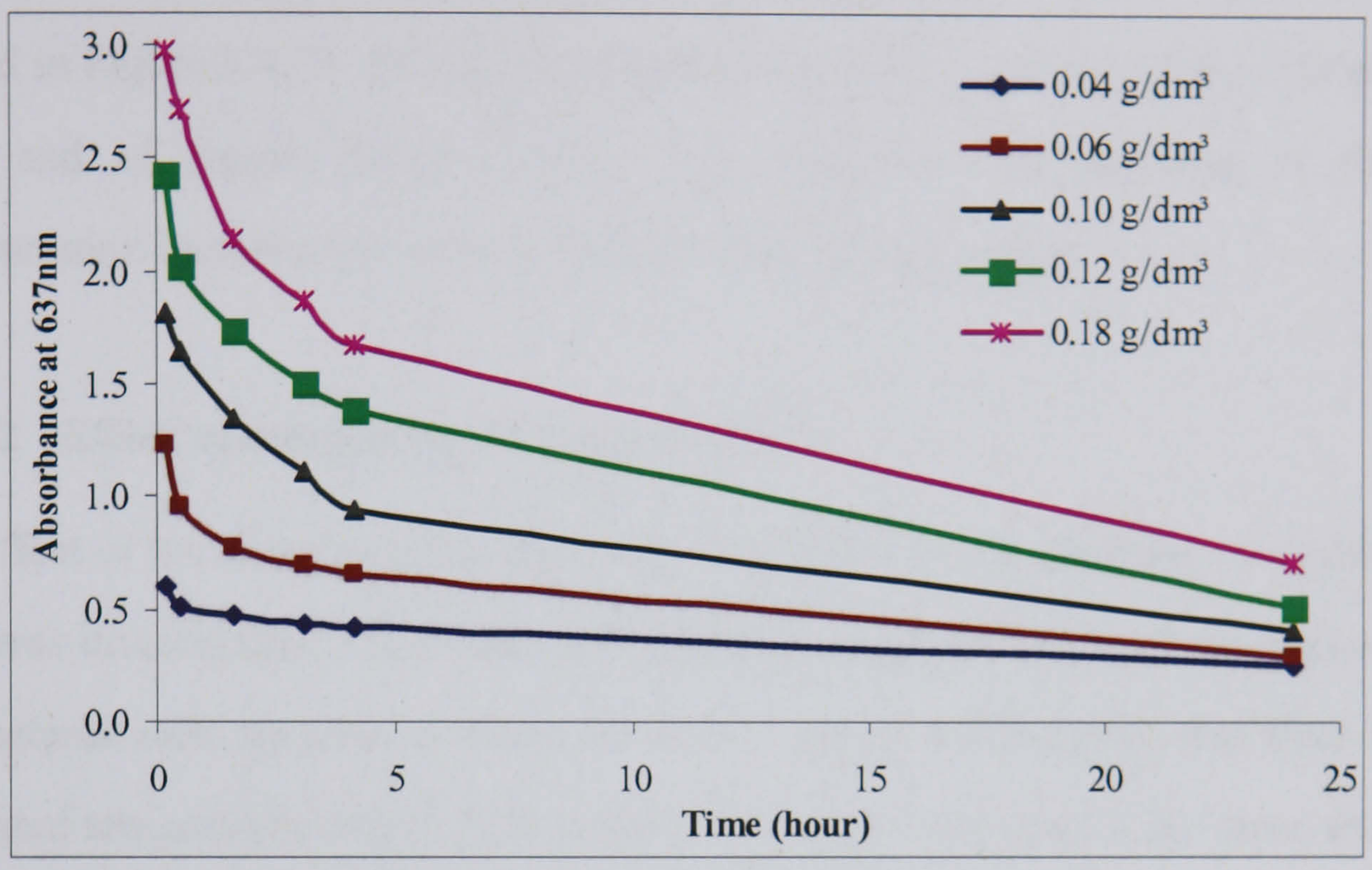


Figure 3.4.14 Reduction of Irgalan Blue 3GL Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C

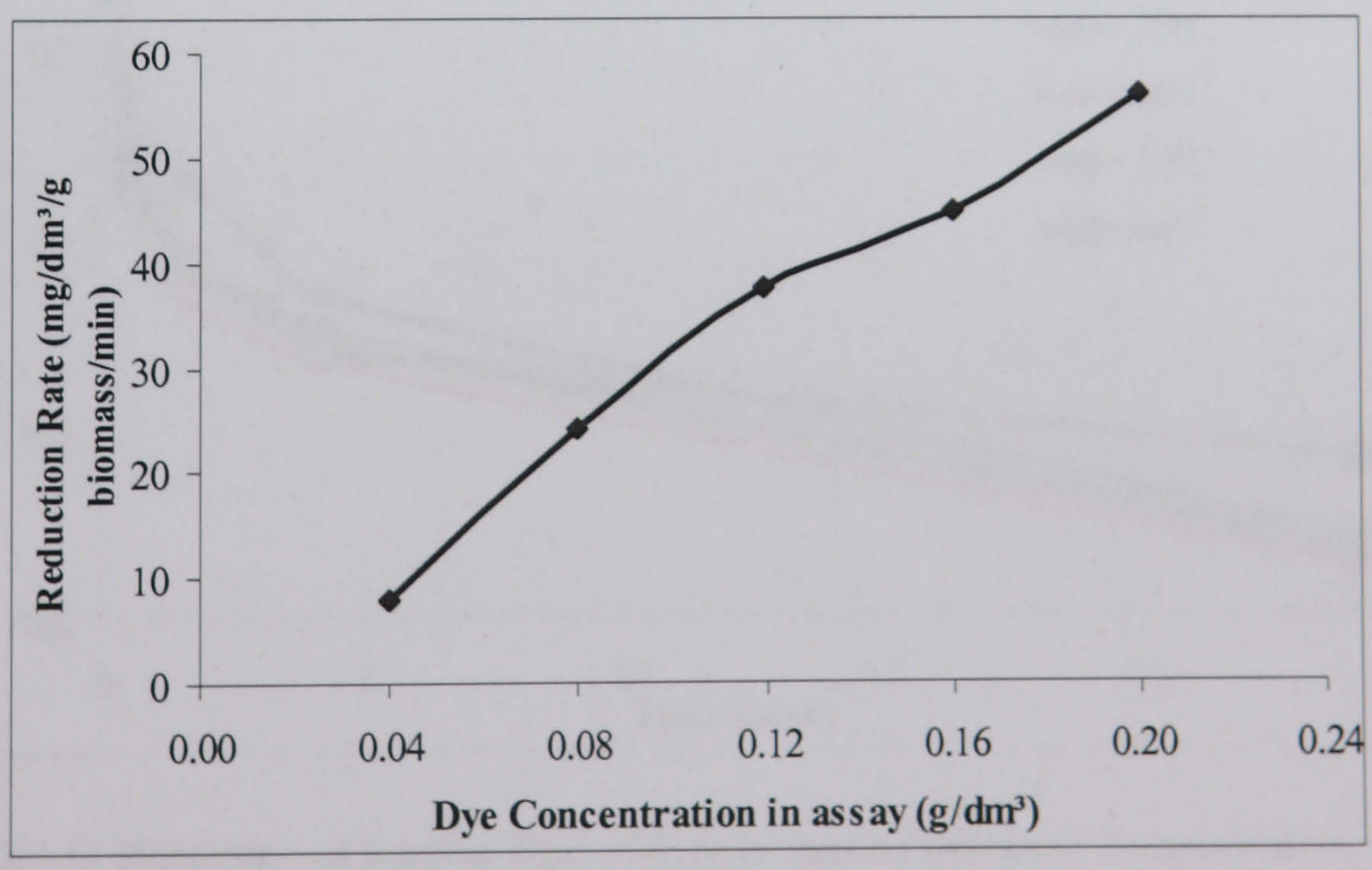


Figure 3.4.15 Effect of Concentration on the Rate of the Irgalan Blue 3GL Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

Figure 3.4.14 shows that the free resting cells of *Shewanella* strain J18 143 provided colour reduction of the dye solutions of Irgalan Blue 3GL over the dye concentration range from 0.04 g dm^{-3} to 0.18 g dm^{-3}. The reduction rate at each concentration was calculated. The reduction rates against the corresponding dye concentrations are plotted in Figure 3.4.15. The results obtained are similar to those of the Irgalan Grey GLN and of Irgalan Black RBLN. The reduction rate increases as the dye concentration increases over the tested dye concentration range.

3.4.4.2 Effect of temperature on dye reduction

The effect of the temperature on the colour reduction of the solutions of Irgalan Blue 3GL was investigated. The results are displayed in Figure 3.4.16. It can be seen that the bacterial cells are able to reduce the colour from solutions of Irgalan Blue 3GL at the tested temperature range, 22°C to 60°C. This result obtained is the same as that of the colour reduction of Irgalan Grey GLN and of Irgalan Blue 3GL.

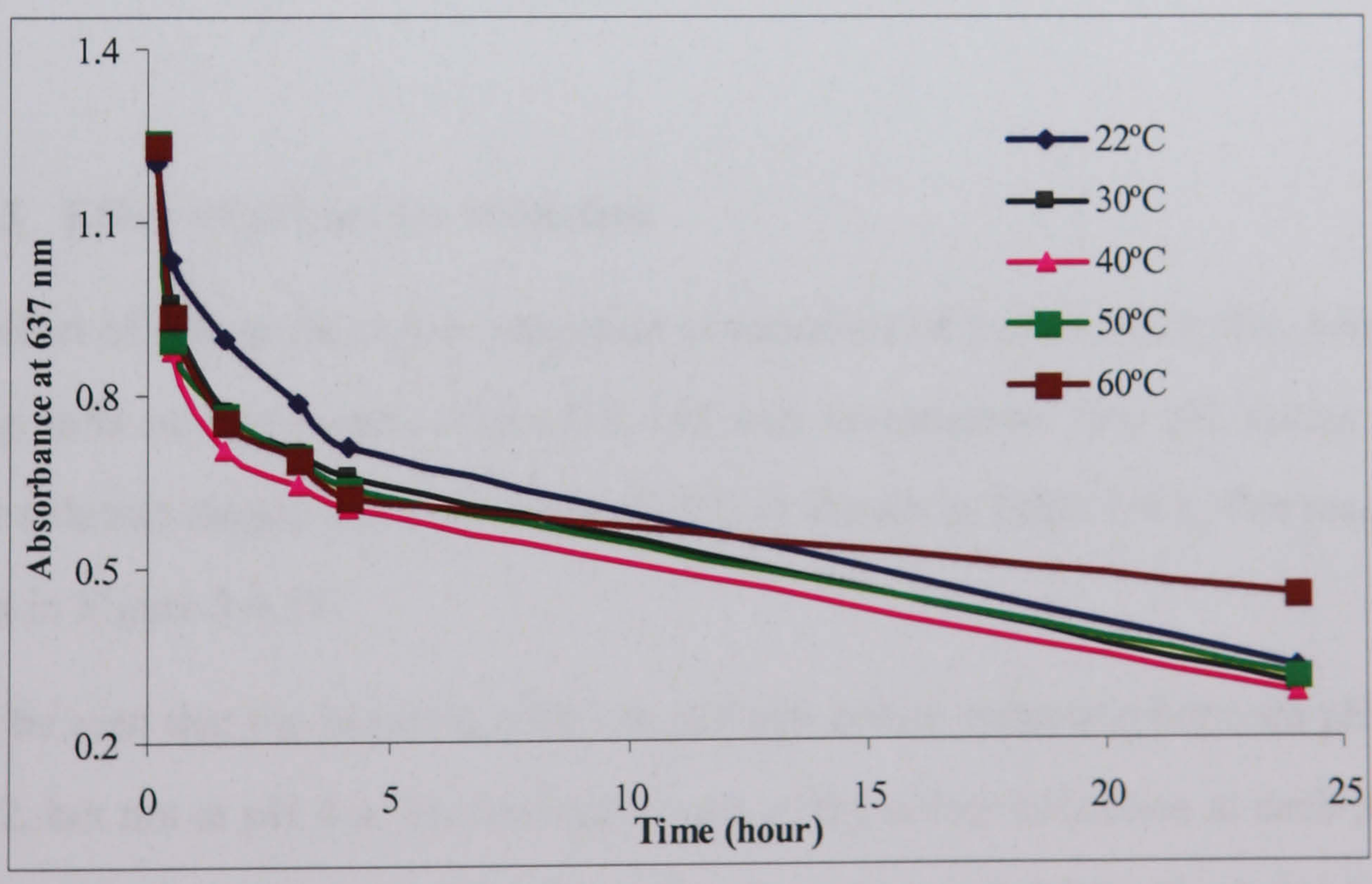


Figure 3.4.16 Reduction of Irgalan Blue 3GL Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8)

The reduction rate of the colour reduction of Irgalan Blue 3GL against the temperature was constructed as shown in Figure 3.4.17. The bacterial cells were more effective at a temperature of 40°C.

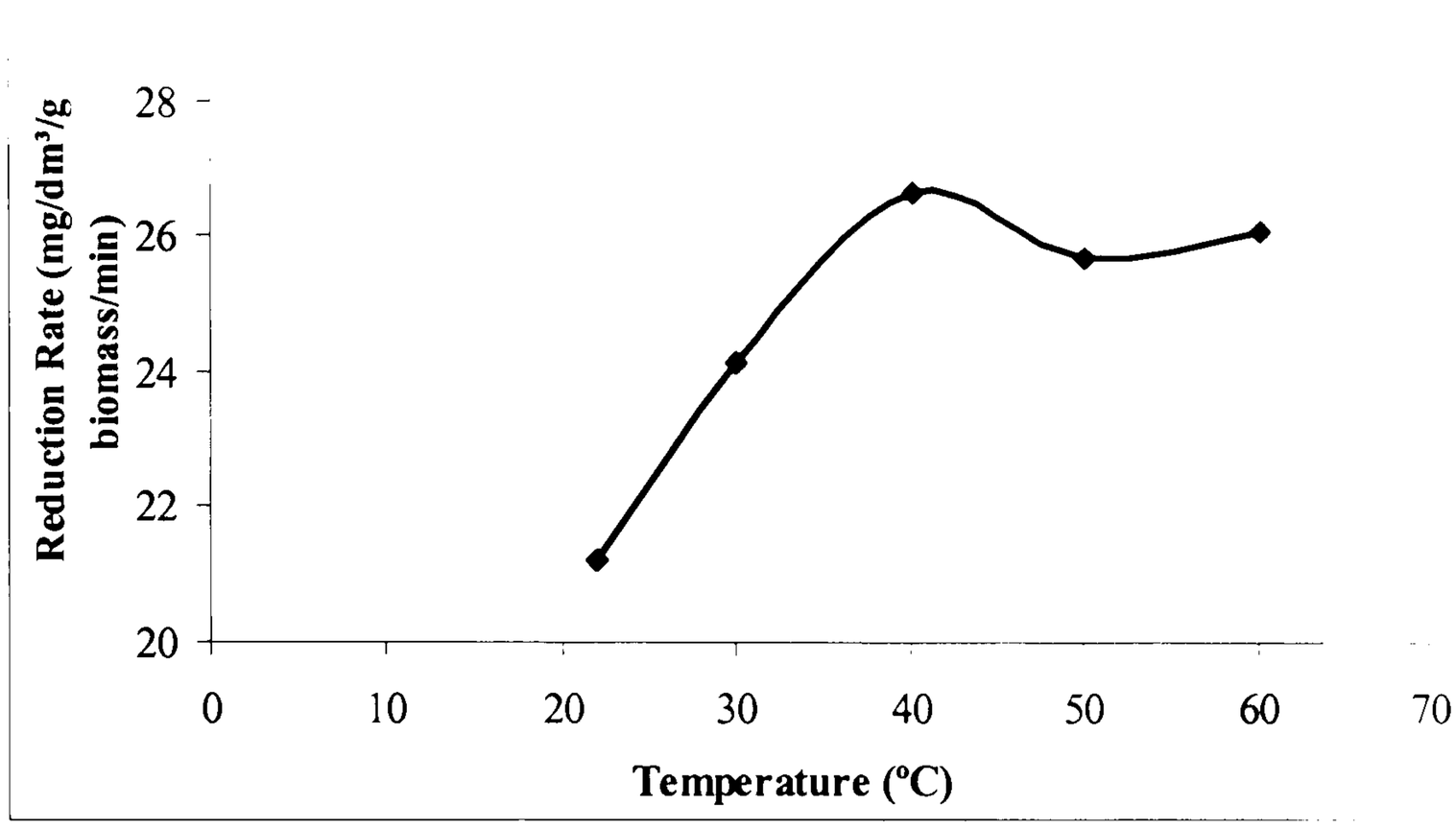


Figure 3.4.17 Effect of Temperature on the Rate of Irgalan Blue 3GL Dye Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

3.4.4.3 Effect of pH on dye reduction

The effect of pH on the colour reduction of solutions of Irgalan Blue 3GL using free resting cells of *Shewanella* strain J18 143 was investigated. The pH values of the buffer solution ranged from pH 4.4 to pH 9.2 as shown in Table 2.4.1. The results are shown in Figure 3.4.18.

It can be seen that the bacterial cells can perform colour reduction between pH 5.6 to pH 9.2, but not at pH 4.4. The reduction rate of the colour reduction at each pH was constructed against the corresponding pH values (Figure 3.4.19). The bacterial cells give more effective colour reduction results from pH 7 to pH 9.

It can be seen that the results obtained, from the investigation of the effect of the dye concentration, the temperature and the pH on the colour reduction of Irgalan Blue

3GL200%, were the same as those of the Irgalan Grey GLN and Irgalan Black RBLN. This provides a confidence in further studies of the colour reduction of metal-complex azo dyes using the bacterial cells of *Shewanella* strain J18 143.

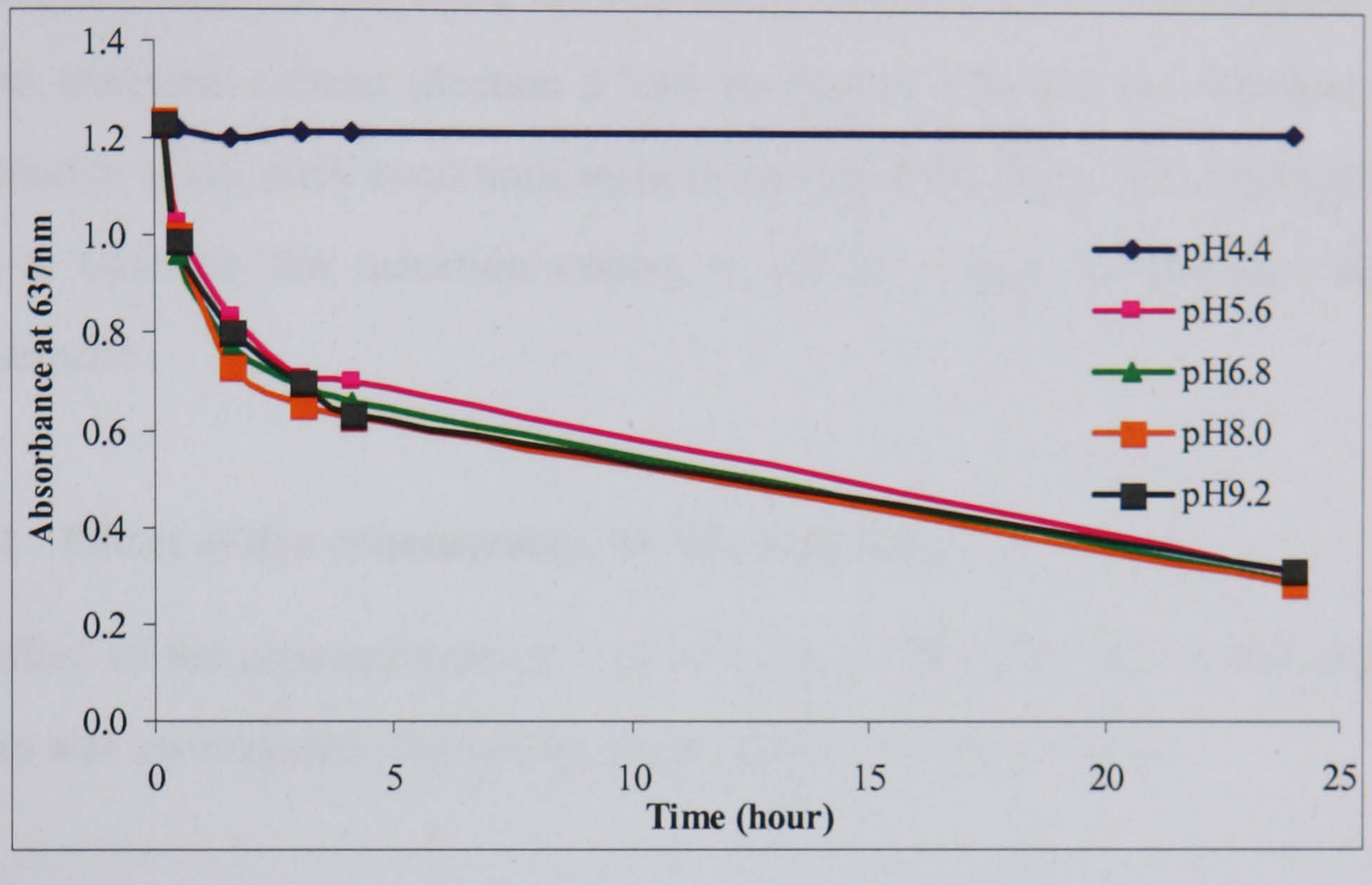


Figure 3.4.18 Reduction of Irgalan Blue 3GL Solutions under Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C)

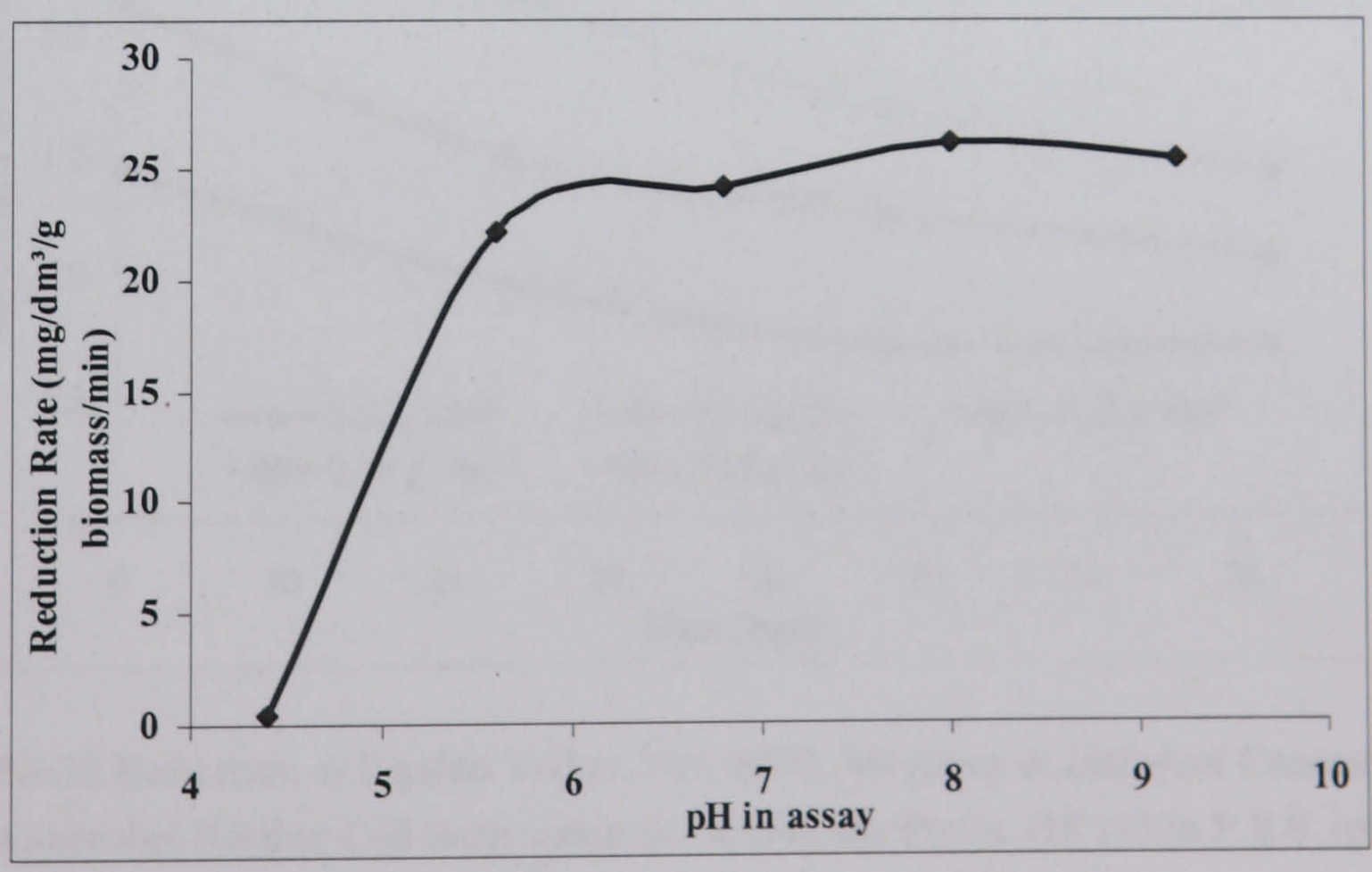


Figure 3.4.19 Effect of pH Conditions on the Rate of Irgalan Blue 3GL Reduction in Solution using a Resting Cell Suspension of *Shewanella* Strain J18 143

3.4.5 Factors affecting the reduction of Irgalan Yellow 3RL KWL solutions using free resting cells of *Shewanella* strain J18 143

Although the reduction of Irgalan Yellow 3RL KWL using free bacterial cells of *Shewanella* strain J18 143 under the conditions chosen was not significant in a total effluent treatment context (Section 3.3.4), the factors affecting the reduction of the dye solution under such conditions were investigated (Section2.3.3). Attempts were made to optimise the reduction system to obtain a more satisfactory reduction environment.

3.4.5.1 Effect of dye concentration on dye reduction

The effect of the concentration of Irgalan Yellow 3RL KWL on the dye reduction process was investigated. The results are presented in Figure 3.4.20.

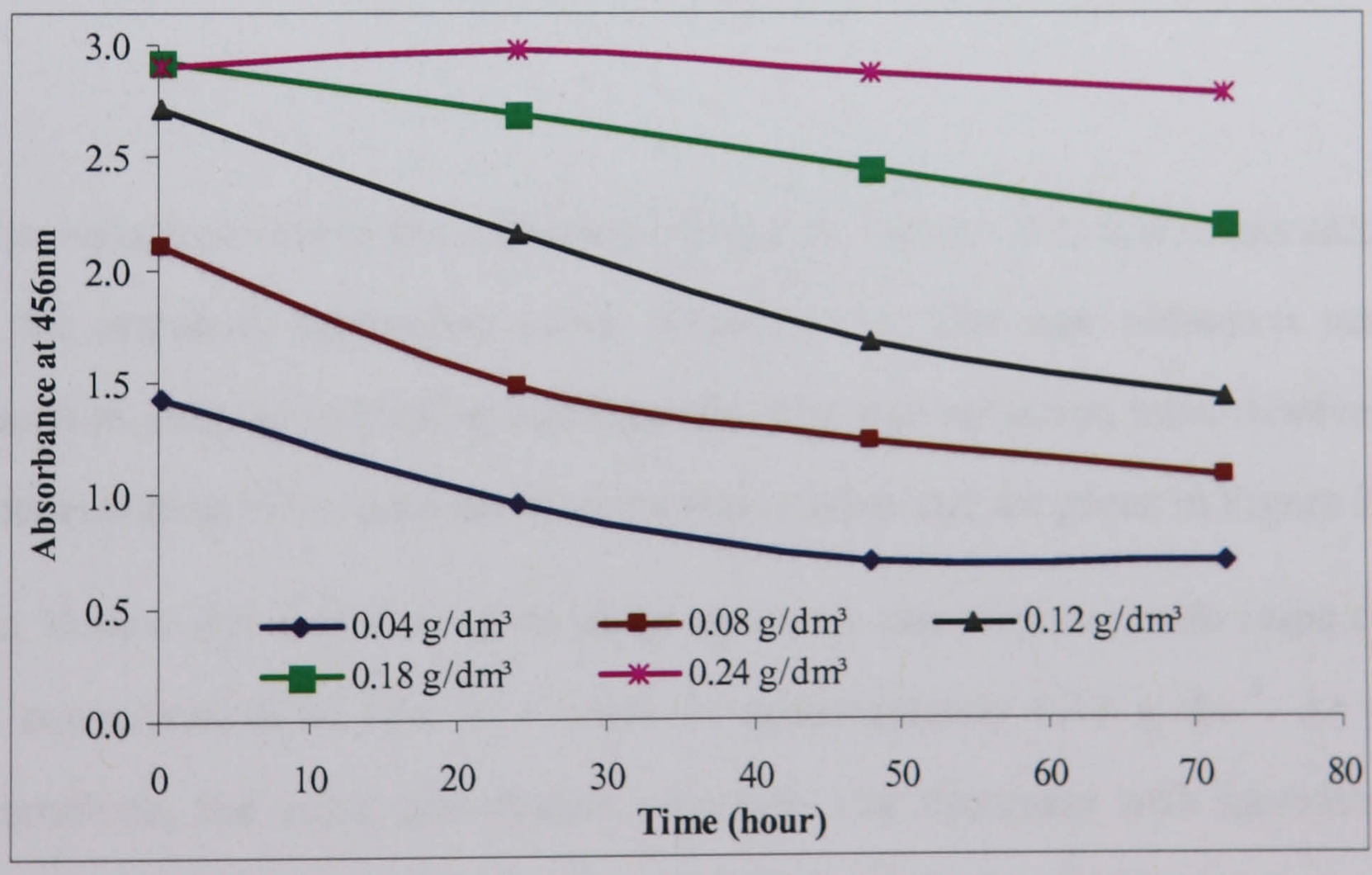


Figure 3.4.20 Reduction of Irgalan Yellow 3RL KWL Solutions at Different Concentrations, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8) at 30 °C

The dye concentrations were prepared in the range from 0.04 g dm^{-3} to 0.24 g dm^{-3}. Figure 3.4.20 shows that almost no reduction occurred at the dye concentration of 0.24 g dm^{-3}. Dye reduction at other dye concentrations was more pronounced.

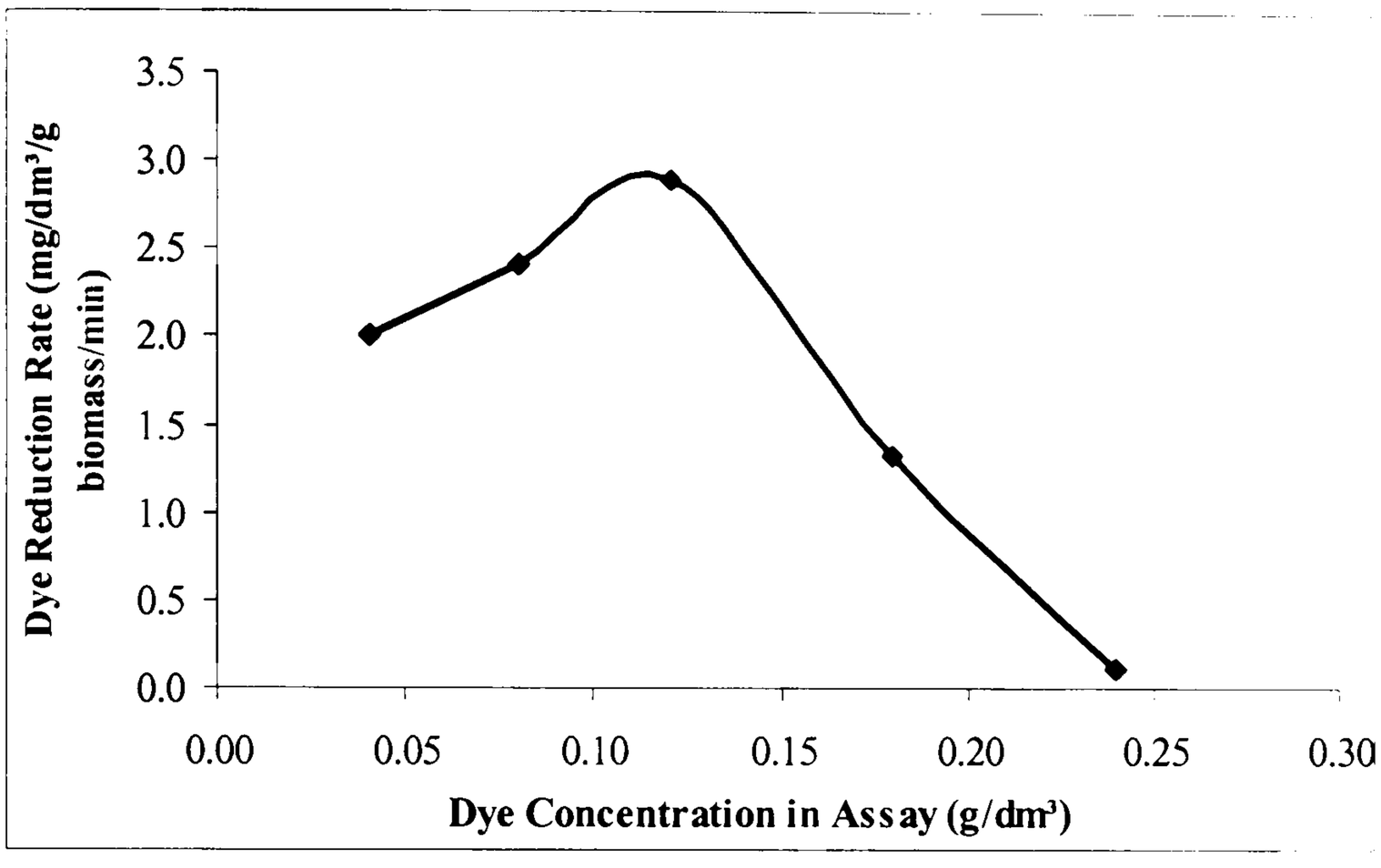


Figure 3.4.21 Effect of Concentration on the Rate of Irgalan Yellow 3RL KWL Dye Reduction in Solution using Resting Cell Suspension of *Shewanella* Strain J18 143

The dye reduction rate in the reduction of Irgalan Yellow 3RL KWL was calculated using the standard calibration curve (Figure3.4.1). The dye reduction rate was expressed in units of mg/dm^3/g biomass/min. The dye reduction rates relative to the dye concentration in the reaction systems were drawn and are given in Figure 3.4.21.

Irgalan Yellow dye reduction showed an optimum rate response with respect to the initial concentration of dye, at a value of approximately 0.12 g dm^{-3}. At greater concentrations, the initial absorbance reduction rate decreases with increasing dye concentration. This is possibly due to toxic effects of the dye on the micro-organisms. Thus, the dye concentration, in the reduction system, of 0.12 g dm^{-3} was chosen as the optimum condition.

3.4.5.2 Effect of temperature on dye reduction

The effect of the system temperature on the reduction of Irgalan Yellow 3RL KWL was investigated. The results are shown in Figure 3.4.22. The temperature range covered from 22° C to 60° C.

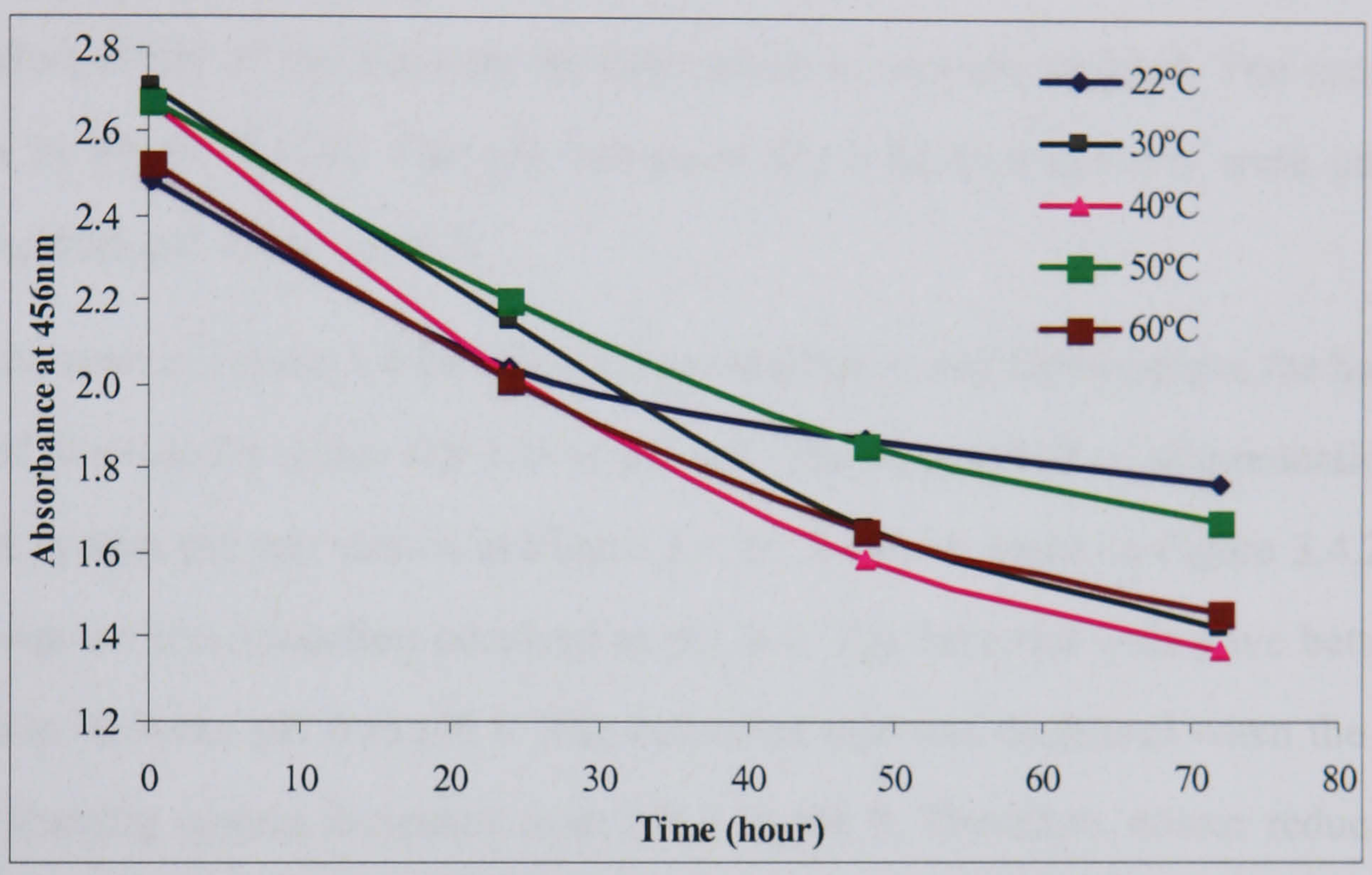


Figure 3.4.22 Reduction of Irgalan Yellow 3RL KWL Solutions at Different Temperatures, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (pH = 6.8)

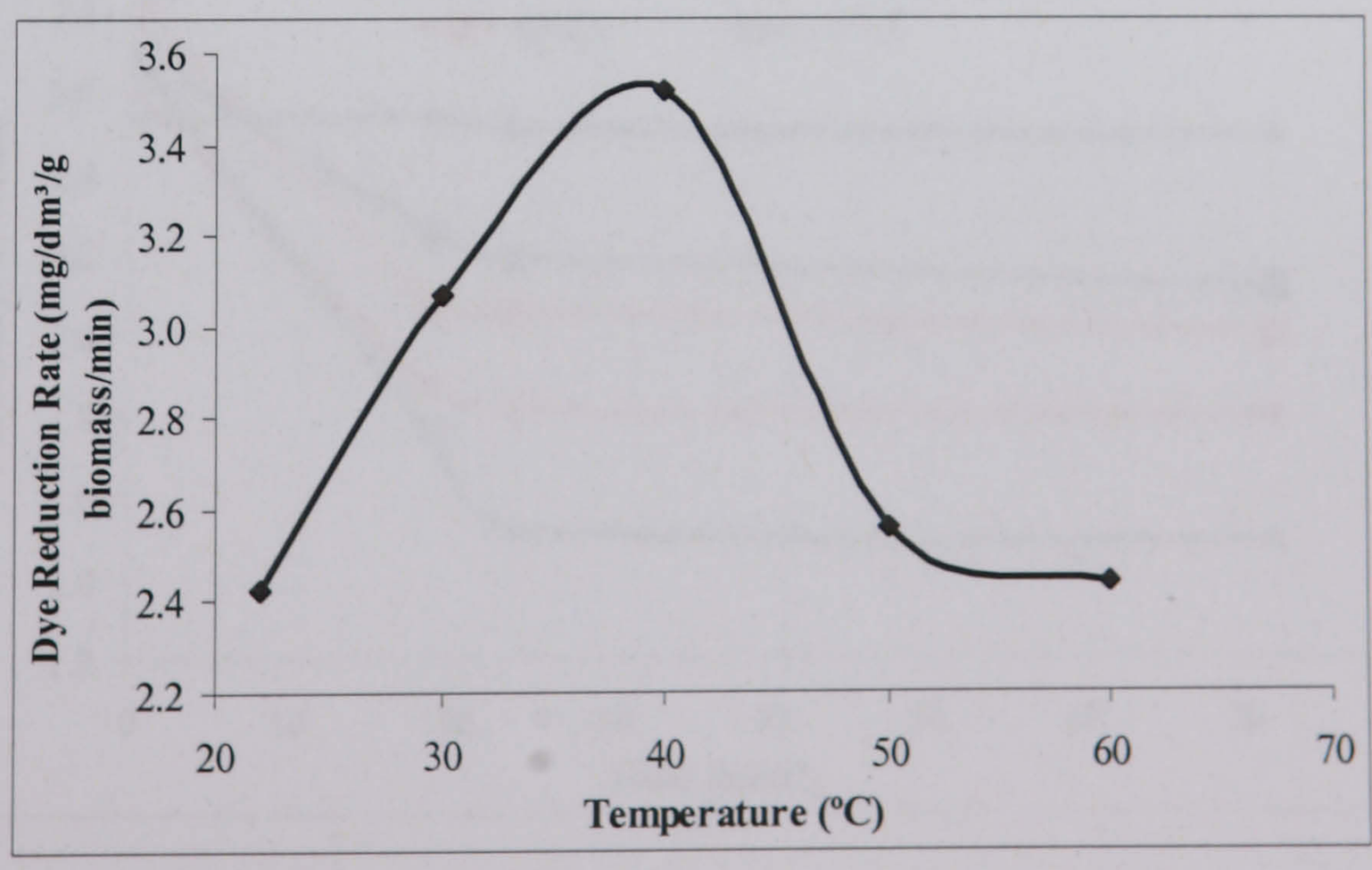


Figure 3.4.23 Effect of Temperature on the Rate of Irgalan Yellow 3RL KWL Dye Reduction in Solutions using Resting Cell Suspension of *Shewanella* Strain J18 143

Figure 3.4.22 shows that the bacterial cells of *Shewanella* strain J18 143 are capable of reducing the dye solutions over a range of temperatures. The dye reduction rate against the temperature was plotted as shown in Figure 3.4.23. An optimum in the reaction temperature was at around 40° C.

3.4.5.3 Effect of pH on dye reduction

The effect of pH of the assay on the dye reduction was investigated. The results are shown in Figure 3.4.24. The pH values of the evaluated systems were prepared ranging from pH 4.4 to pH 9.2.

It can be seen in Figure 3.4.24 that the dye reduction is not favourable to the bacterial cells of *Shewanella* strain J18 143 at pH 4.4. The corresponding dye reduction rate against system pH was drawn in Figure 3.4.25. It can be found in Figure 3.4.25 that there was no dye reduction occurred at pH 4.4. The bacterial cells gave better dye reduction between pH 6 to pH 8. The reduction rate was decreased when the pH of the evaluating system increased from pH 8 to pH 9. Therefore, colour reduction at pH 7 is a practicable option.

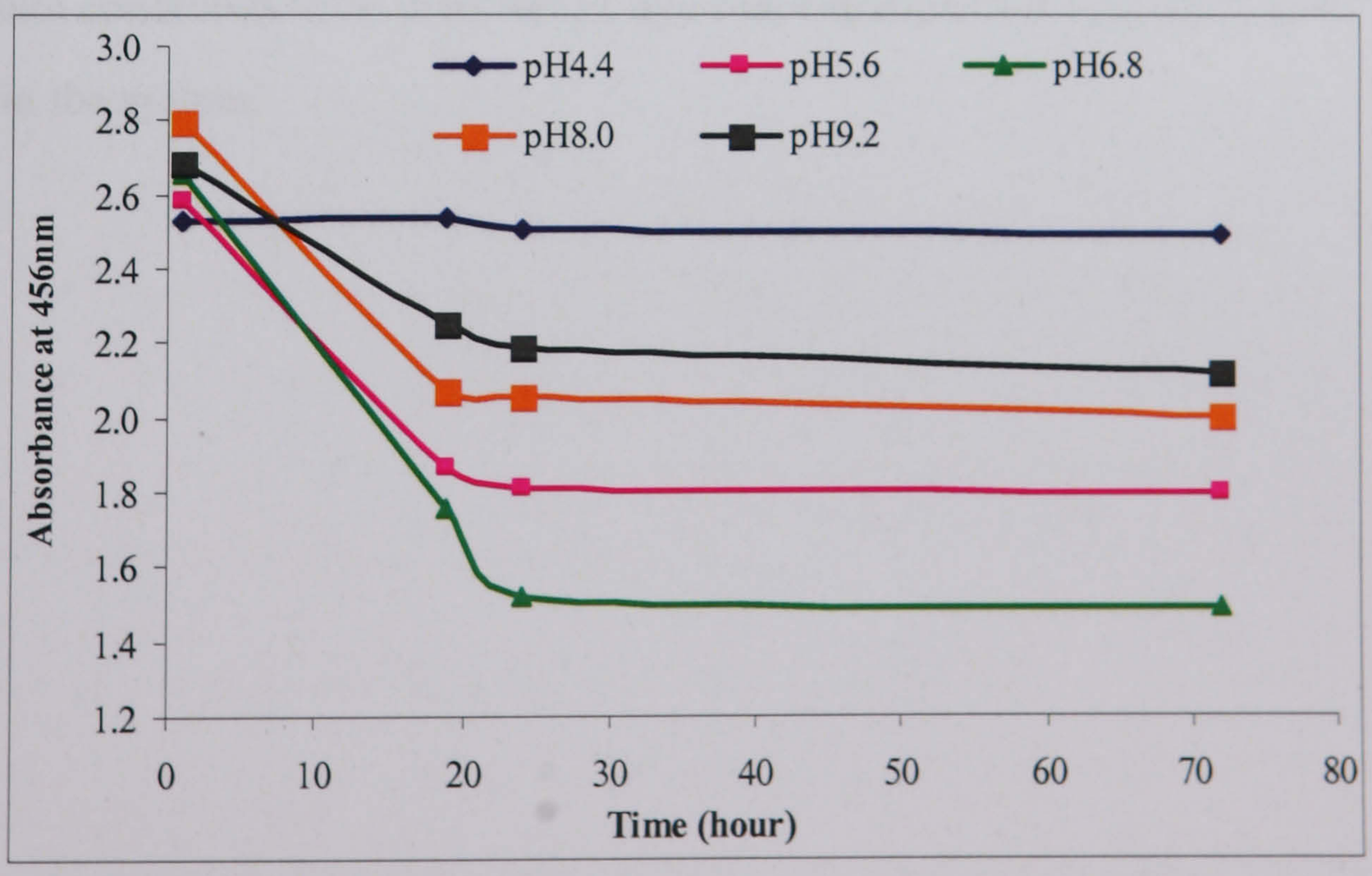


Figure 3.4.24 Reduction of Irgalan Yellow 3RL KWL Solutions at Different pH Conditions, using an Anaerobic Resting Cell Suspension of *Shewanella* Strain J18 143 in P.B.S. (30°C)

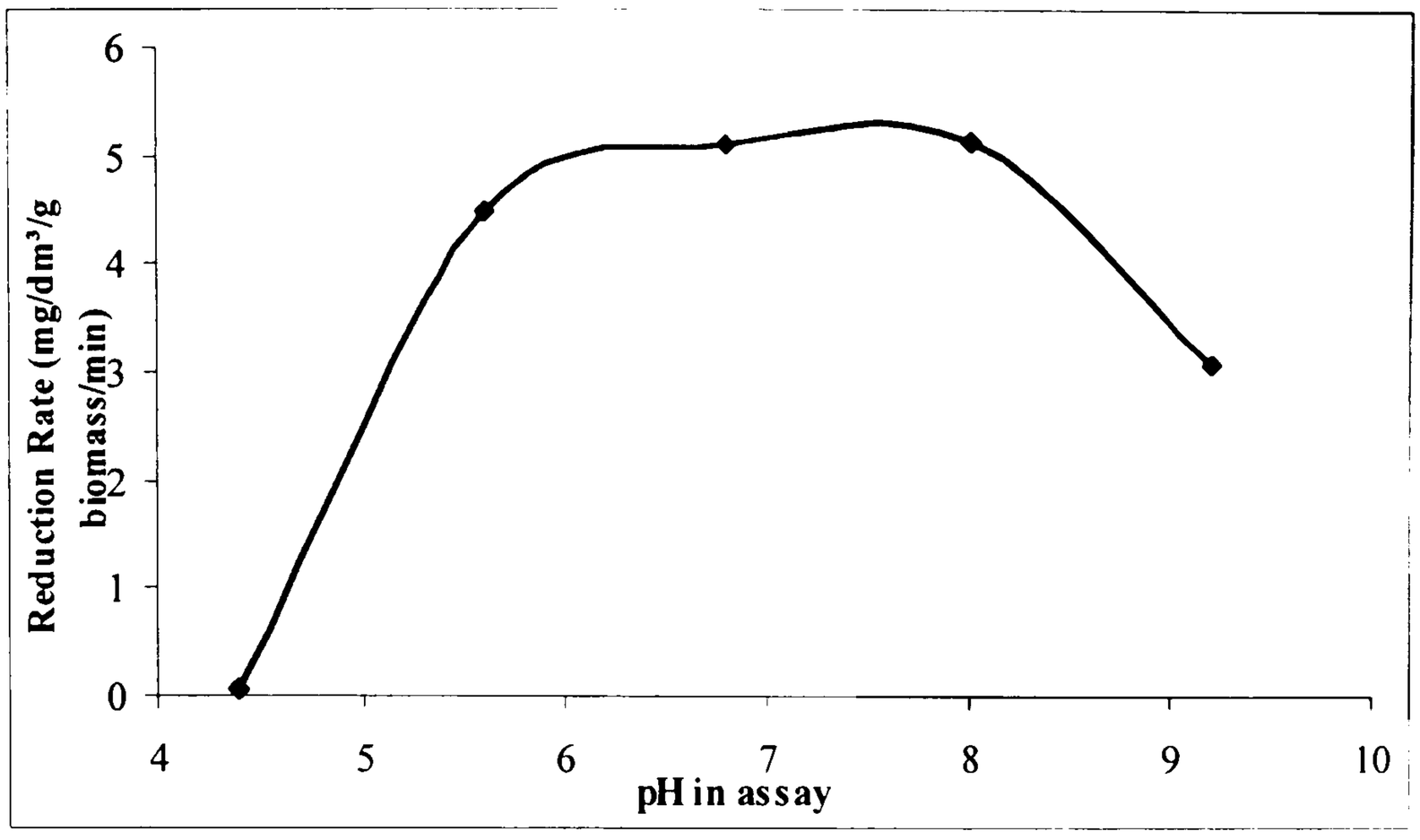


Figure 3.4.25 Effect of pH Conditions on the Rate of Irgalan Yellow 3RL KWL Dye Reduction in Solutions using Resting Cell Suspension of *Shewanella* Strain J18 143

From the results shown in the investigation of the factors may affect the dye reduction of Irgalan Yellow 3RL KWL using free cells of *Shewanella* strain J18 143, an optimum dye reduction can be determined regarding the factors of dye concentration, system temperature and system pH. That is, under the condition of using the same concentration of the bacterial cells as the evaluating systems, the optimum conditions to be used were a dye concentration of 0.12 g dm^{-3}, at 40° C and pH 7 in the system.

4 General discussion

The colour removal studies were carried out with two objectives. One was the decoloration of Remazol Black B using bacterial cells that were immobilised on cellulosic graft copolymeric substrates. Another was the reduction in colour of selected metal-complex azo dyes using planktonic bacterial cells. The bacterial cells used throughout the work were from the *Shewanella* strain J18 143. This is a facultative bacterium that has been reported to decolorise successfully a range of reactive dyes and pigments that contain azo groups (Willmott, 1997; Kamilaki, 2000; Pearce, 2004).

4.1 Studies involving colour removal from Remazol Black B solutions using immobilised cells

In part of the study, whole cells of *Shewanella* strain J18 143 were immobilised onto modified cellulose, graft copolymeric substrates. The composites of bacteria-cellulosic copolymer were viable and active with respect to the decoloration of the Remazol Black B. A number of researchers have reported that immobilised cells can be employed for providing process intensification in wastewater treatment (Section 1.5.2). However, it should be noted that various factors need to be considered including the stability of the immobilised bacterial cells, the activity of the immobilised bacterial cells in aspect of decoloration, the reusability of the immobilised system and the biodegradation of the supporting backbone polymer. Cost and practicality factors are also of relevance.

Preliminary work was undertaken on the immobilisation of the bacterial cells, *Shewanella* strain J18 143, on to a graft cellulosic copolymer substrate. The purpose of the work was to develop a suitable biosupport in the biological decoloration of reactive azo dyes. This suitability was based on factors such as biocompatibility,

accessibility and ease of removal of the by-products of the breakdown. Clearly the copolymer can be influential in other ways (provision of a more gentle microenvironment, localised pH features and so on.

The immobilisation substrate, cellulose-MAA graft copolymer, was firstly prepared. The grafting yield of the graft copolymer used was averaged at 5.5%. The grafting yield of the prepared graft copolymer was deliberately kept low. This was designed to maintain the fibrous nature of the substrate and to create a balanced pH environment for the bacterial cells to be immobilised. The grafting of the monomer of methacrylic acid onto the cotton fabrics were proved by SEM analysis. Representative images are shown in Figure 3.1.2. This grafting event and extent was further proved by the titrimetric analysis of the graft copolymer. The –COOH existing on the graft copolymer was titrated as a concentration of 0.1 ± 0.01 mol dm^{-3}.

The bacterial cells of *Shewanella* strain J18 143 were immobilised onto the cellulose-MAA graft copolymer by three different methods, as described in Section 2.1.3. The methods used were of "growing-in", physical adsorption and chemical coupling. The immobilisation methods were applied by different mechanism as introduced in Section 1.5.1. The effect of colour removal from solutions of Remazol Black B using these three methods was compared among selected methods (Figure3.2.12) and with the use of the planktonic cells (Figure3.2.6). The decoloration of Remazol Black B solution using the planktonic cells was much faster than that achieved using the immobilised cells. This indicates that accessibility is a feature. This point is supported by the calculated reduction rates, using the immobilised cells in the removal of colour of Remazol Black B solutions, as shown in Table 3.2.1. The images given in Figure 3.2.2 show that the bacterial cells immobilised by the method of "growing-in" provided a faster and more complete decoloration extent among the three studied immobilisation methods. However, as shown in the Table 3.1.1, the amount of the bacterial cells presented in each individual decoloration system was different. Accessibility is, again, indicated as being an important feature. The reduction rates, calculated from protein assays and

the reduction calculated from analytic means, were employed (Table 3.2.1). Although the planktonic cells gave faster decoloration than that of the immobilised cells, the reduction rate of the cells immobilised by the method of "growing-in" [66.0 μM dye g^{-1} (biomass) min^{-1}] was at a similar level to that given by the planktonic cells, as indicated from a comparison with the results shown in Pearce's work (2004). It can be concluded that the activity of the immobilised bacterial cells of *Shewanella* strain J18 143 was maintained. There is no doubt that the stability and the reusability of the bacterial cells in application in the decoloration system are improved. The cells immobilised by the method of adsorption and coupling showed lower reduction rates than those given by the two other methods.

All of the three studied immobilisation methods were applied successfully in the removal of colour from the Remazol Black B solutions. The Remazol Black B was almost completely removed using the immobilised cells by the method of "growing-in" (97.7%) and by the method of chemical coupling (96.4%), after 48 hours of treatment. The bacterial cells immobilised by the method of adsorption provided a colour removal of 72.1% after 48 hours of incubation. These differences in the decoloration effeciencies are mainly brought by the stabilities of the bacterial cells that being immobilised by the three different methods. The stability of the bacterial cells, that being immobilised by the method of "growing-in", is proved higher than that provided by the other two methods.

The systems are successful with respect to the objective of effective colour removal. The use of the immobilised cells provides many advantages in the application in decoloration. The immobilised bacterial cells can be used in a continuous system effectively and the control of the decoloration system is much easier, since the stability and the reusability of the bacterial cells are improved. The impact of this approach in the use of the immobilised cells can be seen in two aspects of this work. One is that the activity of the bacterial cells may be inhibited. This observation has been intimated in previous research (Juang *et al.*, 2002; Peralta-Zamora *et al.*, 2003; Lu *et al.*, 2007). Another impact concerned the quantity of the bacterial cells that

were immobilised onto the biosupport. In another words, although the concentration of the bacterial cells existing in the decoloration systems was relatively low the system was effective. However, the selection of the biosupport, and/or the modification of the cellulose may need to be improved.

As stated above, the cellulosic copolymer with its grafted hydroxyl groups has been proved to be suitable as a biosupport for the immobilisation of biological cells. Therefore, the cellulose may need to be modified on the basis of accessibility and the suitability of the microenvironment provided by the copolymeric matrix. The cellulose-MAA copolymer shows potential in use as immobilisation substrate for the bacterial cells of *Shewanella* strain J18 143. Optimisation of the immobilisation system needs to be considered. Such optimisation options include the selection of the monomer, factors relating to the grafting yield, to the homogeneity of grafting and to the length of the grafted chains. Also, optimisation of the immobilisation methods will be needed following such changes.

4.2 Colour reduction of solutions of metal-complex azo dyes using the planktonic cells

The reduction in the colour of solutions of the metal-complex azo dyes, using planktonic bacterial cells of *Shewanella* strain J18 143, was another major aim in this study. The removal of colour from aqueous solutions of the Irgalan Grey GLN, of Irgalan Black RBLN and of Irgalan Blue 3GL was achieved successfully. However, the colour reduction of Irgalan Yellow 3RL KWL was not/less achieved.

Irgalan Grey GLN solutions are toxic to aquatic organisms if the solutions are run directly to surface waters. Irgalan Grey GLN is a metal-complex azo dye that contains cobalt ions and chromium ions in the organo-metal complex. Irgalan Grey GLN has been recognised as being not readily biodegradable (Ciba Specialty Chemicals Safety Data Sheet). The reduction in colour of Irgalan Grey GLN aqueous

solutions (0.08 g dm^{-3} in the final treatment system) using planktonic cells was successful. 91.5% of colour was removed from the dye solution. The by-products from the biological degradation of the dye were not soluble in the buffering solution, and were found as a precipitate at the bottom of the vials that comprised the evaluation system. Some precipitation was also found in the standard dye solution of Irgalan Black B in the buffer. Particle size analysis was carried out on these precipitate/suspensions. The standard dye solutions gave particle size values/ distributions from 20 nm to 7000 nm, after 18 hours of incubation. The planktonic cell suspension standards have a size distribution from 3000 nm to 7000 nm. The biological cells-treated systems have a size distribution from 35 nm to 65 nm and from 4500 to 7000nm. These observations are in an agreement with the results shown in Figure 3.3.2. The by-products of the biological degradation of Irgalan Grey GLN were not identified. Further study on the by-products of Irgalan Grey GLN reduction is recommended for optimisation of the decoloration of metal-complex azo dyes. Clearly the precipitated forms are in a largely energetic state. In reducing this energetic character, precipitation occurs.

Irgalan Grey GLN is a mix of two metal-complex azo dyes, Irgalan Black RBLN and Irgalan Blue 3GL. Irgalan Black RBLN solutions are also recognised as being toxic to aquatic organisms if run directly to surface waters. This system is not recognised as being readily biodegradable (Ciba Specialty Chemicals Safety Data Sheet). Irgalan Blue 3GL solutions are poorly eliminated by adsorption in effluent treatment sludge systems and are harmful to aquatic organisms if run directly to surface waters. Colour reduction of these two dye solution systems was undertaken. The results observed from the colour reduction studies based on these two dyes were similar to those observed in the study of the colour reduction of Irgalan Grey GLN solutions. 76.8% and 76.2% of colour was removed from the solutions of Irgalan Black RBLN and Irgalan Blue 3GL respectively, using the bacterial cells of *Shewanella* strain J18 143. It would appear that the systems are able to tolerate the presence of the central metal cation to a reasonable degree.

Irgalan Yellow 3RL KWL aqueous solutions are identified as being toxic to aquatic organisms if run directly to surface waters. The colour of Irgalan Yellow 3RL KWL dye solutions can be partially eliminated by adsorption on an effluent sludge (Ciba Specialty Chemicals Safety Data Sheet). Colour reduction of Irgalan Yellow 3RL KWL solutions was carried out. However, the dye solution was not fully decolorised using the bacterial cells. 10.5% of colour loss was found. Kandelbauer *et al.* (2004) studied the decoloration of Irgalan Yellow 2GL solutions and Irgalan Yellow 2GI solutions using free laccase, 12% and 9% of colour loss were achieved respectively. The reason of the smaller extent in colour reduction of Irgalan Yellow 3RL KWL have not been identified due to the time and the facility limitations. It is possible that further optimisation of the biological degradation process could lead to a situation where more effective and efficient colorant breakdown could be achieved (nutrient medium, concentration variations, cell loading).

The mechanism of colour reduction of Remazol Black B azo dye solutions using planktonic cells of *Shewanella* strain J18 143 was suggested by Pearce (2004), and shown in Figure 4.2.1.

Complete colour removal from Remazol Black B solution was achieved using the planktonic bacterial cells (Willmott, 1997; Pearce, 2004). The mechanism of the colour reduction of metal-complex azo dyes was considered. It is clear that the colour process is a very complicated matter and requires investigation throughout of the biological reduction procedures and of the reduction by-products. Willmott (1997) demonstrated that extracellular enzymes were not involved in the removal of colour from Remazol Black B solution. *Shewanella putrefaciens* cells have the ability to utilise some minerals, such as U (IV) and Fe (III), as the terminal electron acceptor (Section 1.4.3). From the results obtained relating to the reduction of solutions of the Irgalan series of dyes, some of the metal-complex azo dyes can be assumed to be acting as electron acceptors. Limitations in the extent of colour reduction of the aqueous dye solutions were found in this work. The metal complex Irgalan Yellow 3RL KWL was not successful reduced. Clearly the nature of the continuous medium

(aqueous system) can be a theme for further investigation as can aspects of dye aggregation phenomena.

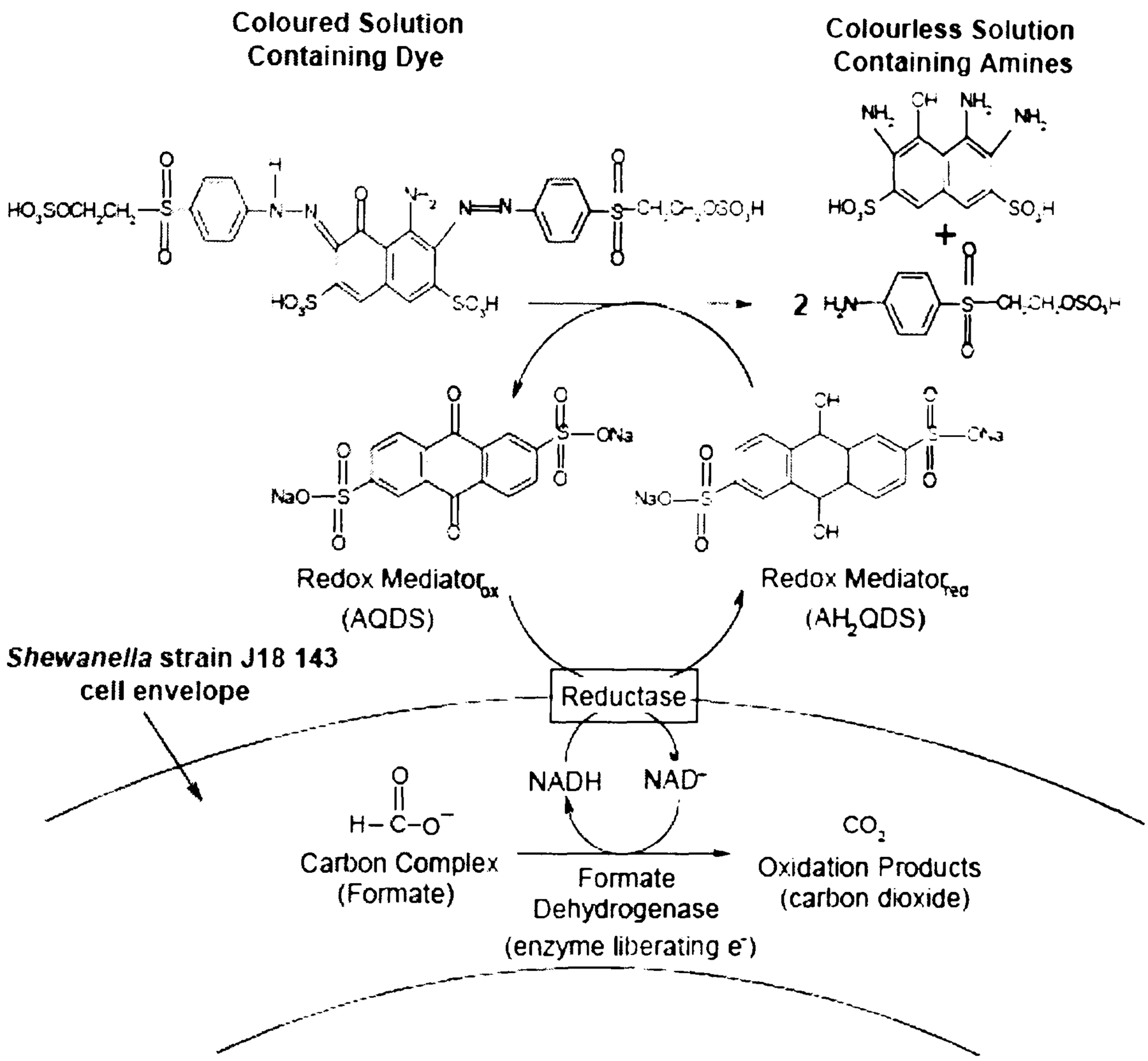


Figure 4.2.1 Suggested Biological Mechanism for the Reduction of Remazol Black B by Bacterial Cells of *Shewanella* strain J18 143 (Pearce, 2004)

4.3 Investigation of the factors affecting the biological colour reduction of the metal-complex azo dye solutions

The influence of temperature, pH and the colorant concentration on the efficiency of the colour reduction were investigated for the solutions of the dyes.

Considering the results achieved from Section 3.3 and Section 3.4, the bacterial cells of *Shewanella* strain J18 143 have shown their ability to reduce colour from solutions of metal-complex azo dyes. The decoloration efficiency varies depending on the individual dye structures, chemical composition, solubility and polarity. Thus, the effect of the general factors, the dye concentration, the incubation temperature and the pH, on the colour reduction efficiency was investigated. The colour reduction rate was greater with increased dye concentration, except in the case of the Irgalan Yellow 3RL KWL. Therefore, there is no particular requirement optimisation of the dye concentration of the targeted metal-complex azo dyes. The decoloration of all of the four metal-complex azo dyes was shown to be more effective in the temperature range from 40 °C to 50 °C. However, the current consent for the temperature of the wastewater at the point of discharge is below 42 °C (Section 1.1.2). From the bacterium species point of view, the organism, *Shewanella* strain J18 143, is mesophilic, with a maximum rate of cell division at 35 °C. Above a temperature of 37 °C, the growth rate of the culture falls sharply (Willmott, 1997). Since the growth rate of the culture was similar at incubation temperatures between 30 °C and 37 °C, the decoloration system could be operated at 30 °C, in order to minimise the risk of protein denaturation (Pearce, 2004; Willmott, 1997). The bacterial cells have been shown to be more effective in colour reduction of the metal-complex azo dyes under slightly higher than neutral pH conditions. Thus, the optimum of the operating pH for maximal growth of *Shewanella* strain J18 143 was at 8.10 (Willmott, 1997). Only slight differences in the reduction rate were found between pH 7 to pH 8. Thus, it is possible to maintain a neutral pH condition for the colour reduction system.

5 Conclusions

From the results that have been achieved, the following conclusions can be drawn.

The cellulose-MAA graft copolymer can be used as a substrate for the immobilisation of species such as the biological cells of *Shewanella* strain J18 143. The grafting yield of the graft copolymer used was averaged at 5.5%, with 0.1±0.01 mol dm^{-3} of –COOH being present on the grafts. These are potentially available for coupling under suitable circumstances.

The bacterial cells were immobilised by the methods of "growing-in", chemical coupling and physical adsorption. The average concentration of the immobilised cells existing on the substrate by these three methods was calculated respectively. "Growing-in" and chemical coupling are better the options for immobilisation.

The decoloration of textile wastewaters using this immobilisation system is feasible. The colour of solutions of Remazol Black B was completely removed using the "growing-in" and the chemical coupling immobilised cells, after 24 hours and after 48 hours of incubation, respectively. 72.1% of colour was removed from the dye solution, after 48 hours, by the adsorption method.

The bacterial cells of *Shewanella* strain J18 143 have been shown to have the ability to reduce the colour of the solutions of some metal-complex azo dyes. This is a successful extension of the ranges of dye solutions that are known to be reduceable. Thus, it is now known that Irgalan Grey GLN, Irgalan Black RBLN and Irgalan Blue 3GL can be reduced biologically. However, not all of the metal-complex azo dyes are able to be reduced by the bacterial cells (i.e. Irgalan Yellow 3RL KWL).

The concept that of using the bacterial cells of *Shewanella* strain J18 143 in the decoloration of dye solutions needs to be extended to other textile effluent treatments. Care will be needed to balance the effluents before treatments can begin.

6 Suggestions for further work

The decoloration of the reactive dye solutions using immobilised biological cells has achieved. The efficiency of the colour removal was compared between results obtained from the free cells and from the immobilised cells, and also among the different immobilisation methods. Effective protein assay of immobilised microbial cells or enzymes in this particular case would be useful to monitor the decoloration processes.

The optimisation of the decoloration of the reactive dye solutions using immobilised cells is recommended, since the results obtained have shown much potential in practice.

Extensive investigations into the mechanism of colour removal of the metal-complex azo dyes are strongly recommended, since it is a very important and determinant factor for the development and the optimisation of the decoloration processes involved with the use of the *Shewanella* strain J18 143 cells. Effective analysis of the by-products from the colour reduction of the metal-complex azo dyes, therefore, would be needed.

It is additionally recommended that the evaluation of whether or not the bacterial cells are acting directly or indirectly with the dyes and, therefore, to establish the importance of water in the events that take place.

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