HYDROGENASES FROM SULPHATE REDUCING BACTERIA AND THEIR ROLE IN THE BIOREMEDIATION OF TEXTILE EFFLUENT

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Of

RHODES UNIVERSITY

in the

Department of Biochemistry, Microbiology and Biotechnology

Faculty of Science

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January 2006

ABSTRACT

The continuing industrial development has led to a corresponding increase in the amount of waste water generation leading to a consequential decline in levels and quality of the natural water in the ecosystem. Textile industries consume over 7 x 10^5 tons of dyes annually and use up to 1 litre of water per kg of dye processed and are third largest polluters in the world, the problem being aggravated by the inefficiencies of the dye houses. An abundance of physio-chemical methods are in use world wide, however, there is increasing concern as to their impact in effectively treating textile effluents as they introduce secondary pollutants during the 'remediation' process which are quite costly to run, maintain and clean up. Research on biological treatment has offered simple and cost effective ways of bioremediating textile effluents. While aerobic treatment of textile dyes and their effluents has been reported, its major draw back is commercial up-scaling and as such anaerobic systems have been investigated and shown to degrade azo dyes, which form the bulk of the dyes used world wide. However, the mechanisms involved in the bioremediation of these dyes are poorly understood. The aims of this study were to identify and investigate the role of enzymes produced by sulphate reducing bacteria (SRB) in bioremediating textile dye and their effluents.

Sulphate reducing bacteria were used in this study because they are tolerant to harsh environmental conditions and inhibit the proliferance of pathogenic micro-organisms. The appearance of clear zones in agar plates containing azo dye concentrations ranging from 10 – 100 mgl^{-1} showed the ability of SRB to decolourize dyes under anaerobic conditions. Assays of enzymes previously reported to decolourise azo dyes were not successful, but led to the identification of hydrogenase enzyme being produced by SRB. The enzyme was found to be localised in the membrane and cytoplasm. A surface response method was used to optimize the extraction of the enzyme from the bacterial cells resulting in approximately 3 fold increase in hydrogenase activity. Maximum hydrogenase activity was found to occur after six days in the absence of dyes but was found to occur after one day in the presence of azo dyes. A decline in hydrogenase activity thereafter, suggested inhibition of enzymatic activity by the putative aromatic amines produced after azo cleavage.

Purification of the hydrogenase by freeze drying, poly ethylene glycol, and Sephacryl – 200 size exclusion- ion exchange chromatography revealed the enzyme to have a molecular weight of 38.5 kDa when analyzed by a 12 % SDS-PAGE. Characterisation of the enzyme revealed optimal activity at a pH of 7.5 and temperature of 40 °C while it exhibited a poor thermal stability with a half-life of 32 minutes. The kinetic parameters V_{max} and K_m were 21.18 U ml^{-1} and 4.57 mM respectively.

Application of the cell free extract on commercial dyes was not successful, and only whole SRB cells resulted in decolourisation of the dyes. Consequently trials on the industrial dyes and effluents were carried out with whole cells. Decolourisation rates of up to 96 % were achieved for the commercial dyes and up to 93 % for the industrial dyes over a period of 10 days.

Keywords: *Sulphate reducing bacteria, azo dye, decolourisation*

Publications from thesis:

1) Mutambanengwe, C.C.Z. and Whiteley, C.G. (2005). Use of sludge biomass in the production of enzymes under sulphidogenic conditions for the treatment of textile effluent. *South Africa Society of Biochemistry and Molecular Biology XIXth Conference, Stellenbosch University, South Africa*.
2) Whiteley, C.G. and Mutambanengwe C.C.Z. (2005). Novel hydrogenases from sulphate reducing bacteria. Decolourisation of industrial waste water effluents. *XVIII Enzyme Engineering, Gyeong-Ju, South Korea*.

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ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation to the following people for the contribution towards this thesis:

- God almighty, for the life and strength He gave throughout the course of this project.
- My supervisor Prof Chris G. Whiteley who supported me through the glorious and difficult times I encountered during my research.
- Rob Van Hille from UCT,
- Chamunorwa A. Togo who supported me during my research and during the writing up of this thesis, and for being the older brother I never had.
- Prof Peter Rose from EBRU, Rhodes University
- Lab 309 members Taurai Mutanda and Cassandra Loux for putting up with me during my studies
- This thesis is dedicated to my parents who prayed for me and checked up on all the time, thank you for believing in me and giving me the strength to prevail.
- A debt of gratitude to the Water Research Council for the funding.

1 LITERATURE REVIEW

1.1 Introduction

Textile demand has been steadily increasing world wide following world population growth and stimulated by a growing Gross Domestic Product (GDP) in many countries, primarily in Pacific Asia (Figure 1.1).

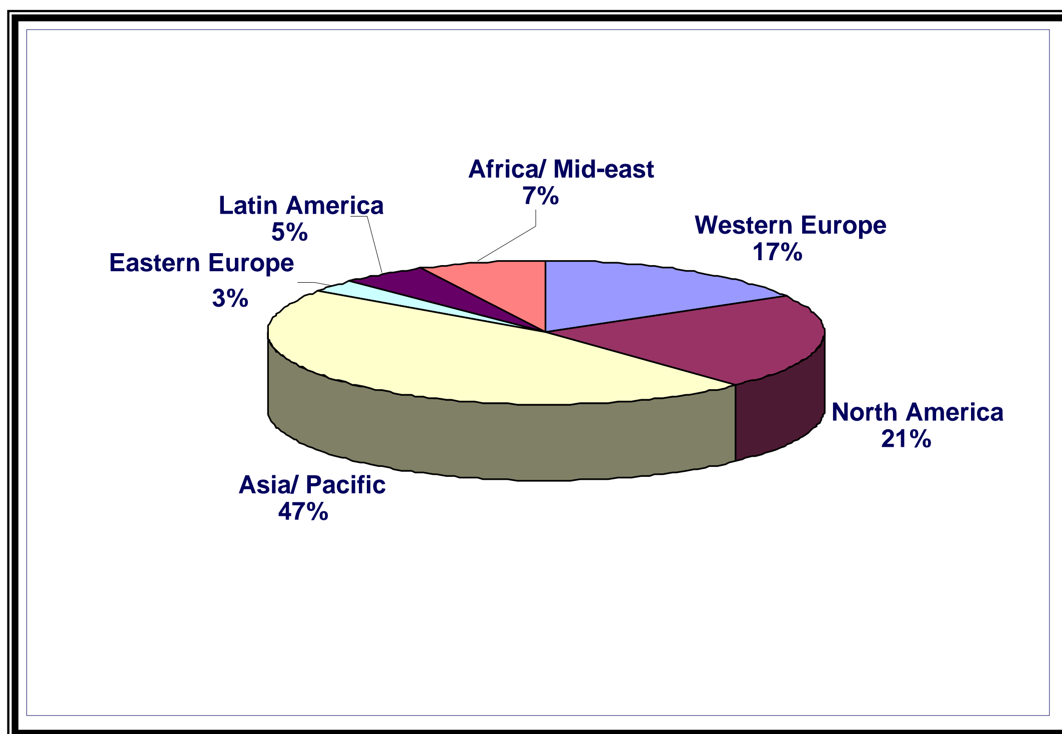


Figure 1.1 World textile demand by region, 2001. (Adapted from, Textile world, 2005)

Cotton and polyester account for 78 % of the world's textile demand. Following this trend, reactive and disperse dyes are the most utilized dyes for colouring cellulosic fibres (Table 1.1). Azo dyes that account for 60 – 80% of the dyes consumed in textile processing (Mendez-Paz *et al.*, 2004; Yang *et al.*, 2004) are characterised by a typical double azo bond linkage (-N=N-), which is the most common chromophore of reactive dyes. The delivery of colour onto fabric is not an efficient process and up to

40 % of the dyes are lost during the dyeing process (Stolz, 2001; Pearce *et al*., 2003; Moreira *et al*., 2004).

Table 1.1 World textile dye market in 1998 (Adapted from Hunger, 2003).

Dye class	**Market share, 10^9 Tons**	**Market share, %**
Disperse dyes	2.3	28
Reactive dyes	2.3	27
Vat dyes	0.8	10
Indigo	0.3	4
Sulphur dyes		6
Direct dyes		10
Naphthols		4
Others (anionic, cationic, etc)		11
Total	**8.7**	**100**

Dyeing, desizing and scouring are the major sources of water pollution in textile effluent. Textile and dyestuffs waste waters are characterised by their highly visible colour, high chemical oxygen demand (COD), suspended solids and alkaline pH (9 – 11) (Manu and Chaudhari, 2001) thus effluent discharge from these industries into the environment is a major cause for concern. As dyes are designed to colour various substances and solutions indefinitely, there is great potential for these dyes to accumulate in the environment as many of them are recalcitrant to normal bioremediation (Bell *et al*., 2000).

Several chemical and physical decolourisation methods that are available include: adsorption, precipitation, coagulation/ flocculation, oxidation, electrolysis and membrane extraction. These techniques are effective for colour removal but are energy intensive and introduce chemicals which are not wanted in the first place. They also concentrate the pollutants into solid or liquid side streams which require additional treatment or disposal thus escalating cost of effluent treatment (Bell *et al*., 2000; Robinson *et al*., 2000; Shaw *et al*., 2001).

Biological decolourisation is the most common and widespread technique used in textile effluent treatment (Hunger, 2003). There are two types of biological treatment: aerobic and anaerobic. Aerobic systems require oxygen for fungi and bacteria to

perform the degradation process whereas, anaerobic operate in the absence of air and under static conditions. Activated sludge has been shown to remove a moderate amount (10 – 20 %) of the colour by adsorption to cell and sludge biomass (Stolz, 2001; Hunger, 2003). The efficiency of biological treatment systems in textile effluent treatment has stimulated investigation into the actual mechanism behind the process. As such there has been a growing recognition that enzymes can be used in many remediation processes to target specific pollutants for treatment. In this direction, recent biotechnological advances have led to the production of cheaper and more readily available enzymes through improved isolation and purification procedures (Nuran and Esposito, 2000). The potential advantages of enzymatic treatment as compared with conventional treatments include: application on recalcitrant materials, operation at high and low contaminant concentrations over a wide pH range, temperature and salinity range, acclimatization to biomass and the easy control process (Nuran and Esposito, 2000).

1.2 Textile processing and dyeing

In order to understand the impact of textile effluent, it is necessary to gain an insight into the processes that bring about the effluent. The major sources of textile effluent are the dye houses and the major processes involved are desizing, scouring, bleaching, mercerizing, dyeing and finishing. A schematic representation of these main stages involved in processing textile fibres is shown (Figure 1.2).

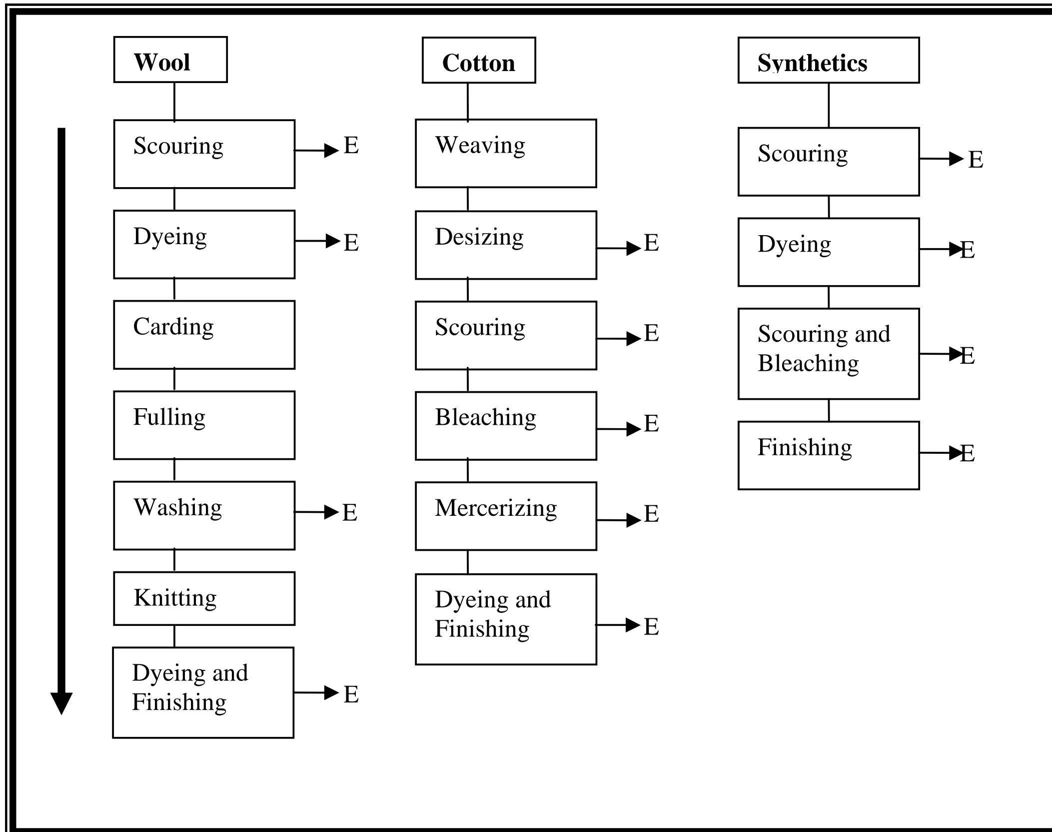


Figure 1.2 Diagrammatic representation of textile fibre processing and the major stages of effluent discharge (adapted from Barnes *et al.*, 1992). E denotes the effluent discharge at each stage.

The major stages involved in the colouring of cotton and synthetics, using azo dyes are:

a) **Desizing**. This is the preliminary stage of cotton processing. Basically it involves the removal of size from cotton with the aid of enzymes or detergents (i.e. acid or alkali) to produce a homogenous fabric in preparation for the subsequent processing. Sizes are organic compounds such as starch, and its derivatives, cellulose-derivatives, polyacrylates and polyvinyl alcohol (PVA). Therefore desizing effluent is characterised by high organic compounds and Chemical Oxygen Demand (COD), (Carliell, 1993). Generally size effluents represent the main component (~ 60%) of the organic load of effluents from the textile finishing mills (Schluter, 1991).

b) **Scouring**. The purpose of this stage is to remove oils, fats, waxes, soluble impurities and any other solid dirt that may have been left over by desizing. The fabric is treated with detergents. Scouring effluent is therefore

characterised by high COD and pH values. The effluent is coloured by a strong yellow-brown colour which is often referred to as "Brown Yorkshire Grease" (Trotman, 1968).

c) **Bleaching**. This process removes the natural colouring matter that cannot be removed by scouring in a purely chemical reaction. The overall objective of this stage is to improve the whiteness of the textile fabric, through redox reactions. The main chemical used is sodium hypochlorite and as a result the effluent has high levels of halogens (Carliell, 1993).

d) **Mercerizing**. Involves the treatment of cotton fibres with approximately 20 % caustic soda, to increase tensile strength and improve dye affinity of the fabric. High pH is the major characteristic of mercerising effluents.

e) **Dyeing and finishing**. There are three methods of dyeing textiles namely, mass dyeing, which involves dying of synthetic polymer before fibre formation; pigment dying where an insoluble colorant is affixed on to the fibre surface with the aid of a binder; and exhaustion dyeing from an aqueous bath with dyes that have an affinity for the fibre. Of the three methods, the exhaustion method is the most commonly used (Hunger, 2003).

   Dyes used for imparting colour include cationic, non-ionic or anionic. The cationic and anionic dyes are direct, acid and reactive dyes (Mishra and Tripathy, 1993). It is these water soluble reactive and acid dyes that are the most problematic as they tend to pass through conventional treatment systems (Willmott *et al*., 1998). Non-ionic dyes refer to disperse dyes because they do not ionize in aqueous media and, if they are not adsorbed onto the fabric, they end up being discharged as waste water (Robinson *et al*., 2000). Application of dyes can be continuous or batch. The volume of dye-containing effluent resulting from batch dyeing is greater than that generated from a continuous process, as between four and ten rinses are required after batch dyeing (Carliell, 1993). Finishing involves final rinsing of the fabric after dyeing to improve quality. Therefore colour is the major noticeable characteristic in dyeing and finishing effluent.

1.3 Dyes and Pigments

1.3.1 History of textile dyeing

The first steps into the world of textile and dyeing were started when man discovered the use of animal hides and skins to interlace vegetable derivatives such as hemp, creeper, mannilla, sisal and even pulp bark in water to form tape cloth (Robinson, 1969). This led to the subsequent development of wool, flax, linen, cotton and silk. Once the 'material' became available there was a need to add colour to the material. The art of dyeing and fabric patterning with dyes and pigments dates back to 800BC (Robinson, 1969), however, scientists have been able to date the black, yellow, white and reddish pigments made from ochre used by primitive man in cave paintings to over 15000BC (Grierson, 1989).

In order to understand the art and history of dyeing, it is important to understand how the process of dying started. By definition the term 'dyeing' refers to the process of colouring fibres, yarns or fabrics using a liquid containing colouring matter for imparting hue to a substance. The substances that were used for decorating or imparting a particular hue in the earliest times may be classified into four categories (Robinson, 1969). The first and most primitive method was to use natural products such as leaves, flowers, fruits, sticks, wood, shells, hair, and similar objects without modification. These were merely interlaced and pressed into or stuck on the body or fabric with albumen or clotted blood to impart a temporary coloration (Robinson, 1969).

The second method involved physical rubbing of the desired colours onto the cloth. The pigment colours were derived from crushed or burnt lime, gypsum and clay to give white and creams; haematite, ochre, iron rust from iron minerals and earths generated yellow, reds and browns; soot and coal were used to give black and grey shades. The pigments were bound to the fabric by mixing them with resins obtained from trees, egg albumin, clotted blood or saliva. In some cases the pigments were roasted with turpentine, glues or wax to enhance fastness of pigments to the fabric.

The third method in the development of the dyeing industry entailed use of a true dye, where the colour of a substance was deposited onto the fabric in an insoluble

form. Many of these dyes were obtained from crushing fruits, flowers, roots and even bark to yield a thin coloured paste which was then brushed onto the fabric. These true dyes were also boiled or steeped in water to give a dye-type concoction to improve fastness (Robinson, 1969).

The fourth method employed the use of the sun, fire or smoke to impart colour in the form of patterns onto the fabric. Light rays from the sun tend to bleach out the natural colour of fabric such as bark, grass, and cloth as well as many dyed fabrics. Addition of colour onto the fabric was achieved by placing stencil plates over the fabric and exposing it to direct heat from the sun. A dark pattern then emerges from underneath the plates when treated with heat from fire, to give a similar result, however, extra soot (almost pure carbon) was deposited as an additional pattern through the stencil or the whole fabric would be heavily stained to yield a deep, even black colour often difficult to obtain from natural dyestuffs. This technique was widely applied in East African tribes where the soot was scraped off a collecting plate and mixed with cooking fat, resin, clay or soil onto bark fibres to yield a beautiful deep black stain (Trotman, 1968).

The biggest drawback to all these methods of fabric dyeing was inefficiency of the fixation process. All these pigments although fast to light, they were not fast to water, humidity or sheer wear. Therefore there was a serious demand for a permanent fixation of dyes onto fabric. This stimulated a whole range of experiments leading to the discovery of 'magic' properties being found in certain rivers, sea water, saliva and even urine. These provided a more permanent effect upon the fabric. As technology progressed, further experiments gradually isolated the 'magic' quality leading to discovery of mordanting and chemical conversions.

Interestingly, not all the fabric was meant to maintain colour, some ceremonial gowns for royalties were brightly coloured with non-permanent stain dyes (Robinson, 1969).

1.3.2 The basis of colour in dyes

Dyes are characterised in accordance with their capacity to absorb the energy of a particular part of the electromagnetic radiation to which the human eye is sensitive (Rys and Zollinger, 1972). Dyestuffs give colour to the material onto which they have

been anchored, by selectively retaining some of the wavelengths out of the light falling upon the surface. However, there are only a few organic molecules that possess this property of absorbing light selectively (Trotman, 1990). Therefore if a dye absorbs strongly at the red end of the spectrum, the light which is reflected is of a bluish hue (Table 1.2).

Table 1.2 Colour absorbance spectrums (Adapted from Trotman, 1990, p254).

Wavelength of Light absorbed nm	*Absorbed light*	*Visible colour*
400 – 435	Violet	Yellowish Green
435 – 480	Blue	Yellow
480 – 490	Greenish Blue	Orange
490 – 500	Bluish Green	Red
500 – 560	Green	Purple
560 – 580	Yellowish Green	Violet
580 – 595	Yellow	Blue
595 – 605	Orange	Greenish Blue
605 - 750	Red	Bluish Green

A coloured compound is built up from three subsystems, namely aromatic groups called chromophores which are characteristically electron accepting molecules and secondly, nucleophiles commonly referred to as auxochromes. These are electron withdrawing and contain substituted groups like amino, hydroxyl, sulfonic and carboxylic groups. The third constituent of a typical coloured compound is a system of conjugated double bonds that joins the chromophore and auxochrome to form a colour specific compound known as a chromogen (Wallace, 2001). Besides supplementing the chromophore in the production of colour, they also enhance solubility of the dye and increase its affinity towards fibres (Trotman, 1990). Both auxochromes and chromophores shift the higher wavelength absorption bands of the conjugated system to longer wavelengths (bathochromically) and both can be classified empirically in order of increasing bathochromic effect on a particular conjugated system (Rys and Zollinger, 1972).

1.3.3 Classification of dyes

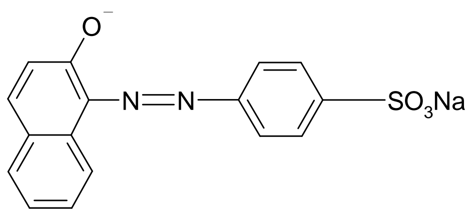
Dyes may be classified according to either chemical structure (chemical classification) or by usage (colouristic classification) (Rys and Zollinger, 1972; Trotman, 1990). Chemical classification is predominantly used by the practising dye chemists who use terms such as azo dyes, anthraquinone dyes, or phthalocyanine

dyes. On the other hand, the dye technologist uses colouristic classification and speaks of “reactive dyes for cotton” and “disperses dyes for polyester” (Trotman, 1990).

However, a review of the whole field of technical dyes reveals that the two classifications are interlinked, since there is hardly a chemical class of dyes which occurs solely in one colouristic group and vice versa. Similarly some colouristic groups can be applied to two or more substrates, whilst others are more specific to a single substrate (Rys and Zollinger, 1972).

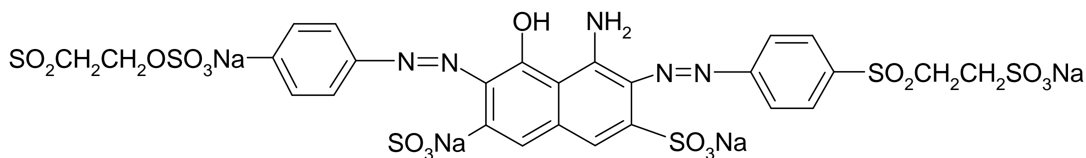
1.3.3.1 Chemical classification

This is probably the most appropriate classification and offers advantages over colouristic classification. Firstly it readily identifies dyes belonging to a particular group that has characteristic functional properties, e.g. azo dyes are strong, have good all round properties and are cost effective. Anthraquinone dyes on the other hand are not as effective and are more expensive (Trotman, 1990; Robinson *et al*., 2001). Secondly this classification is manageable with respect to the number of different chemical groups (about a dozen). Most importantly, this classification is widely applied by both the synthetic dye chemist and the dye technologist. Thus both parties can readily identify with phrases such as ‘azo yellow’, anthraquinone red, and phthalocyanine blue (Rys and Zollinger, 1972; Hunger, 2003). Figure 1.3 shows the structural formulas of the dyes that were used in this study.

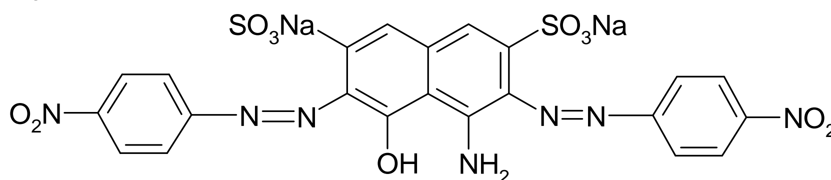


Orange II

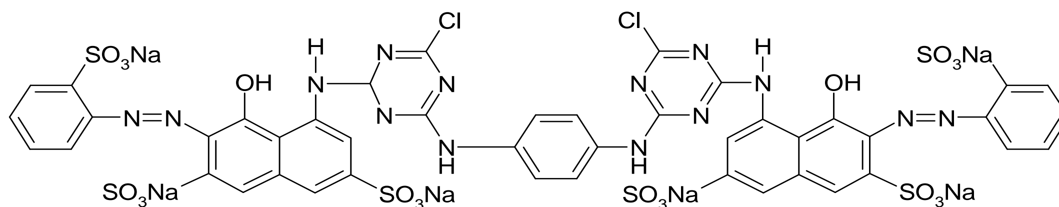
Reactive group: mono-azo,
Molecular weight – 351.34



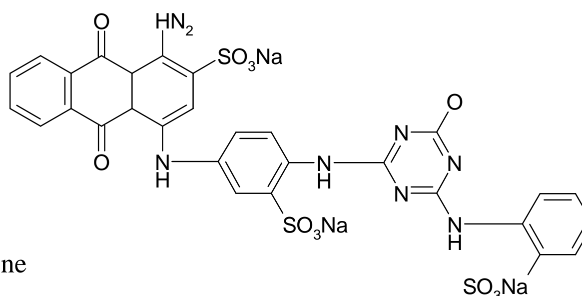
Reactive Black 5
Reactive group: di-azo
Molecular weight – 989.93



Amido Black 10
Reactive group: di-azo
Molecular weight – 624.56



Reactive Red 120
Reactive group: di-azo
Molecular weight – 1496.2



Reactive Blue 2
Reactive group: Anthraquinone
Molecular weight – 558.65

Figure 1.3 Structural representation of textile dye diversity

1.3.3.2 Colouristic classification

Classification by usage or application is the principle system adopted by the colour index (Trotman, 1990). The most important and widely used textiles fibres are cotton and polyester, and because of this the dyes predominantly used are those for dyeing these two fibres, and to some extent polyester-cotton blends. Dyes classified according to method of application maybe anionic, direct or disperse depending on their terminal application i.e. either uses on protein, cellulose or polyamide fibres (Hunger, 2003).

1.3.4 Azo Dyes

Azo dyes are by far the most important and versatile class of dyes which have been studied and used more than any other class (Stolz, 2001; Hunger 2003). They are water-soluble synthetic organic compounds possessing the characteristic –N=N–, which links the chromophore and auxochrome to form coloured molecules of great structural diversity. Generally azo dyes contain between one and three azo linkages, linking phenyl and/or naphthyl rings that are usually substituted with some combination of functional groups including triazine amino, chloro, hydroxyl, methyl, nitro and sulphonate (Bell *et al*., 2000). Azo dyes exist in the *trans* form (Figure 1.4) in which the bond angle is ca. 120º. The nitrogen atoms are sp^2 hybridized and the designation of A and an E group is consistent with colour index (C.I.) usage (Colour index, 1971).

A—N

N—E

Figure 1.4 The azo chromophore

Typical properties of an azo dye are the groups which A represents electron accepting substituents particularly hydroxyl and amino groups, while E represents electron withdrawing groups such as halogens and carboxylic groups. Dyes without any substituents that carry only aromatic groups such as benzene are known as carboxylic azo dyes while those with heterocyclic groups are referred to as heterocyclic azo dyes (Hunger, 2003). The fact that the azo bond can link together various chromophores

and auxochromes illustrates the enormous structural diversity that is possible with azo dyes leading to a wide spectrum of shades, mainly within the scale of red. Unfortunately, the disadvantage limiting their function is that none of the azo dyes are green (Rys and Zollinger, 1972).

1.3.5 Anthraquinone dyes

These dyes constitute the second largest dyes (~15 % of the entries in the colour index (Van der Zee, 2002). Anthraquinone dyes are characterised by a quinoid ring which has either hydroxyl or amino groups attached to the general chromophore structure. Hydroxy-anthraquinones are more common due to their histological application and include mordant dyes such as alizarin and alizarin red S. The amino-anthraquinones are less common and include dyes like nuclear fast red which is used in staining nuclei red.

1.4 Textile effluent and waste water

Textile industries consume large volumes of water and chemicals during wet processing of textiles. The chemical reagents used during manufacture and processing are diverse in chemical composition ranging from inorganic compounds to polymers and organic products (Banat *et al*., 1996; Juang *et al*., 1996). There are over 100 000 commercially available dyes with about 700 000 tons being consumed in the textile industry (Robinson *et al*., 2001; Parac-Ostermann, 2004). By design the majority of dyes are recalcitrant so that they can confer color onto the designated materials (Plumb *et al*., 2001) and resist fading on exposure to sweat, soap, water, light or oxidizing agents (Banat *et al*., 1996; Robinson *et al*., 2001).

Dye waste waters enter the environment from manufacturers and consumers (i.e. textile, leather and food industries) usually in the form of dispersion or a true solution (Seshadra *et al*., 1994; Bell *et al*., 2000) and often in the presence of other organic compounds originating from operational processes. Colour is the first contaminant to be recognized in textile waste water and has to be removed before discharging into water bodies or onto land (Banat *et al*., 1996; Kumar *et al*., 2005). The presence of small amounts of dyes in water (even < 1 ppm) is highly visible and it affects the aesthetic merit, causes significant loss in luminosity and any increase in the temperature will greatly deplete the dissolved oxygen concentration in waste water. This results in subsequent alteration of the aquatic ecosystem (Moreira *et al*., 2004).

The removal of colour from textile waste water is often more important than the removal of the soluble colourless organic substances which usually contribute to the major fraction of Biochemical Oxygen Demand (BOD). Methods for the removal of BOD from most of these effluents are fairly well established (Banat *et al*., 1996; Sponza and Isik, 2002; Pearce *et al*., 2003). On the other hand textile waste waters exhibit low BOD to COD ratios (< 0.1) indicating their difficulty to bioremediate or breakdown (Pagga and Brown, 1986). On the whole, textile waste water is characterised by unfixed dyes, organic pollutants (much higher than regular domestic waste water), large amounts of COD (organic compounds), high conductivity due to salts, high amounts of sulphide and heavy metals due to chlorinated bleaching agents and halogen, sulphur or heavy metal dyes (Porter, 1998; Balidia, 2001).

1.5 Environmental legislation on textile effluent

In the last few years, environmental legislature about the appearance of colour in discharges, combined with increasing cost of water in the industrial sector has made treatment and re-use of dying effluents increasingly attractive to the industry (Maier *et al*., 2004). Effluent discharge from textiles and dyestuff industries into water bodies and waste water treatment systems is currently causing significant health concerns to environmental regulatory agencies. Government legislation is increasingly becoming more stringent especially in the more developed countries, regarding the removal of dyes from industrial effluents. Environmental policy in the United Kingdom (UK) has, since September 1997, stated that zero synthetic chemicals should be discharged into the marine environment (Willmott *et al*., 1998; Robinson *et al*., 2003). Effluent discharge regulations in Germany are amongst the most stringent in the world. Textile effluent discharge has to meet the following guidelines: coloration, toxicity, Total Organic Carbon (TOC) content, absorbable of organic halogens, presence of metals, and salt content (Carliell, 1993; Robinson *et al*., 2003).

However in developing countries there is lack of legal enforcement of environmental legislation, mainly as a result of limiting financial resources. This results in significant contamination of the environment by large industries that can afford to pay municipal fines. Protection and preservation of the environment in South Africa is increasingly becoming a national concern. The Department of Water Affairs and Forestry (DWAF), which is the custodian of South Africa's water resources (Zokufa, 2001) has

policies and laws that are based on integrated watershed management. Its fundamental objectives include the achievement of equity in access of water resources, and its sustainable and efficient use; and to minimise the potential risk of environmental contamination (Fresh water country profile, 2002). Legislation in South Africa states that discharged effluent must conform to a general standard of zero colour, although in practise the measurement of colour is often complicated by interference from natural colouring agents and suspended solids in the water bodies (Carliell, 1993). There are no stringent limits set for effluent TOC and COD in South Africa, thus allowing textile industries to get away with dumping high organic content effluents to municipal waste water treatment plants (Quibell *et al*., 1997). The municipalities calculate effluent charges based on the organic load of the effluent (usually determined by COD), and therefore the discharge of desizing and scouring effluents usually results in extremely high effluent treatment penalties. However, the cost of pre-treating these effluents versus the penalties is always debatable as some companies prefer to dump their waste at the expense of the environment. Therefore, there is need to develop cheap cost effective methods for removal of these contaminants before discharge into the environment.

1.6 Textile effluent treatment methods

Effluents from textile industries are the most expressive from an ecological and physiological perspective (Parac-Osterman *et al*., 2004). In order to achieve satisfactory and acceptable quality levels that allow recycling of textile waste water, removal of dyes and related compounds is very crucial. Based on the fact that azo dyes constitute the largest percentage of textile dyes, most treatment methods are based on the decolourisation of azo dyes (Raghavacharya, 1997). Currently the main operational methods used in treatment of textile waste water involve physical and chemical processes (Shaw *et al*., 2001; Liu *et al*., 2005).

There are several factors that determine the technical and economic feasibility of each dye removal technique. These include; dye type, composition of the waste water, dose and cost of required chemicals, operational costs, environmental fate and handling costs of generated waste (Van der Zee, 2001). Each dye removal technique has its limitations and one individual process may not be sufficient to achieve complete

decolourisation (Table 1.3). To overcome this problem, dye removal strategies involve a combination of different techniques (Raghavacharya, 1997).

Table 1.3 Advantages and disadvantages of physical and chemical textile effluent treatment.

Physical/ Chemical Methods	Advantages	Disadvantages
Fenton's reagent	Effective decolourisation of both soluble and insoluble dye	Sludge generation
Ozonation	Applied in gaseous state; no alteration of volume	Short half life
NaOCl	Initiates and accelerates azo bond cleavage	Release of aromatic amines
Photochemical	No sludge production	Formation of bi – products
Cucurbituril	Good sorption capacity for various dyes	High operating cost
Electrochemical destruction	Breakdown compounds are non hazardous	High cost of electricity
Activated carbon	Good removal of a variety of dyes	Very expensive to operate
Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	Regeneration; no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2
Electro kinetic coagulation	Economically feasible	High sludge production

1.6.1 Physical methods

These include traditional physical-chemical techniques such as ultra filtration, reverse osmosis, ion exchange and adsorption on various adsorbents (activated carbon, peat, fly ash, and coal, wood chips, and corncob) (Azhar *et al*., 2003). Adsorption techniques are more popular due to their efficiency in the removal of pollutants too stable for conventional methods (Lin, 1993; Choy *et al*., 1999). Decolourisation of azo dyes is a result of two mechanisms; (1) adsorption onto a solid matrix and (2) ion exchange as a result of differential charges between dyes and the matrix (Slokar and Le Merchal, 1998). These two mechanisms are influenced by physical-chemical factors such as dye-sorbent interaction, sorbent surface area, particle size, temperature, pH and contact time (Liu *et al*., 2005).

Adsorption with activated carbon is also a widely used technique especially for decolourisation of mordant and acid dyes and to a slightly lesser extent disperse, vat,

pigment and reactive dyes (Raghavacharya, 1997; Basava Rao and Ram Mohan Rao, 2005).

Physical treatment methods have the advantage that they are non destructive, but the major set back is that they do not effectively remove the colour but they simply transfer the pollutant from the liquid phase (water) to a solid matrix, (adsorbent). This normally requires expensive regeneration operations of the adsorbent materials thus post treatment of the solid waste is required (Shaw *et al*., 2001; Liu *et al*., 2005).

1.6.2 Chemical methods

Widely used techniques include oxidation reactions, photochemical oxidations and electrochemical treatment (Shaw *et al*., 2001; Basava-Rao and Mohan-Rao, 2005), of which, oxidation processes are the most common. This is primarily due to its simplicity of application with hydrogen peroxide being the main oxidizing agent. Chemical oxidation removes the dye from the dye –containing effluent by oxidation resulting in aromatic cleavage of the molecules (Raghavacharya, 1997). Other newer emerging techniques which include oxidation with Fenton's reagent (Liu *et al*., 2005), photo catalysis (Yang *et al*., 1998), and ozonation have shown great potential for effectively removing dyes from waste water. These techniques, however, involve complicated procedures and are economically unfeasible for application on a commercial scale (Chang and Lin, 2000). Use of ultra-violet light in photo catalysis is not effective for dark coloured effluents as the UV cannot penetrate the depth of the waste water. Chemical treatment methods also pose a serious threat of introducing secondary pollution due to excessive chemical use (Pearce *et al*., 2003).

1.7 Bioremediation of textile effluent

A wide range of structurally diverse dyes are consumed within a very short time during textile processing. Therefore, textile effluents are extremely variable in composition (Correia *et al.,* 1995). This underlines the need for a largely non-specific treatment process of textile effluents. Alternative approaches utilizing microbial biocatalysts to remove dyes in textile effluent offers potential advantages over conventional processes due to minimal impact on the environment and cost effectiveness (Hiroyuki *et al*., 2002). These treatment textile processes are essentially self sufficient and do not require rigorous monitoring. Options for biological

treatment of textile effluent may be single phase aerobic or anaerobic or a combination of the two (Stolz, 2001).

1.7.1 Fungal biodegradation

Lignin degrading (White Rot) fungi have been shown to degrade several aromatic compounds due to the presence of three lignin modifying enzymes, namely laccase, lignin and manganese peroxidase (Fu and Viaraghavan, 2001; Wesenberg *et al*., 2003). The most studied white rot fungi in regards to xenobiotic degradation is *Phanaerochaete chrysosporium*. Several researchers have shown the ability of this fungus to degrade dioxins, polychlorinated biphenyls (PCBs) and several azo dyes (Banat *et al*., 1996; Wesenberg *et al*., 2003).

Although stable operation of continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved (Mielgo *et al*., 2001; Zhang and Yu, 2000), commercial utilization of lignolytic fungi in the treatment of dye containing waste water is faced with several problems:

a) traditional sewage treatment plants are not ideal habitats for lignolytic fungi and therefore their cultures require special bioreactors,
b) lignin modifying enzymes require an external source of carbon for cultivation because neither lignin nor any of the pollutants degraded by the enzymes has been shown to be utilized as a carbon source. Moreover, they are thought to be expressed during secondary metabolism, following growth when carbon and nitrogen sources become limiting (Swamy and Ramsay, 1996b),
c) the enzymes function optimally at low acidic pH values (4.5 – 5). This would require extensive acidification of the characteristically highly alkaline textile waste water which may result in the inhibition of other useful micro-organisms like bacteria (Zhang and Yu, 2000),
d) turnover rates of lignolytic fungi are rather low. Zhang *et al* (1995) reported the decolourisation of Acid Orange 7 by a fungus in two months. In other experiments dye decolourisation rates ranging from 50 – 150 mg per litre within 5 – 10 days have been reported (Paszczynski *et al*., 1992; Swamy and Ramsay, 1996a, b; Hardin *et al*., 2000). Therefore application on a commercial level is economically feasible when the turnover time is long, and

e) lignin modifying enzymes are highly unspecific for the oxidation of aromatic and xenobiotic compounds. Therefore, their application in a typical textile effluent that contains a complex mixture of dyes and chemical compounds would be extremely inefficient.

1.7.2 Bacterial biodegradation

1.7.2.1 Aerobic

Investigations into bacterial dye biotransformation have been mainly focussed on the most abundant class (azo) of dyes. The electron withdrawing nature of the azo linkages obstructs the susceptibility of azo molecules to oxidative reactions (Fewson, 1988). It has been reported that aerobic sewage treatment plants reduce the level of dyes by adsorption to the sludge biomass, in a non enzymatic process (Pagga and Taeger, 1994). Therefore conventional aerobic sewage treatment systems are not useful for the decolourisation of effluents containing azo dyes and related coloured compounds (Schönberger, 1997). Specialized peroxidase producing bacterial strains of the genera *Streptomyces* and *Sphingomonas* were shown to decolorize azo dyes (Cao *et al*., 1993; Paszczynski, 1992). Later research revealed some limitations of bacterial aerobic azo dye decolourisation. Zissi *et al* (1997) observed that these bacterial strains could only decolorize azo dyes in the presence of additional carbon and energy sources when they used *Bacillus subtilis* to decolourize *p*-amino-azo-benzene in the presence of glucose to yield aniline. This is not economically viable on a commercial scale since the carbon source is expensive.

1.7.2.2 Anaerobic

Under anaerobic conditions many bacteria reduce the highly electrophilic azo bond in the dye molecule through non-specific enzymatic action (Stolz, 2001; Pearce *et al*., 2003). The non-specific action of anaerobic bacteria allows the biodegradation of a wide range of textile dyes thus making this process more suitable for application on a commercial scale. Anaerobic reduction of azo dyes by bacteria seems to be better suited for the decolourisation in sewage treatment systems (Stolz, 2001). This process offers the following advantages:

a) reactions take place at neutral pH and are extremely non-specific when low molecular weight redox mediators are available,

b) in static cultures the depletion of oxygen is easily accomplished thereby allowing obligate and facultative anaerobic bacteria to reduce azo dyes, and
c) sewage systems often provide additional carbon sources which generally increase the reduction rates. These carbon sources also facilitate formation and regeneration of reducing equivalents through their oxidation.

All processes, however, tend to have disadvantages. The major restriction of anaerobic azo dye reduction is that the aromatic amines formed from reductive cleavage cannot be further mineralized (Rafii *et al*., 1990; Chung *et al*., 1992; Van der Zee, 2002). Their accumulation is a serious cause of concern since they are presumed carcinogenic (e.g. naphthylamine or benzene derivatives) and the fact that these amines maybe formed in the lower intestine after ingestion of dye containing foods (Chung *et al*., 1992). Fortunately the potential carcinogenic producing dyes have been banned from the market (Reife and Freeman, 2000).

1.7.3 Anaerobic – aerobic (combined) biodegradation

The complete mineralization of aromatic amines and sulfonated amino aromatics by aerobic bacteria (Nortemann *et al*., 1994; Feigel and Knackmuss, 1993) has led to the suggestion of combining the anaerobic cleavage of azo dyes with aerobic treatment to mineralize the aromatic amines generated in the anaerobic step. This can be done sequentially or simultaneously. In the former processes the anaerobic phase and aerobic phases may be combined alternatively in the same vessel or in a continuous system with separate vessels (Glasser *et al*., 1992; Sponza and Isik, 2002). Simultaneous treatment systems utilize anaerobic zones within aerobic bulky phases as demonstrated in bio-films, granular sludge or biomass immobilized in other matrices (Jiang and Bishop, 1994; Kudlich *et al*., 1996). Both treatments require auxiliary substrates to feed the bacteria in the anaerobic zones with carbon and energy sources as well as reducing equivalents for azo bond cleavage.

Generally it can be concluded that, in continuous anaerobic – aerobic systems, there is complete decolourisation of dyes and significant reduction in BOD and COD levels. In the subsequent aerobic stage the remaining BOD (which includes the aromatic amines) from the auxiliary substrates may be completely mineralized (Zaoyan *et al*., 1992; O'Neill *et al*., 2000a, b).

1.8 Sulphate reducing bacteria

1.8.1 Classification of sulphate reducing bacteria (SRB)

Sulphate reducing bacteria classification is primarily based on morphological and nutritional characteristics, which is supported by some chemotaxonomy such as guanine: cytosine ratios of the genetic make-up (Zehender, 1988). Occasionally, the presence of certain pigments has also been used in the classification of SRB (Zehender, 1988; Feio *et al*., 1998). The diverse nature and abundance of SRB in the environment tends to complicate the classification of the sulphate reducers. Morphologically similar types may have different nutritional requirements, as well as the presence of different types of pigments which do not compliment the nutritional and phenotypic traits. This complexity of features compromises the classification of SRB. Because of this, it is important to note that species and genera of this group that have been established thus far, for determinative purposes and do not necessarily indicate phylogenetic relationships (Zehender, 1988).

1.8.1.1 Morphological classification

Like any other bacteria, the genus names of sulphate reducers, apart from the prefix 'desulfo' have been derived from optical observation through microscopy studies (Zehender, 1988). The varying environment conditions, however, that these sulphate reducers grow under heavily impacts on the cell shape and motility. Therefore it is not adequate to classify these bacteria based on morphology, motility and nutritional requirements, but rather on a combination of all the above characteristics (Postgate, 1984). As more and more sulphate reducers were discovered, it could not be avoided that the genus names were used less precisely than before, as can be exemplified by the designation *Desulfovibrio* which was originally given to sulphate reducers with curved cells and a distinct vibrating motility. Later with the advent of technology, the rod-shaped *Desulfovibrio* strains 'Norway 4' (Postgate, 1984); *D. thermophilius* (Rozanova and Kudyakova, 1974); *D. baculatus* (Rozanova and Nazina, 1976) were all discovered. This classification was later reversed in the classification of *D. baarsii* (Pfennig *et al*., 1981; Postgate, 1984) which had the same motile cells typical of *Desulfovibrio* sp but nutritionally it was distinctly different.

1.8.1.2 Nutritional classification

Typical *Desulfovibrio* sp. has been shown to grow well using lactate but not acetate as a carbon source (Mudryk *et al*., 2000). They are also referred to as incomplete oxidizers since they can only oxidize lactate to acetate. In contrast *D. baarsii* grows on fatty acids up to stearate that are completely oxidized but it cannot utilize lactate as a carbon source. *Desulfovibrio* sp. can also be cultured with H_2 or formate but it requires acetate as a carbon source for cell synthesis. However, *D. baarsii* requires no additional C-source when grown on formate (Jansen *et al.,* 1984). Most of the different types of SRB have been isolated from batch enrichment cultures that are specific for one type of sulphate reducer depending on the following factors: carbon source and electron donor, temperature profile, salt concentration, and source of inoculum.

1.8.1.3 Chemotaxonomic classification

Comparative studies on the chemistry of cell components and on nucleic acids from SRB highly complement the classification based on morphology and nutrition. At molecular level some sulphate reducers appear to be less related to members of their own genera. Chemotaxonomic studies have revealed relationships of sulphate reducers with non-sulphate reducing bacteria, which have so far been explained as being evolutionary (Scheid and Stubner, 2001).

The DNA base ratios (molecular % guanine + cytosine) of known sulphate reducers within the genus tend to vary depending on the method of determination and calculation (Zehender, 1988; Castro *et al*., 2000; Scheid and Stubner, 2001). The G+C values of the 'classical' *Desulfovibrio* sp isolated with lactate range from 49 – 65 mol %. Phylogenetic classification of SRB by rRNA sequence analysis has a variety of advantages, including providing insights into the evolutionary origins of sulphate reduction in distantly related species and in facilitation of development of group specific phylogenetic probes and PCR primers for use in ecological studies (Castro *et al*., 2000; Lloyd *et al*., 2004). This led to the observation that there was a significant gap between the sporing and non-sporing sulphate reducers, where the spore forming *Desulfotomaculum nigificans* and *D. acetoxidans* resemble and cluster with *Clostridium* sp that are gram positive; contain a cell wall structure and are capable of

forming spores. The analysis of rRNA sequences has facilitated the broad classification of SRB into four distinct sub-groups: (1) gram negative mesophilic; (2) gram positive spore forming; (3) thermophilic SRB and (4) thermophilic archaeal SRB (Ogram *et al*., 2000) (Table 1.4).

Table 1.4 Important characteristics in the classification of representative sulphate reducing bacteria (Castro *et al*., 2000).

	Shape	Motility	GC content of DNA (%)	Desulfovirin	Cytochromes	Oxidation of acetate
Gram-negative mesophilic SRB						
Desulfobulbus	lemon to rod	−/+	59 - 60	−	b,c,c_3	I[a]
Desulfomicrobium	ovoid to rod	+/−	52 - 67	−	b,c	I
Desulfomonas	rod	−	66	+	c	I
Desulfovibrio	spiral to vibroid	+	49 - 66	+/−	c_3,b,c	I
Desulfobacter	oval to rod	+/−	44 - 46	−		C[b]
Desulfobacterium	oval to rod	+/−	41 - 52	−	b,c	C
Desulfococcus	spherical or lemon	−/+	46 - 57	+/−	b,c	C
Desulfomonile	rod	−	49	+	c_3	C
Desulfonema	filaments	gliding	35 - 42	+/−	b,c	C
Desulfosarcina	oval rods or coccoid, packages	+/−	51	−	b,c	C
Gram-positive spore-forming SRB						
Desulfotomaculum	straight to curved rods	+	48 - 52	−	b,c	I/C
Bacterial thermophilic SRB						
Thermodesulfobacterium	vibroid to rod	−/+	30 - 38	−	c_3,c	I
Archaeal thermophilic SRB						
Archaeoglbus	coccoid	+/−	41 - 46	−	n.r.[c]	I

[a]I, incomplete, [b]C, complete, [c]n.r., not reported

1.8.2 Ecology of SRB

Sulphate reducers are a mixed group of morphologically and nutritionally diverse, strictly anaerobic bacteria which utilize sulphate or other sulphur compounds as an electron acceptor for the dissimilation of organic compounds (Widdel and Pfennig, 1981; Gibson, 1990 Castro *et al*., 2000). SRB are environmentally important micro-organisms which regulate sulphate levels and play a pivotal role in both carbon and sulphur cycles, and thus are extremely important components of the global microbial community(Mudryk *et al.,*2000; Purdy *et al*.,2002;) They are primarily found in the anaerobic regions of marine, estuarine and mine waste water sediments as well as saline ponds due to the high sulphate content (Gibson, 1990; Zuo and Wood, 2004).

SRB's occurrence has been detected in other habitats such as sour whey digesters (Zellener *et al*., 1989); anaerobic purification plants (Nanninga and Gottschal, 1987); sewage effluents (; Gibson, 1990; Buismanman *et al*., 2000). SRB derive their carbon source from activities of heterotrophic micro-organisms in the water column overlying the sediments, or from a direct organic influence especially habitats that are contaminated by a secondary nutrient input such as sewage effluent (Gibson, 1990; White and Gadd, 1996). The majority of SRB are neutrophiles with maximum growth obtained in the pH range 7.0 – 8.0, although two strains belonging to the genus *Archaea* have been found to grow in acidic pH ranges of 4.5 – 6.5 while *Desulfonatronovibrio hydrogenovorans* and *Desulfonatronum lacustre*, can grow at alkaline pH 8.0 - 10 (Pikuta *et al*., 1998; 2000). Because of the vast diversity of SRB and occurrence in varying ecosystems, they can grow over a broad temperature range from 4 – 98 ºC although optimum temperature for growth is around 35 ºC (Holmer and Storkholm, 2001).

1.8.3 Physiology

The physiological properties of SRB were first investigated by Baars (1930) who isolated the sulphate reducing bacterium *Desulfovibrio rubetschickii* that was capable of directly oxidizing acetate to carbon dioxide. Subsequent attempts, however, to isolate species capable of utilizing acetate led Selwyn and Postgate (1959) to the conclusion that the original culture was part of a mixed comensal population. Earlier research on SRB concentrated on strains that utilized lactate as a carbon and energy source which were classified into two subgroups; (Equation 1) spore-forming straight or curved rods belonging to the genus *Desulfotomaculum* and (Equation 2) non-spore forming *Desulfovibrio* containing motile vibrios or rods (Postgate and Campbell, 1966; Gibson, 1990). The preferred carbon sources for bacteria belonging to each of these genera include organic acids, lactate, pyruvate, and malate, and alcohols such as ethanol, propanol and butanol which are incompletely oxidized to acetate.

SRB were the first non-photosynthetic bacteria that were shown to generate ATP through electron transfer coupled phosphorylation, using sulphate as a final electron acceptor, from respiration of H_2 or the various organic acids mentioned above resulting in the production of hydrogen sulphide as an end product (Heidelberg *et al.,* 2004).

1.8.4 Electron donors and degradative capacities of SRB

Specific electron donors oxidized by SRB are always low molecular weight compounds predominantly derived from fermentation products of anaerobic bacterial primary degradation of macro-molecular compounds i.e. carbohydrates, proteins, and lipids and other components of dead biomass(Zehender, 1988).

Inorganic sulphate is the highest oxidation state and is the most stable form of all sulphur compounds which is used as an external electron acceptor to perform net oxidation of the organic substrates through electron transfer phosphorylation. Sulphate respiration, however, is only ATP yielding if the SRB are grown in the presence of H_2 or acetate (Nicolet *et al*., 2000).

There are two major metabolic groups that can be distinguished in the nutritionally diverse SRB which are ;(1) species oxidizing their substrates incompletely to acetate and (2) species which in principle are able to completely oxidize their organic substrates including acetate to CO_2. The equations of incomplete and complete lactate oxidation are as follows:

$$2CH_3CHOH\ COO_- + SO_4^{2-} \longrightarrow 2CH_3COO^- + 2HCO_3^- + HS^- + H^+ \ldots\ldots\ldots\ldots (1)$$

$$2CH_3CHOH\ COO_- + 3SO_4^{2-} \longrightarrow 6HCO_3^- + 3HS^- + H^+ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

1.8.4.1 Incomplete lactate oxidizing SRB

The classical lactate oxidizing species of the genera *Desulfovibrio* (Postgate and Campbell, 1966) and *Desulfotomaculum* (Zehender, 1988)) are not capable of terminally oxidising their organic substrates. Other incomplete oxidisers which physiologically resemble the ‘classical’ *Desulfovibrio* sp include *Desulfovibrio thermophilus, Thermodesulfobacterium commune* and some named fatty-acid sulphate reducers with vibrio-shaped cells. The inability to completely oxidize organic substrates is because they lack an operating enzymatic mechanism similar to the citric acid cycle, which allows complete oxidation of the acetate unit (Acetyl-CoA) originating from substrates and is thus excreted as acetate (Pfenning and Widdel, 1981; Pfennig *et al.,* 1981; White and Gadd, 1996).

These organisms cannot oxidize acetate when it is added externally as enrichment or if naturally present in the medium. Only C_2 compounds such as oxalate and glycine (which are more oxidized than acetate) and C_1 compounds such as formate and methanol are completely oxidized. Acetate may only be assimilated as an organic source for cell growth when the species are grown with H_2 or formate as electron donors (Gibson, 1990). Some electron donors yield products or fragments other than acetate that may not be metabolized further by the respective sulphate reducer (Zehender, 1988). On the whole, the incompletely oxidizing sulphate reducers are nutritionally less versatile than completely oxidizing species. One of the few versatile incomplete oxidizers is a *Desulfovibrio* species that utilizes glycerol, choline and a number of amino acids, in addition to the traditional H_2, lactate, pyruvate malate, fumarate and ethanol (Zehender, 1988). Incomplete oxidizers may grow significantly faster than the complete oxidizers; therefore batch enrichments with electron donors that can be utilized by both types usually select the incomplete oxidizers (Badziong and Thauer, 1980).

1.8.4.2 Complete lactate oxidizing SRB

Species of the genera *Desulfobacter, Desulfococcus, Desulfosarcina, Desulfonema, and Desulfobacterium* are, in principle, able to oxidize all their substrates completely to CO_2. Some *Desulfovibrio* sp (*D. Baarsii*) and *Desulfotomaculum* (*D. acetoxidans, D. sapomanders*) are complete oxidizers and differ from other representatives of the same genera (Klemps *et al*., 1985). The degree of assimilation, however, varies among these genera. Specialized obligate acetate oxidizers (*Desulfobacter*) grow relatively fast (doubling time of ~ 20 h) and generate higher cell yields as they utilize acetate as a carbon source. Versatile complete acetate oxidizers tend to prefer other organic acids or alcohols and only utilize acetate from the medium very slowly with low cell yields (Zehender, 1988). It is interesting to note that these completely oxidizing sulphate reducers are capable of generating acetate from other organic compounds as exemplified by *D. acetoxidans* growing on ethanol or butyrate (Widdel and Pfennig, 1981) and *Desulfobacter* sp growing on ethanol (Laanbroek, *et al*., 1984). This pathway is favourable probably because the oxidation of these substrates to the level of acetyl-CoA is faster than its terminal oxidation, so that acetate

excretion via acetyl phosphate and a substrate level phosphorylation are favoured (Carepo *et al*., 2002).

1.8.4.3 Bioenergetics of sulphate reduction

Sulphate reducing bacteria are capable of using different carbon sources as electron donors and the sulphate molecule is often implied as a 'respiratory' substrate (Hansen, 1994; Carepo *et al*., 2002). Sulphate is the most abundant form of sulphur available to micro-organisms. It exists as a stable non-reactive molecule that needs to be activated before being used up in subsequent metabolic reactions (Schiff and Saidha, 1987). There are two types of sulphate metabolism: assimilation and dissimilation. In the former, sulphate is reduced to sulphide and is then incorporated in the biosynthesis of sulphur amino acids while in the latter termed dissimilatory sulphate reduction, in the absence of O_2 the oxidized forms of sulphur are used as electron acceptors for the biological oxidation of organic substrates by specialized group of anaerobic bacteria (SRB).This reduction of the organic compounds serves for energy conservation (Peck and LeGall, 1982; Peck and Lissolo, 1988). For purposes of this study, the latter process of sulphate reduction will be discussed.

Sulphate reduction occurs primarily in the cytoplasm of SRB (Nicolet *et al*., 2000), Warthmann and Cypionka (1990) investigated the transport of sulphate in *Desulfovibrio sulfuricans, Desulfobulbus propionicus* and *Desulfococcus multivorans,* and their findings suggested the presence of a regulatory system to conserve the internal chemical balance and avoid sulphate overload. This regulatory system was also found to be coupled to enzyme expression (Jansen *et al.*, 1984).

Dissimilatory sulphate reduction starts with ATP activation of the non-reactive sulphate molecule by the enzyme ATP-sulphurylase (Peck, 1959; Hansen, 1994), to yield adenosine-5´-phosphosulphate (APS) and pyrophosphate (*PPi*), which may be subsequently cleaved by pyrophosphatase to yield inorganic phosphate (Figure 1.5) (Trudinger and Loughlin, 1981; Peck and Lissolo, 1988). APS serves as an electron acceptor which is rapidly converted to bisulphite and adenosine monophosphate (AMP), catalysed by APS reductase. Müller and Massey (1969), in agreement with studies on sulphite- flavin adducts proposed APS reduction involves the transfer of a

sulphonate group from APS to the N-S of the enzyme bound reduced $FADH_2$. The sulphite is thought to be cleaved off leaving the protein with the oxidized FAD (Peck and Bramlett, 1982). At physiological pH, the sulphite is partially protonated to bisulphite and then reduced to sulphide by the enzyme bisulphate reductase (Peck and Lissolo, 1988; Gavel *et al*., 1998).

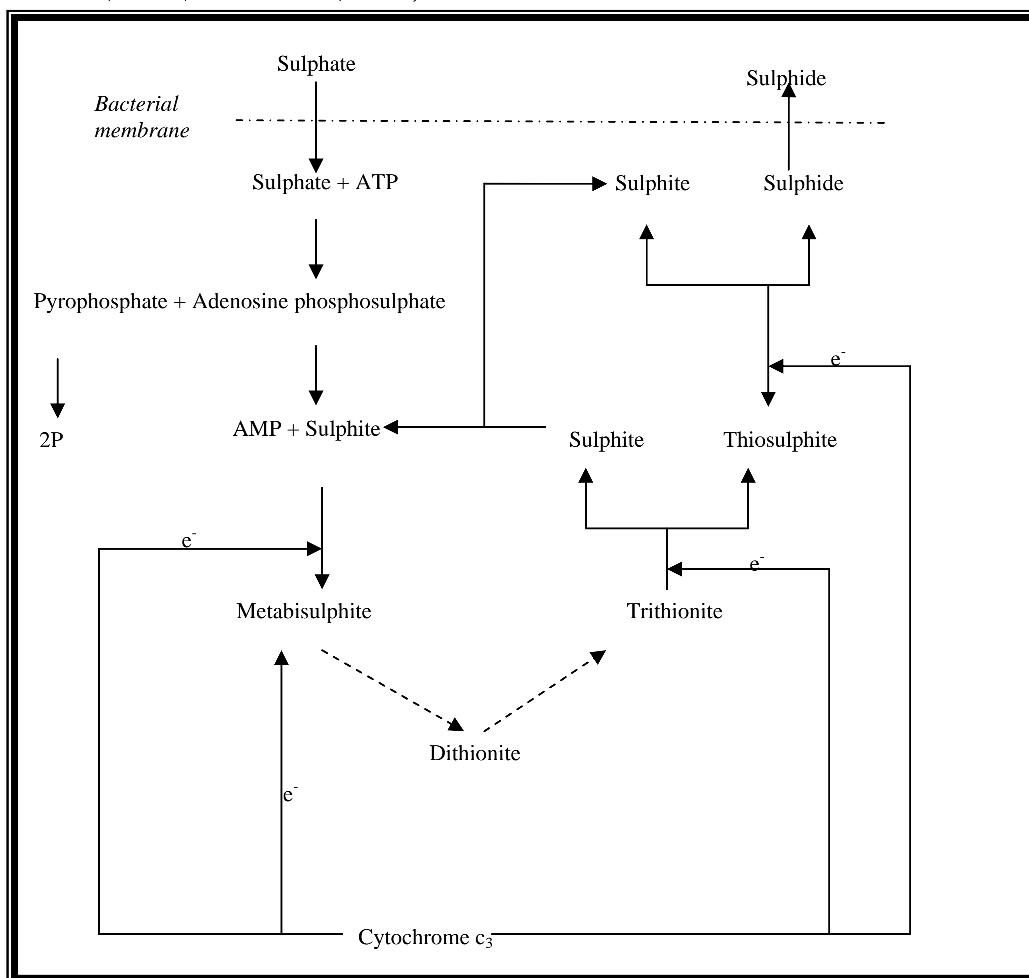


Figure 1.5 Dissimilatory sulphate reduction and sulphide generation pathway (adapted from Gibson, 1990)

Sulphate reducing bacteria have shown a unique ability to couple dissimilatory sulphate reduction with net ATP synthesis via electron transfer linked phosphorylation (ETLP) or substrate level phosphorylation (SLP) to the reduction of sulphate, depending on the oxidation state of the electron donor (Peck, 1966). However, SLP cannot generate the sufficient ATP levels required for sulphate reduction and cell growth. Therefore, another mechanism of energy conservation is required for cell growth and oxidation of organic substrates (Rashamuse, 2003; Heidelberg *et al*., 2004). The solution to this problem is derived from the hydrogen

coupled metabolism which plays a central role in the energy generating mechanism in various micro-organisms. Studies on the genus *Desulfovibrio* have proposed hydrogen cycling with vectorial electron transfer as a general energy coupling mechanism, which occurs when cells are grown on organic substrates in the presence of sulphate as demonstrated by Heidelberg *et al* (2004). They showed that during growth *D. vulgaris* derives energy from oxidative phosphorylation by coupling the reduction of sulphate to sulphide with oxidation of H_2. *Desulfovibrio* sp capable of consuming or evolving H_2 can carry out intracellular transfer of molecular H_2 between cytoplasmic fermentation and a membrane-bound respiratory chain. This recycling of H_2 within the same organism bestows energetic and adaptive advantage on the organism (Rashamuse, 2003).

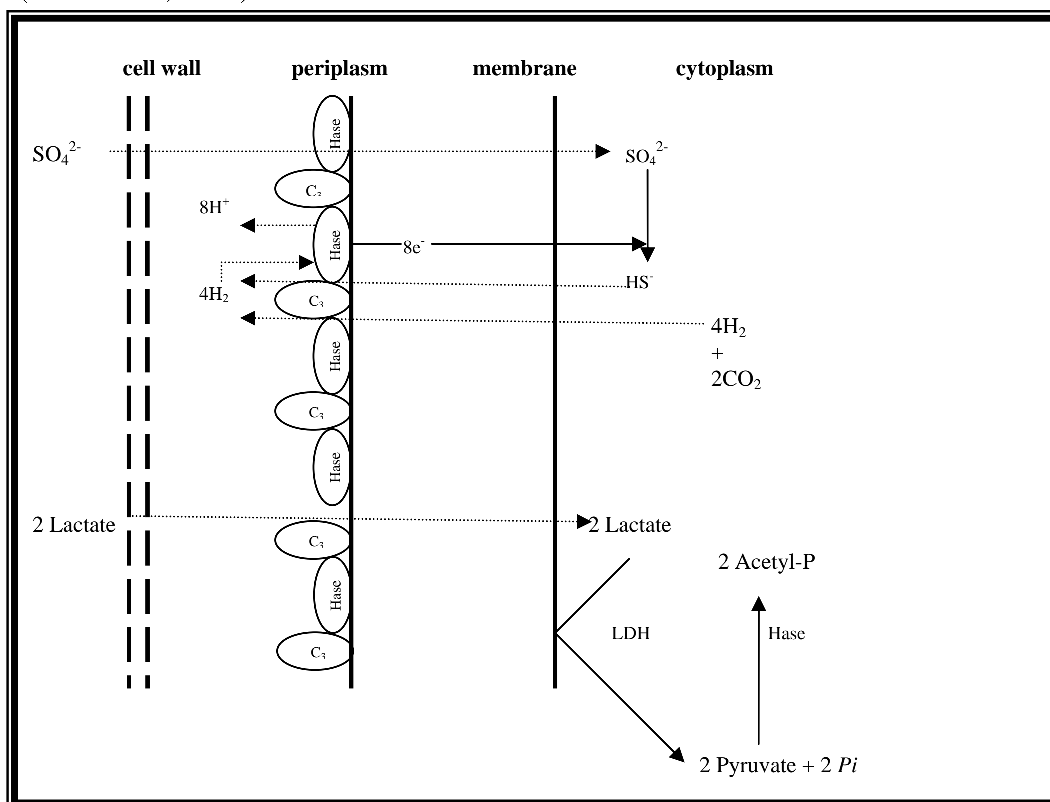


Figure 1.6 Proposed mechanism of H2 cycling in D. vulgaris [Hase=Hydrogenase; c3= cytochrome C3; PDH = lactate dehydrogenase] (Adapted from Odom and Peck, 1984).

Work on *D. vulgaris* by Odom and Peck (1984) and more recently Heidelberg *et al* (2004) suggests intracellular H_2 is coupled to energy production during cell growth on organic substrates such as lactate. They postulated a mechanism in which molecular H_2 produced in the cytoplasm from oxidation of lactate or other organic substrates diffuses across the cytoplasmic membrane (Figure 1.6). The H_2 is then oxidized by

periplasmic hydrogenases which are coupled to vectorial electron transfer across the cytoplasmic membrane which is simultaneously used in the reduction of APS and involves consumption of protons. On the outside of the cell wall, lactate enters the cell and is rapidly oxidized to pyruvate by lactate dehydrogenase enzymes located on the internal aspects of the cytoplasmic membrane (Heidelberg *et al*., 2004). The electrons generated from this oxidation step are used to generate the H_2. Pyruvate is then converted to acetyl-phosphate, CO_2 and lower potential electrons that serve as electron donors to the molecular H_2. This reaction is facilitated by an internal hydrogenase (Odom and Peck, 1984). This hydrogenase also distinguishes electron transfer leading to the production of H_2 and the electron transfer leading to reductases involved in respiratory sulphate reduction. The H_2 produced in the cytoplasmic membrane rapidly diffuses across the cytoplasmic membrane, but in the presence of electron acceptors like sulphate, it is re-oxidised to protons and electrons by periplasmic hydrogenases and its cofactor cytochrome c_3 (Aubert *et al*., 2000). The electrons are transferred across the cytoplasmic membrane whilst the protons are left at the external surface of the membrane, thus establishing a proton gradient and subsequent energy that can be utilized for transport and ATP synthesis.

1.9 Dye degrading enzymes

Some of the enzymes that are currently known in biotechnological processes such as amylases and proteases that are being used in the synthetic and biochemical reactions have evolved over millions of years to become efficient and selective for specific reactions taking place in living systems. The potential to use these biocatalysts in industrial applications has been compromised by several obstacles as these processes involve substrates, organic solvents, metallic ions and other reactions conditions that are not normally encountered in their natural conditions (Bylina *et al*, 1999). The introduction of protein engineering has facilitated the adaptation of potentially useful enzymes so that they can be used commercially. Extensive research has been conducted to try and optimize the application of natural enzymes in industrial reactions by directing their evolution *in-vitro*. Controlling *in-vitro* mutation efficiencies and screening for enhanced catalytic properties over multiple generations has led to the development of new enzymes that have 100-fold more activity than the natural enzymes. Different microbial populations (i.e. fungi, bacteria, and algae) with

different chemical and physiological characteristics have been investigated for their potential to produce dye degrading enzymes.

The physiology and production of lignin modifying enzymes (LME) by white-rot fungi has shown great potential in degrading azo dyes and related effluents. The enzymatic system that constitutes lignin degrading enzymes transforms aromatic dyes, polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, pesticides and even explosives (Rodriguez *et al*, 1999). The main LME include two types of peroxidases lignin (LiP) and manganese (MnP) and a phenol oxidase, also called laccase. Production of these enzymes occurs during the secondary stage of white-rot fungi metabolism since oxidation of lignin does not produce any net energy. Thus synthesis and secretion of these enzymes is induced in conditions where nutrients (carbon or nitrogen) are limiting. It is interesting to note that the production of LiP and MnP is optimal at high oxygen tension but is repressed by agitation whilst on the other hand laccase production is enhanced by agitation. Research over the years has shown that expression of LME varies according to taxonomy and culturing conditions (Wesenberg *et al*, 2003).

In the course of investigating the degradation of azo dyes by lignolytic fungi, Paszczynski *et al* (1992) and Cao *et al* (1993) discovered the decolourisation of azo dyes by some peroxidase-producing bacterial strains (mainly *Streptomyces* species and gram-negative *Sphingomonas chlorophenolics or Flavobacterium*). The oxidation of azo dyes by *Streptomyces chromofuscus* A11 involved an extracellular peroxidase that showed restricted substrate specificity analogous to that of *Phanaerochaete chrysosporium* or horse radish peroxidase. The only significant difference with the lignolytic fungi peroxidase was that bacteria peroxidase produced insignificant amounts of $^{14}CO_2$ from industrially relevant ^{14}C-labelled azo dyes (Stolz, 2001).

1.9.1 Laccases (benzenediol: oxygen oxidoreductases, EC.1.10.3.2)

These phenol oxidase enzymes have broad substrate specificity with respect to their electron donor. The mechanism of laccase involves removal of an H^+ atom from the hydroxyl and amino groups of the *ortho-* and *para-* substituted mono and poly-phenolic substrates, and aromatic amines. This is achieved through a one electron step abstraction to form free radicals capable of undergoing further re-polymerization, and

demethylation or quinone formation (Abdullah *et al*., 2000). As a result laccases have found application in the treatment of a wide range of industrial effluents such as paper and pulp, textile, tannery and other industrial effluent containing chloro-lignins or phenolic compounds. Laccases have been shown to render phenolic compounds less toxic via degradation or polymerization reactions and/or cross-coupling of pollutant phenols with naturally occurring phenols (Huttermann *et al*., 1980).

The application of laccases to such processes however demands the production of significant amounts of enzyme, usually cultivated in laboratory shake flasks. This requires development of efficient production systems and is costly (Kahramann and Yasilada, 2001; Mougin *et al*, 2002).

1.9.2 Peroxidases

Lignin peroxidase (diarylpropane: oxygen H_2O_2 oxidoreductases EC.1.11.1.14 and manganese peroxidase (II) H_2O_2 oxidoreductases EC.1.11.1.13) are glyco-proteins containing one iron protoporphyrin IX heme prosthetic group. They have molecular weights ranging between 32 – 62.5 kDa and are secreted in multiple isoforms (Wesenberg *et al*, 2003). The two enzymes have a similar mechanism to laccase but generally have lower oxidation potential.

Studies have shown that LiP catalyzes the oxidation of non-phenolic aromatic lignin moieties and structurally similar compounds. It also catalyzes several oxidations in the side chain of lignin and related compounds. It has been reported that LiP has the ability to catalyze the cleavage of aromatic ring structures including azo dyes (Umezawa and Higuchi, 1987; Wesenberg *et al*, 2003). This enzyme was also found to mineralize a variety of recalcitrant 3- and 4- ring polycyclic aromatic hydro-compounds (PAHs) (Gunther *et al*., 1998), while Chivukula *et al* (1995) successfully decolorized azo dyes.

LiP degrades azo dyes by oxidation of the phenolic group to produce a radical at the carbon bearing the azo linkage. The phenolic carbon is then attacked by a water molecule resulting in its break down to produce phenyldiazene which is easily oxidised by a one electron reaction to generate nitrogen (Rodriguez *et al*., 1999).

Manganese peroxidase is the most commonly produced enzyme by white-rot basidiomycetes. This enzyme has generated a lot of interest in enzyme biotechnology because of its high degradative potential. It is a specific enzyme that oxidizes Mn^{2+} to Mn^{3+} ions which are highly reactive (Ziegenhagen and Hofrichter, 2000). However, these reactive ions give the MnP an apparent unspecificity which is caused by the secondary reactions of the reactive Mn^{3+} ions. As a result this reactivity is stabilized by chelation with organic acids such as oxalate, malonate, malate or lactate which are produced and excreted into the microenvironment by fungi under natural conditions. The chelated Mn^{3+} ions act as redox mediators which oxidize a wide range of aromatic compounds including lignin, humic substances, organo-pollutants (i.e. dyes and pesticides). The resultant products consist of low molecular mass fragments, quinones, ring-fission products, organic acids and carbon dioxide, all of which can serve as substrates for other reactions (Ziegenhagen and Hofrichter. 2000). MnP production is regulated by the concentration of Mn^{2+} ions (Brown *et al*., 1990; Perez and Jeffries, 1992) and a 24 –fold increase in enzyme activity has been reported when fungi are grown in the presence of $MnSO_4$ or $MnCl_2$.

1.9.3 Azoreductases

These are non-specific cytoplasmic enzymes that catalyze the cleavage of the characteristic (-N=N-) azo bonds. The physiology and mechanism of azoreductase is still unclear as many mechanisms and theories have been proposed. For purposes of the present study, a few of the mechanisms postulated will be discussed. There are two broad classes of azoreductases; - the true azoreductases produced under aerobic conditions and those that are said to be produced under anaerobic conditions.

Aerobic azoreductases catalyze reductive metabolism of azo dyes in the presence of molecular oxygen. Previous studies conducted by Zimmermann and co-workers (1982, 1984) on *Pseudomonas* strains K22 and KF46 revealed two azoreductases capable of decolorizing Orange I and II respectively. The two enzymes were purified, characterised and compared. It was found that both azoreductases were monomeric flavin-free enzymes that preferentially used NADPH and to some extent NADH as co-factors. They also demonstrated the ability to reductively cleave sulfonated substrates in addition to the carboxylated growth substrates. The only unique aspect of

these two enzymes was in their mechanism of azo dye degradation. Orange I azoreductase from strain K22 strictly required the presence of a hydroxyl group in the *para-* position to the azo bond while on the other hand, Orange II azoreductase from KF46 strain required the hydroxyl group to be in the *ortho-* position relative to the azo bond for catalytic activity (Stolz, 2001) (Figure 1.7).

Azoreductases transfer the reducing equivalents originating from the oxidation of organic substrates to the azo dyes. Studies with some aerobic and facultative aerobic bacteria demonstrated that azo compounds can be utilized as the sole source of carbon, with azo bond cleavage being facilitated by enzymes that are thought to be either intracellular or membrane bound (Van der Zee *et al*, 2002). These specifically adapted strains synthesize true azoreductases which reductively cleave the azo group in the presence of molecular oxygen.

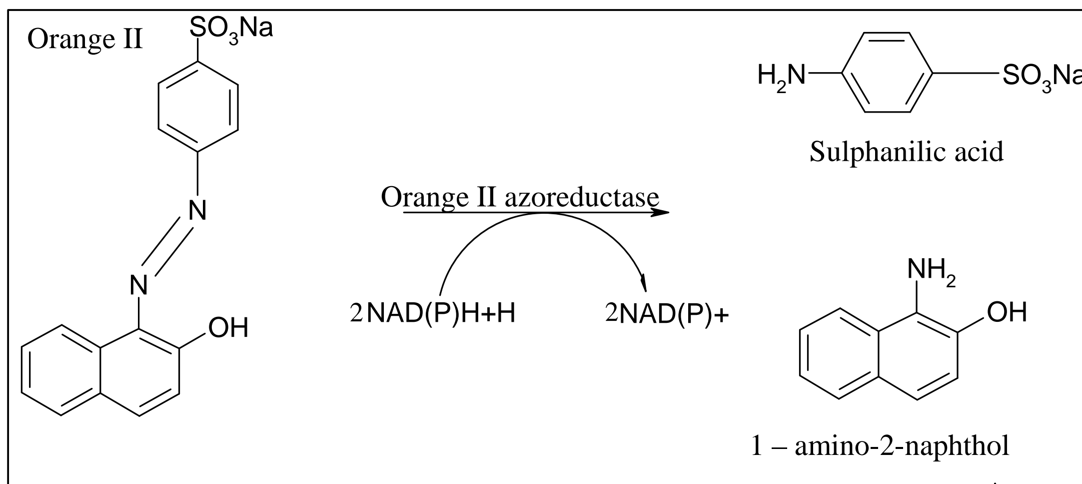


Figure 1.7 Proposed mechanism for the aerobic degradation of Orange II azo dye by Orange II azoreductase produced by Pseudomonas strain KF46 (Zimmermann *et al*., 1982).

The reductive cleavage of azo dyes under anaerobic conditions is still unclear and quite interesting, as it has generated several theories as to how the mechanism works. Previous studies have indicated that azo reduction is enzymatically catalysed by an 'azoreductase' (Kahn *et al*., 1983. cited by Kremer, 1989), but it still remains uncertain whether a single enzyme or composite system of enzymes is responsible for these reactions. The only evidence of the so called 'azoreductase' has only been demonstrated in studies with some aerobic (*Bacillus)* and facultative aerobic

(*Pseudomonas)* bacteria (Zimmermann *et al*., 1982; Van der Zee, 2002). However azo dye reduction under anaerobic conditions has been reported, where reductive cleavage of azo dyes readily occurs. These reactions usually occur with rather low specific activities but are extremely unspecific in terms of the organism involved and the dyes that are converted (Stolz, 2001). There is therefore more potential for dye degradation in anaerobic systems as opposed to the highly specific azoreductases from aerobic bacteria. These unspecific processes make use of low molecular weight mediators (e.g. flavins or quinones) which are enzymatically reduced in the cells. In earlier studies with facultative anaerobic bacteria, it was repeatedly suggested that reduced flavins generated by cytosolic flavin-dependent reductases were responsible for the unspecific reduction of azo dyes (Roxon *et al*,. 1967). Russ *et al* (2000) later reinforced these earlier findings by demonstrating the ability of cytosolic flavin reductases to act as *in-vitro* azoreductases using a recombinant flavin reductase in different genetic backgrounds. It was also shown that the addition of external redox mediators e.g. benzene viologen, or quinones to strictly anaerobic bacteria resulted in a significant increase in decolourization of azo dyes (Stolz, 2001). This was also supported by the findings of Kudlich *et al* (1997) and Keck *et al* (1997) who postulated that azo reduction by bacteria does not require a transport system for the dyes or the reduced equivalents. Instead they suggested that the quinones (anthraquinone-2-sulfonate or 2-hydroxyl-1-4-naphthaquinone) which acted as redox mediators were enzymatically reduced by the cells and the hydroquinone formed subsequently reduced the dyes in a purely chemical reaction. Cell fractioning experiments demonstrated that the quinone reductase activity was located in the cell membrane of *Sphingomonas xenophaga* BN6. Therefore there was no need for the sulfonated azo compounds or the hydroquinone/quinone redox mediators to be transported via the cell membrane (Kudlich *et* at, 1997).

In summary, it appears that under anaerobic conditions in the environment, or sewage treatment systems, specific azoreductases, if they exist at all, are of very little significance in the reduction of azo dyes. This statement does not hold for true azoreductases which are produced under aerobic conditions.

1.9.4 Hydrogenases

Hydrogenases are a class of enzymes that catalyze the reversible reduction and oxidation of molecular hydrogen (Guirl-Brugna *et al*., 2001) in the reaction (Equation 3):

$$H_2 \longleftrightarrow 2H^+ + 2e^- \quad \ldots\ldots (3)$$

Hydrogen metabolism plays a central role in the energy – generating mechanisms of various micro-organisms in particular sulphate reducing bacteria (Brugna *et al*., 2000). It is important to know that the various hydrogenases found in different microbial species differ in molecular weight, quaternary structure, active site orientation, and specificity with respect to the type of electron carriers (Zadvorny *et al*., 2004). The catalytic processes of hydrogenases involves the following steps: (a) activation or production of hydrogen at the active centre, (b) transfer of two electrons between the active site and the redox partner of the hydrogenase and (c) transfer of two hydrogen atoms between the active centre and the medium solvent (De Lacey *et al*., 2000).

Three types of hydrogenases are known to date: on the basis of their metal content, two groups have been described, namely [Fe] and [Ni-Fe] (which includes [Ni-Fe-Se]) hydrogenase. The third type does not contain any metals, instead they attribute their catalytic activity to the presence of an organic factor (Zadvorny *et al*. 2004). Studies have shown that all three types of enzymes can be found in the same bacteria. A survey of genomes of several sulphate reducing bacteria indicated that only the [Ni-Fe] enzyme is uniformly present (Brugna *et al*., 2000).

Iron only hydrogenases have high specific activities and usually function to evolve hydrogen. Amino acid sequence analysis and electromagnetic resonance (EPR) studies have demonstrated that Fe – only hydrogenases generally contain two [4Fe – 4S] clusters (the F-clusters) in a ferredoxin-like domain. In addition an unusual EPR signal has been attributed to a novel 6F cluster proposed to be the active site called the H-cluster (Nicolet *et al*., 2000). Studies on the *Desulfovibrio desulfuricans* Fe hydrogenase have shown that it is a periplasmic protein made of two different

subunits of molecular masses 43 and 10 kDa. It has been demonstrated that the location of hydrogenases in bacterial cells is representative of its function. According to Nicolet *et al* (2000), the *D. desulfuricans* hydrogenase's physiological role is hydrogen uptake. In *Desulfovibrio* sp investigated, protons resulting from the oxidation of molecular hydrogen by Fe – hydrogenase in the periplasm create a gradient across the membrane that is thought to be coupled to ATP synthesis in the cytoplasm. The resulting electrons generated are also transferred to the cytoplasm by a redox trans-membrane system where they are used in a stepwise manner to reduce sulphate to sulphide or generate reducing power for the cell (Nicolet *et al*., 2000).

Nickel-Fe hydrogenases have low specific activities and have been shown to function in the oxidation of hydrogen. They are found predominantly in all *Desulfovibrio* sp and a lot of research has been done on this type of enzyme from *D. gigas* (Albracht, 1994). They are periplasmic proteins consisting of two subunits of molecular masses 60 and 28 kDa. Other subunits may also be present which allow the hydrogenase to interact with dedicated electron carriers such as NAD^+, F_{420}, and cytochrome *b* (Albracht, 1994). This metallo-protein contains 12 Fe atoms, 12 acid labile sulphides and one Ni atom per molecule (De Lacey *et al*., 2000). The Fe- and sulphide atoms are arranged as $2[4Fe4S]^{2+/1+}$ clusters and $1[3Fe4S]^{1+/0}$ cluster. X-ray structure studies revealed that the active site is located in the large subunit near the interface with the small subunit. The active site consists of a binuclear NiFe centre and accessibility of hydrogen to the active site of this protein seems quite clear, as x-ray crystallographic and molecular dynamic studies have shown the existence of hydrophobic channels that connect the molecular surface of the enzyme with the active site. However there is still some great controversy on whether the splitting of the hydrogen occurs on the nickel or iron atom (De Lacey *et al*., 2000).

Nickel-iron-selenium hydrogenases occur less frequently as compared to the other two metallo-enzymes, although their existence has been demonstrated in some *Desulfovibrio* sp. NiFeSe hydrogenases differ from their NiFe counterparts by replacing one cysteine amino acid with a seleno-cysteine amino acid complex. They also contain equimolar amounts of nickel, iron and 4Fe - 4S centres. The location and functional physiology is similar to NiFe hydrogenases (Fauque *et al*., 1988).

1.9.5 Role of hydrogenase in azo dye reduction

Previous reports that there is no clear evidence for the existence of specific azoreductases in anaerobic bacteria have generated a lot of interest and have led into the investigation of the enzymes that are responsible for the non-specific azo dye reduction. Evidence to suggest the presence of non-specific enzymes that are responsible for the almost ubiquitous capacity to reduce azo dyes in many bacterial strains has been reported recently by Van der Zee (2002). Earlier research by Rafii and Cerniglia (1995) with purified cell free extracts from intestinal bacterial strains showed that a ‘flavoprotein’ was capable of catalysing the reduction of azo dye as well as other nitro-aromatics. These observations may indicate that enzymatic anaerobic azo dye reduction is more or less a fortuitous reaction catalysed by enzymes such as hydrogenases which are usually used for other reactions (Van der Zee, 2002). It is not known whether these hydrogenases are directly involved in azo bond cleavage or whether they facilitate the reduction of reduced equivalents (i.e. FAD → $FADH_2$). The latter seems to be more evident as some observations from studies with *Desulfovibrio vulgaris* has shown their ability to reduce artificially added quinones (Tatsumi *et al*., 2000). A mechanism for the involvement of hydrogenases in azo dye degradation has been proposed in SRB (Figure 1.8).

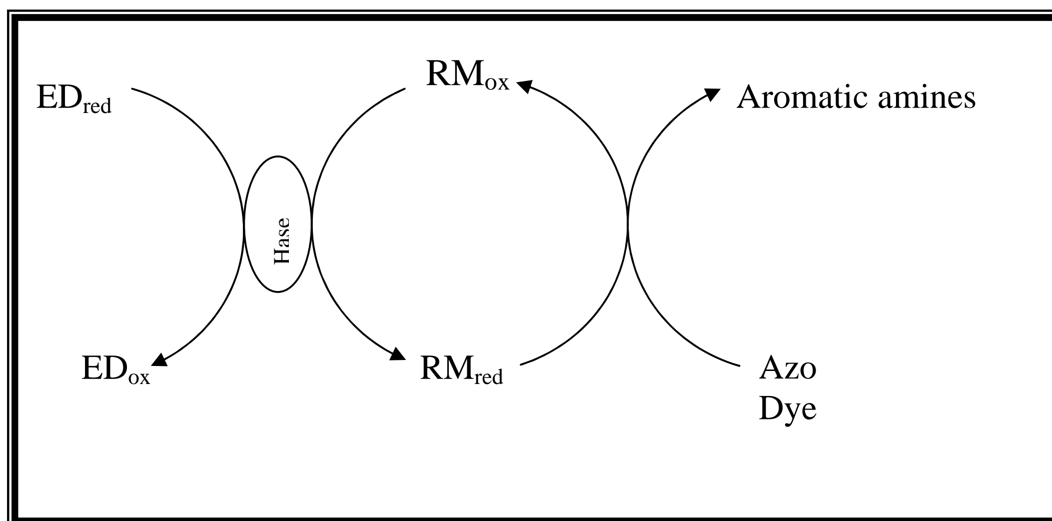


Figure 1.8 Proposed schematic representation of anaerobic azo degradation, mediated by hydrogenase reduced electron donors. RM = redox mediator; ED = electron donor; Hase = Hydrogenase enzyme (Modified version adapted from Van der Zee, 2002).

1.10 Current research study

1.10.1 Hypothesis

Enzymes produced within a biosulphidogenic bioreactor are capable of bioremediating textile industrial effluent.

1.10.2 Objectives

The aim of this study was to investigate the enzymes responsible for the anaerobic degradation of azo dyes and effluent. Biological treatment is fast becoming the most popular and efficient method of industrial effluent treatment. As a result there is need to seek cheaper alternative ways of bioremediating textile effluent. Sewage sludge, which is an effluent itself, has been reported to serve as a good carbon source for anaerobic bacteria including SRB and methanogens (Van der Zee, 2002).

While the treatment of textile effluent has been studied extensively, most research has been centred on fungal aerobic systems, which are quite difficult to upscale commercially due to the sensitivity of the culturing conditions (Stolz, 2001; Wesenberg *et al*., 2003). On the other hand SRB have been shown to be robust bacteria that can withstand harsh environmental conditions (Zehender, 1988), and because of these properties, it was decided to investigate their potential to produce enzymes that can bioremediate textile effluent while utilizing sewage sludge as a carbon source, thereby leading to an overall and effective treatment of the two waste waters. The following objectives were made up to guide the investigation:

a) to identify enzymes that can degrade textile dyes under biosulphidogenic conditions,
b) to isolate, purify and characterise the selected dye degrading enzymes,
c) to optimize enzyme activity,
d) to optimize enzyme production – by inducing the bacteria to express the target enzymes in the presence of substrate and,
e) to carry out field trials on industrial effluent from Da Gama Textiles.

2 PRODUCTION OF DYE DEGRADING ENZYMES BY SULPHATE REDUCING BACTERIA

2.1 Introduction

The ability of micro-organisms to degrade textile azo dyes has been studied extensively in both aerobic and anaerobic processes (Banat *et al*, 1996; Pearce *et al*, 2003). More and more recalcitrant dyes are manufactured with the hope of improving the delivery of colour onto fabric, at the expense of becoming increasingly difficult to bioremediate. This has created a need to investigate and understand the actual mechanisms behind the biodegradation of textile waste water. Several enzymes from fungi and bacteria have been identified and used in the breakdown of azo dyes.

White – rot fungi produce Lignin Modifying Enzymes (LME) namely; laccase, lignin and manganese peroxidase. These enzymes have been shown to oxidize coloured phenolic compounds and aromatic amines via a one step electron oxidation (Have *et al.*. 1997; Couto *et al*., 2004). The wood degrading white rot basidiomycete *Phanaerochaete chrysosporium* is the only one that has been shown to completely degrade a number of azo dyes (Chivukula and Renganathan, 1995). Lignin and manganese peroxidase produced by *P. chrysosporium* appear to initiate the azo degradation while the laccases oxidize aromatic compounds such as aniline and phenols in the presence of oxygen. The substrates are oxidized by one electron to generate corresponding phenoxy radicals, which either polymerize to yield a phenolic polymer or are further oxidized by laccase to produce a quinone (Bollag, 1992). The electrons generated from these reactions are transferred onto oxygen which is reduced to water. Although several white rot fungi have shown great potential to degrade azo dyes, they are difficult to apply in the actual bioremediation of textile waste water. The major disadvantage is that they can only be

used in lab scale operations where they are cultured in shaking flasks. Their ability to adapt and survive in a sewage waste water treatment plant is minimal.

Earlier research in this field identified bacterial species that are capable of reducing and degrading azo dyes include *Bacillus, Xanthomonas,* and *Pseudomonas* (Zimmermann *et al*., 1982; Sugiuira *et al*., 1999). Azoreductase enzymes produced by *Pseudomonas* sp catalyze reductive cleavage of azo dyes in the presence of molecular oxygen (Nakanishi *et al*., 2001), thus making them favourable for the development of azo dye bioremediation systems. These enzymes have high substrate affinity but can only degrade a small fraction of the vast range of azo dyes that are manufactured annually. They are therefore, not readily applicable to industrial treatment of textile effluent.

Research on bacterial azo dye reduction has traditionally been focused on the activity of facultative anaerobic bacteria from mammalian intestines. As the removal of dyes from waste water became a cause for concern, bacteria from other origins which included pure cultures, mixed consortia, anaerobic sediments, digester sludge, anaerobic granular sludge and activated sludge were investigated (Razo Flores *et al*., 1997; Van der Zee, 2002). Since a large proportion of azo dyes can be reduced by different bacteria under anaerobic conditions, this indicates that azo dye reduction is a non specific reaction. There are two main enzymatic mechanisms that are thought to be involved in the anaerobic reduction of azo dyes, namely: direct enzymatic azo dye reduction and indirect azo dye reduction.

2.1.1 Direct enzymatic azo reduction

In this mechanism, enzymes transfer reducing equivalents generated from the oxidation of organic substrates (e.g. lactate) to the azo dyes (Figure 2.1). Enzymes that catalyze azo dye reduction may either be specific or non-specific enzymes to catalyze a wide range of compounds including azo dyes (Van Der Zee, 2002). The latter seems more likely as the existence of specific azo reducing enzymes (azoreductases) have only been found in studies with aerobic and facultative aerobic bacteria grown on simple azo compounds as the sole source of carbon and energy. On the other hand, non specific enzymes catalyzing

azo dye reduction which were isolated from aerobically grown cultures of *Shigella dysenteriae, Escherichia coli* and *Bacillus* were found to be flavin-proteins (Ghosh *et al*., 1992, 1993; Suzuki *et al*., 2001).

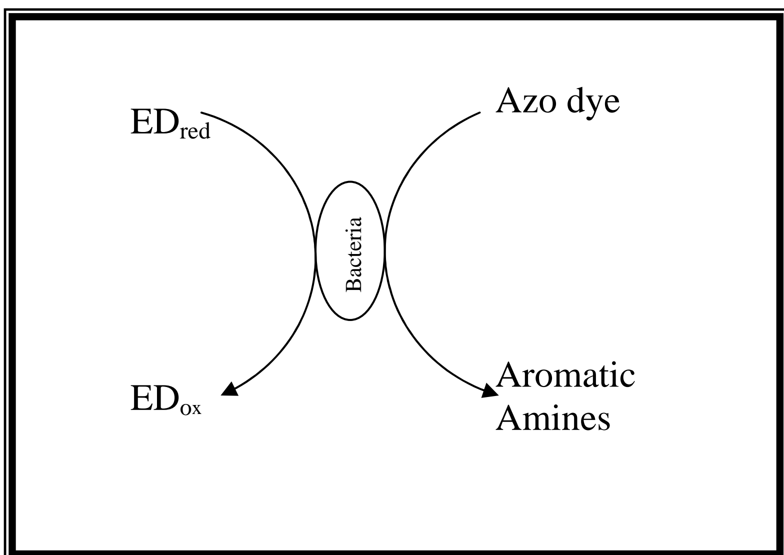


Figure 2.1 Mechanism of direct enzymatic azo dye degradation (Adapted from Van der Zee, 2002)

2.1.2 Indirect biological azo dye reduction

The second mechanism of azo dye reduction involves mediated reduction of the double azo bond by enzymatically reduced electron carriers which include FADH2, FMNH2, NADH2, or NADPH2 (Section 1.9.5, Chapter 1, Figure 1.8) Previous studies have demonstrated that a periplasmic hydrogenase enzyme from *Desulfovibrio vulgaris* can reduce several quinone compounds as exogenous electron acceptors coupled to hydrogen oxidation (Tatsumi, *et al*., 2000; Ngwenya, 2004).

2.1.3 Localisation of hydrogenase enzymes

The cellular localisation of bacterial enzymes is directly related to its function (Nicolet *et al*., 2000). Fauque and co workers (1988) suggested that the localisation of hydrogenases in *Desulfovibrio* species is dependent on the particular growth conditions, which in turn determines the mechanism of metabolic regulation. Studies with *Desulfovibrio vulgaris*

Hildenborough have demonstrated that, during hydrogen metabolism: the protons and electrons produced in lactate and pyruvate oxidation react with cytoplasmic hydrogenase to form hydrogen which diffuses across the membrane where it is re-oxidized by periplasmic hydrogenases to form a proton gradient. This proton gradient is further used for additional ATP synthesis (Heidelberg *et al.*, 2004). Therefore presence of distinct hydrogenases, with different intracellular localisations implies that appropriate cell lysis techniques should be applied to release most of the activity into the aqueous extract (Walker, 2000).

2.1.4 Cell disruption and extraction

Several standard procedures for lysis of bacterial cells are available (Rotman, 1956; Bollag, 1992; Borthwick *et al.,* 2005), but it is crucial to make use of the most compatible and efficient method of cell disruption so as to harvest maximum activity of target protein. Common methods of cell disruption used are summarized (Table 2.1). Previous studies on hydrogenase enzymes in sulphate reducing bacteria have shown that maximum enzyme yield can be achieved by using sonication. Cell disruption methods such as French press and cell lysis with detergents resulted in loss of activity between 20 – 30 % relative activity (Rashamuse, 2003; Ngwenya, 2004). Therefore, enzymes were harvested using the sonication method throughout this study. Despite previous workers (Rashamuse, 2003; Ngwenya, 2004) from our research group having established that sonication was the preferred method for cell disruption of SRB, it was still necessary to optimise the conditions of this method, which had not been done before. A surface response methodology of determining the optimal amplitude and exposure time was used in this study.

2.1.5 Principles of the surface response method

Surface response methodology allows the comparison of more than two variables that affect the optimal performance of enzymes or chemical reactions. The method selects the best points from three dimensional maps that will provide the best operating conditions based on a series of experiments with the variable parameters. A two dimensional contour map can thus be drawn from the surface response map to provide a detailed account of

the methodology (Box and Wilson, 1951). This technique was used in the current study to investigate the best conditions of harvesting target enzymes for down stream application. The variables compared were sonication time, power (watts) versus the enzyme activity.

Table 2.1 Cell disruption methods for various tissues (adapted from Bollag, 1992)

Disruption type	Cell Lysis Technique	Type of Tissue
Physical/ Mechanical	Sonication	Bacteria, yeast, animal tissue
	French Pressure Cell	Bacteria, Yeast, plant cells
	Blade Homogenization	Animal and Plant Tissue
	Grinding	Bacteria, plant tissues
	Freeze –Thaw Lysis	Cell suspensions
	Osmotic shock	Erythrocytes, bacteria
Chemical	Detergent Lysis (Triton-X-100)	Tissue culture cells
	Organic Solvent Lysis	Bacteria, yeast
Biological	Enzyme Digestion (lysozymes)	Bacteria, yeast

2.2 Methods and materials

2.2.1 Source of micro-organism and culturing conditions

A mixed consortium of sulphate reducing bacteria previously isolated from an acid mine drainage (Grootvlei mine, South Africa) and from the Environmental Biotechnological Research Unit (EBRU) Bio-sure® process (Grahamstown, South Africa) was used. The culture was maintained in a 10 L stock batch bioreactor, containing modified Postgate medium B (Postgate, 1984). The bioreactor was kept anaerobic by purging regularly with N_2: CO_2 (80:20 %) (Afrox, South Africa) for 15 minutes followed by H_2 (99 %), (Afrox) gas for 5 minutes at room temperature. The bioreactor was covered with aluminium foil to minimise exposure to light and was sub-cultured every 3 weeks with new Postgate medium. A 15 % (v/v), (750 ml) inoculum was taken from this stock bioreactor and used as a starter for a smaller 5L bioreactor (Figure 2.2) which was used for all experiments carried out during the research. The metabolism of SRB as a measure of growth was monitored by measuring pH, sulphide, sulphate and COD concentrations. Sulphate and sulphide concentrations were determined using Spectroquant® Nova 60 (Merck, South Africa) while COD was measured using a Thermospectronic Aquamate (Aquamate,

England). Protein concentration was determined by the Bradford assay (Bradford, 1976) (Appendix B), and protein absorbance was measured using a PowerWave X (Bio-Tek, Instrumental inc., USA). The Level 1 Inolab pH meter (WTW Ltd) was used for pH measurement.

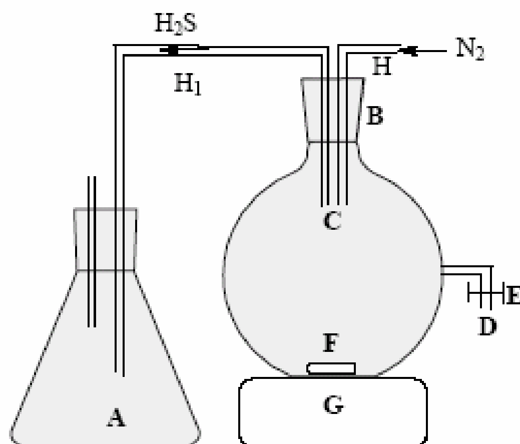


Figure 2.2 A diagrammatic representation of the laboratory-scale biosulphidogenic reactor. [A = Zinc acetate trap; B = rubber stopper; C = 5L flat bottom flask reactor vessel; D = sampling port; E = Metal clamp; F/G = magnetic stirrer; H/H1 = Tygon tubing.

2.2.2 Harvesting cells and preparation of cell free extracts

SRB was harvested during the mid-stationary stage (6 – 8 days). The cells were centrifuged (7000 rpm, JA-10 rotor, 15 minutes, 4ºC). Bacterial cell disruption was achieved using a Vibra-cell ™ Sonicator (Sonic & Materials, Inc, USA) at 4ºC. The cell free extract was separated from the cell debris by centrifuging (10 000 rpm, JA-20 rotor, 25 minutes, 4ºC).

2.2.3 Dye degrading ability of sulphate reducing bacteria

Dye degrading ability was determined using a protocol adapted from Rafii *et al* (1990). One millilitre of sulphate reducing bacteria was diluted in nine millilitre of de-ionized water (dH_2O). Tenfold serial dilutions of the SRB were made and 0.1 ml of 10^{-5} to 10^{-9} dilutions were plated on nutrient agar plates containing Orange II azo dye (final concentration, 100 mg/l). The nutrient agar was enriched with 2 % (w/v) glucose as a carbon source. The agar plates were sealed with para-film, placed in an anaerobic jar which was degassed with oxygen free nitrogen gas for 15 minutes and placed in an incubator (30 °C, 48h). Plates showing zones of decolourisation and clearing were taken to possess dye degrading enzymes. Cultures exhibiting dye degrading ability were scaled up and inoculated in modified Postgate medium as described in section 2.2.1, Chapter 2.

2.2.4 Enzyme screening assays

2.2.4.1 Laccase assay

Laccase enzymes have been widely shown to catalyze the degradation of azo dyes through a one step electron oxidation using molecular oxygen as a terminal electron acceptor (Abdullah *et al*., 2000; Stolz, 2001). In light of this, it became necessary to investigate the potential of terminal electron acceptor to replace the oxygen under anaerobic conditions.

Laccase activity was determined using a modified protocol from Zarvazina *et al* (2004), which used 2, 2´- azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) as a substrate. The reaction mixture contained ABTS (2 ml) dissolved in citrate buffer (50 mM, pH 6.0). The reaction was started by adding 1 millilitre (ml) of sample and was monitored at 30 °C for 3 minutes. The change in absorbance was monitored spectrophotometrically at 436 nm. One unit of activity was regarded as the amount of enzyme capable of converting 1 µmole ABTS per min per ml.

2.2.4.2 Lignin peroxidase assay

Lignin peroxidase activity was measured by recording the increase in absorbance at 310 nm, at 30 °C due to the oxidation of veratryl alcohol (VA) to veratraldehyde (VAD) (Have *et al*., 1997). The reaction mixture contained in 2 ml, 1 ml of sample and 1 ml of VA (2 mM) dissolved in 50 mM citrate buffer, pH 3.0. The reaction was started by adding 100 µl of 0.5 mM H_2O_2, and monitored over 10 minutes. One unit of activity was regarded as the amount of enzyme capable of converting 1 µmole VA per min per ml.

2.2.4.3 Manganese peroxidase assay

Manganese peroxidase was determined spectrophotometrically at 270 nm by monitoring the oxidation of $MnSO_4$ (1 mM) in 50 mM sodium malonate buffer, pH 4.5. The reaction was started by addition of 0.1 mM H_2O_2 (Wariishi *et al*., 1992; Tychanowicz *et al*., 2004). The mechanism of this reaction is based on the oxidation of Mn^{2+} to Mn^{3+} which forms a complex with malonate that absorbs maximally at 270 nm (ε = 11.59 $mM^{-1}cm^{-1}$). Enzymatic activity was expressed as international units (U), which is defined as the amount of enzyme required to produce 1 micromole product per min.

2.2.4.4 Azoreductase assay

The assay was carried out using a modified protocol by Maier *et al* (2004), and was carried out in a four millilitre cuvette. All reagents used were dissolved in 50 mM phosphate buffer, pH 7.0 which has been boiled, cooled, and degassed with oxygen free nitrogen for five minutes. The reaction mixture contained in 3 ml;1.2 ml of phosphate buffer; 0.6 ml of Reactive Black 5 (final dye concentration 16.3 µM) and 0.6 ml of sample. The reaction was initiated by the addition of 0.6 ml of $NADH_2$ (7.1 mgl^{-1}; final concentration 2 mM) and the decrease in absorbance was monitored spectrophotometrically at 595 nm for five minutes. The reaction was kept anaerobic by continuous purging with O_2 free N_2. The slope of the initial linear decrease in absorbance (Δmin^{-1}) was used to calculate azoreductase activity based on the molar absorption coefficient of reactive black 5 (ε = 35.5 $mM^{-1}cm^{-1}$). One unit of activity was defined as the reduction of 1 nanomol of dye per second.

Dye decolourization in the presence of FAD was measured in a similar fashion by addition of 1.0 mM of FAD solution to 50 mM of phosphate buffer, pH 7.0 to give a final concentration of 0.2 mM in the assay.

2.2.4.5 Hydrogenase assay

Hydrogenase activity was measured using a protocol described by (De Lacy *et al* 2000; and Rashamuse, 2003). The assay was determined spectrophotometrically at 604 nm, at room temperature by the reduction of methyl viologen ($\varepsilon_{604\ nm}$ = 13.9 $mM^{-1}cm^{-1}$; electron acceptor) with H_2 gas (electron donor) in the presence of Tris-HCl buffer (7.6, 50 mM). The assay was carried out in a stoppered cuvette, and contained in a final volume 3.105 ml; 3 ml (1 mM) methyl viologen which was pre-activated by bubbling with H_2 gas for 15 minutes at 1 atm 30 $ml^{-1}min^{-1}$, sodium dithionite (100 mM; 5 µl) which was added into the cuvette to eliminate residual O_2 and the reaction was started by addition of 100 µl of pre-activated cell free extract. The kinetics of methyl viologen reduction was monitored at 604 nm. One unit of activity was defined as the amount of hydrogenase that catalyzes the reduction of 1 µmol of methyl viologen per min per ml in the presence of excess hydrogen.

2.2.5 Enzyme localisation

SRB cells (5.0 grams wet weight) were harvested as described in section 2.2.2, Chapter 2. The pellet and supernatant were separated and designated P1 and S1 respectively and were assayed for protein and hydrogenase activity. The pellet (P1) was then re-suspended in Tris-HCl buffer (pH 7.6, 50 mM, 100 ml) and then disrupted by sonication at 30 second intervals for 4 minutes at 4 ºC. Cell debris designated (P2) and cell free extract designated (S2) were obtained by centrifugation (10 000 rpm, JA -20 rotor, Beckman, USA, 25 minutes, 4 ºC) Both P2 and S2 were once again assayed for protein concentration and enzyme activity.

2.2.6 Optimization of enzyme extraction

SRB cells were harvested as described in section 2.2.2, Chapter 2 to yield a pellet (9.5 grams wet weight) supernatant. The pellet was washed with ddH_2O and then re-suspended in Tris-HCl buffer (pH 7.6, 50 mM, 200 ml). The cells were divided into 5 separate samples of equal volume and subjected to sonication 30W amplitude for different times ranging from 1 – 5 minutes. Cell free extracts were obtained by centrifugation (10000 rpm, JA-20 rotor, Beckman, USA, 25 minutes, 4 ºC). All sonicated samples were tested for protein concentration and hydrogenase activity.

The re-suspended cells were subjected to a sonication time of 5 minutes with varying power amplitude ranging from 10 – 50W. The sonicated samples were centrifuged and cell free extracts harvested as previously described in section 2.2.2, Chapter 2. The cell free extracts were assayed for protein concentration and hydrogenase activity.

2.3 Results and Discussion

2.3.1 Growth of sulphate bacteria

The growth of SRB in a batch bioreactor was monitored over a period of 15 days. The rate of sulphate utilization and concomitant sulphide production was used to measure the metabolic activity of the actively respiring bacteria. There was a rapid rate of sulphate reduction with an efficiency of 90 % at a conversion rate of 194 mgl^{-1} a day for the first nine days (Figure 2.3), and decelerated to 29 mgl^{-1} a day from day 10 to 15. The decrease in the rate of conversion could be due to depleting carbon source (lactate); as a result, sulphate was no longer required as a terminal electron acceptor. Another reason could be that the bacteria starts to generate energy for maintenance instead, when they enter the stationary phase of growth thereby, uncoupling sulphate reduction. Moreover, the sulphate concentration becomes limiting after 10 days.

The initial rate of sulphide production was slow during the first four days corresponding to the lag phase of SRB which can be between 0 – 70h (Mohanty *et al*., 2000). This was followed by an accelerated rate with the highest concentration being produced after nine

days (354 mgl^{-1}). This also relates to the log phase of SRB which compares well to SRB isolated from Aska sugar mill effluent that showed a log phase of up to 25 days (although the sulphate levels were much higher; 1.3 gl^{-1}) by Mohanty and co-workers (2000). The sulphide concentration decreased after day nine. This was probably due to speciation of sulphide which is strongly dependent on pH (Rashamuse, 2003). The chemical equilibrium of the three sulphide species is represented below (Equation 4):

$$H_2S \longleftrightarrow HS^- + H^- \longleftrightarrow S^{2-} + 2H^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

At pH 8.0 and greater most of the sulphide is dissolved in the aqueous medium, while at pH values below 7.0 most of the sulphide is in the gaseous form ($H_2S_{(g)}$) (Okabe *et al.,* 1995). This can be related to the decrease in the detection of sulphide levels after day 10 when the pH also starts to decrease (Figure 2.4). Therefore, though the sulphide may still be present it cannot be detected using the standard sulphide test. Van Hille (2001) demonstrated with the MinteqA2 software package that at pH 7.0, 56 % of the sulphide exits as HS^- and 44 % as $H_2S_{(g)}$. It has been shown that at certain concentrations the sulphide becomes toxic to the cells and may also attribute to the decrease in sulphide production (Mohanty *et al., 2000*).

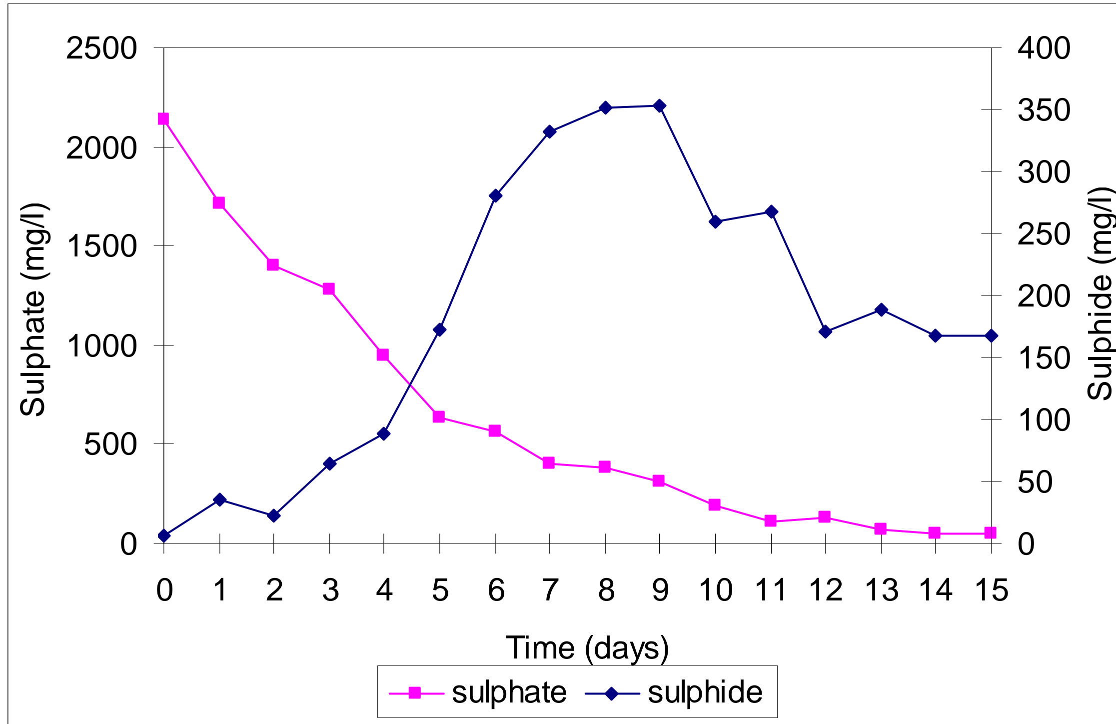


Figure 2.3 Characteristic sulphate reduction and sulphide generation in actively growing sulphate reducing bacteria.

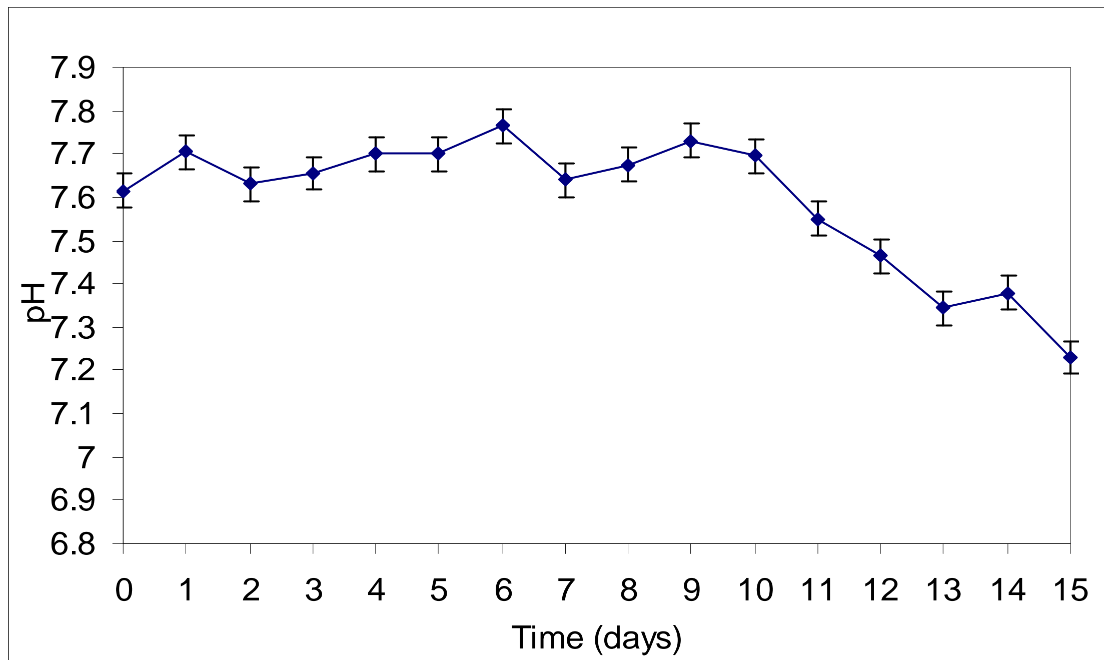


Figure 2.4 pH profile of SRB bioreactor during growth.

The pH is stable during the first 10 days and declines thereafter (Figure 2.4). The optimal pH for SRB growth is in the range 7.5 – 8.0 (Zehender, 1988.). The decrease in pH could be due to the production of volatile fatty acids (e.g. acetic acid, propionic, and butyric acid) that are produced during lactate oxidation (Elefsiniotis and Oldham, 1994). In addition, as the SRB enter the autolysis stage, there is generally a decrease in pH.

2.3.2 Dye degrading ability of SRB

Following a 48 h incubation period in nutrient agar containing orange II under anaerobic conditions, colonies of sulphate reducing bacteria were observed. Colourless zones around the colonies indicated that these bacteria had reduced the azo dye Orange II (Figure 2.5). The first catabolic step in the reduction of azo dyes which is accompanied by a decrease in the visible light absorbance of the dye and then decolourization of the dye is the reduction of the (-N=N-) azo bridge (Rafii *et al*., 1990).

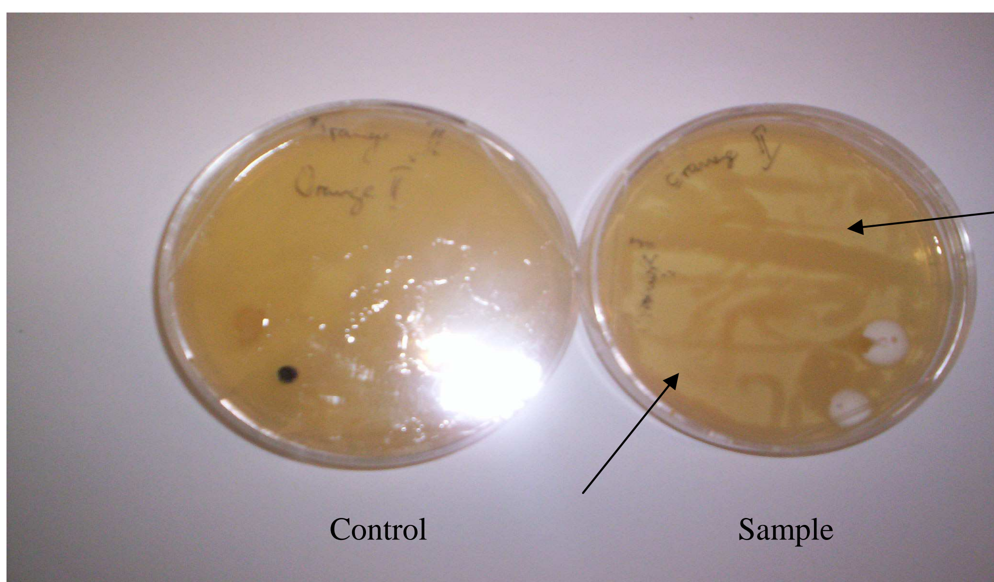


Figure 2.5 Illustration of the ability of SRB to decolourize the azo dye Orange II under anaerobic conditions. In the control, there was no decolourization as there was no SRB to reduce the azo bond of Orange II. (Arrows indicate zones of clearing).

2.3.3 Screening for dye degrading enzymes.

Once the ability of SRB to grow in the presence of an azo dye had been demonstrated, the next step was to identify the enzymes responsible for the reductive cleavage of the azo bond. Lignin modifying enzymes (laccase, lignin and manganese peroxidase) were

investigated in this experiment with the aim of finding a substitute enzyme that would operate under anaerobic conditions, maybe in the form of sulphate which serves as a terminal electron acceptor in SRB metabolism. Unfortunately, but expectedly the activities for the laccase and the peroxidases were 0.026 and 0.047 Umg^{-1} respectively which were significantly lower than that for hydrogenase (Figure 2.6). This led to the conclusion that the enzymes that are normally produced by white-rot fungi are not usually produced in SRB. It must be highlighted that an interesting result was observed with the laccase assay, which uses ABTS as a substrate. This substrate is an azo compound, and the "normal" oxidation of ABTS in the presence of laccase results in the development of a darker green colour. In our case, however, there was a rapid decolourization of the green colour with the cell free extract from SRB. This decolourisation points to further evidence of a hydrogenase or 'azoreductase' present in the cell free extract, which catalyses the reductive cleavage of this azo bond molecule under the aerobic conditions of the assay.

Azoreductase assays resulted in a specific activity of 0.082 Umg^{-1} which was also low in comparison with the hydrogenase activity. These enzymes have been successfully isolated and purified under aerobic conditions using *Pseudomonas* strains K22 and KF46 (Zimmermann *et al*., 1982, 1983). It was demonstrated by Zimmermann and co-workers (1982; 1983) that the enzymes orange I azoreductase and orange II azoreductase were highly specific and required molecular oxygen as terminal electron acceptor for the reduction of the respective azo dye. Under anaerobic conditions, it has been reported that azoreductases require reducing equivalents to provide the electrons for the reductive cleavage of the dyes which act as the terminal electron acceptors. In our current experiment, NADPH was used as a redox mediator since previous work indicated that azoreductase enzymes strictly require NADPH to provide the hydrogen and electrons required for reductive cleavage of the azo bond (Zimmermann *et al*, 1982; 1983; Stolz, 2001*)*. Attempts to use other redox mediators like NADH and FADH did not yield any significant azoreductase activity. This led to the conclusion that the azoreductases were either not produced by SRB or if they were, the redox potentials (E_0') of the mediators used in this experiment were well outside the range acceptable for suitable coupling. Unfortunately, reliable E_0' values for the azo dyes could not be found. On the other hand,

the $E_0´$ values for the redox mediators used in this study were -440,-324, -320 and -219 mV for methyl viologen, NADP+, NAD+ and FAD+ respectively (Faro *et al*., 2002; Hanson *et al*., 2004). The redox potential ($E_0´$) of a redox mediator (i.e. the $E_0´$ of the couple oxidized/reduced redox mediator) for azo reduction should lie in between the $E_0´$ of the primary electron donor and the $E_0´$ of the azo dye i.e. ~100mV (Haderlein, 1995).

Significant amount of hydrogenase activity relative to the other enzymes investigated was observed (Figure 2.6). The occurrence and production of hydrogenases have been extensively studied in *Desulfovibrio* sp (Brugna *et al*., 2000; Zadvorny *et al*., 2004). Ni-Fe hydrogenase occurs universally in most bacteria, while Fe-only and non-metal containing hydrogenase depends on carbon source and culturing conditions (Nicolet *et al*., 2000). The enzyme activity observed in this experiment co-relates with the activity of Ni-Fe hydrogenases because of the low specific activities and low substrate affinities (Sapra *et al*., 2000). This characteristic is applicable to the anaerobic non-specific reduction of azo dyes. Therefore, further studies on the decolourization of azo dyes in SRB were based on the hydrogenase enzyme.

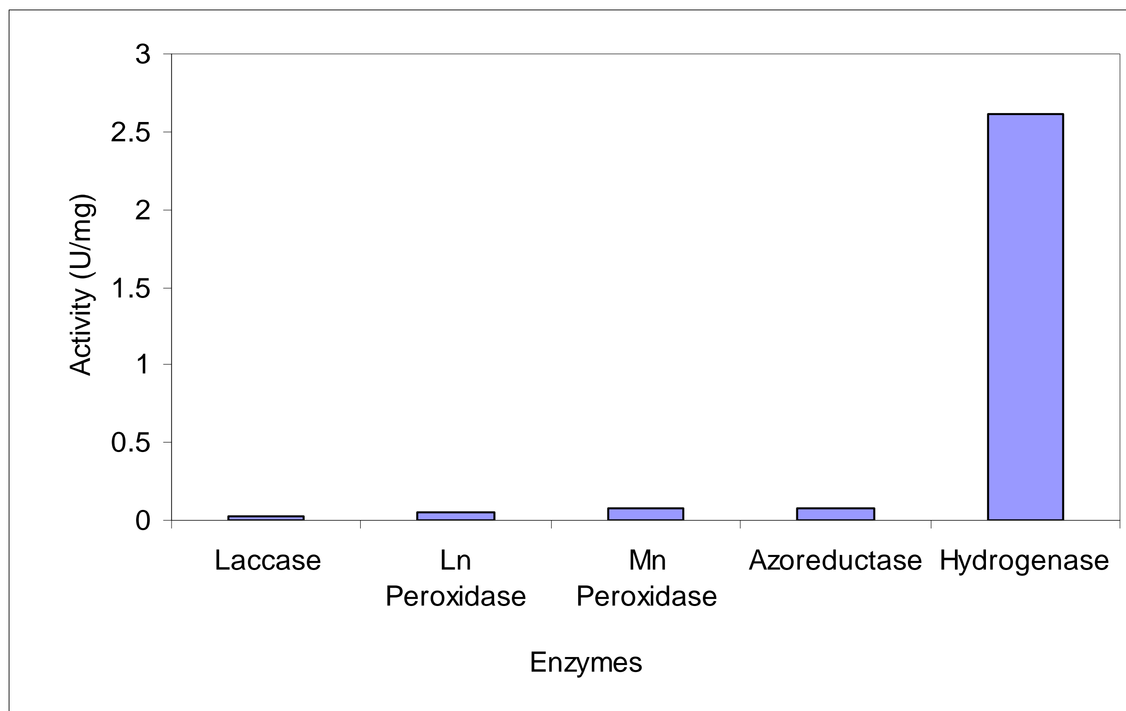


Figure 2.6 Identification of enzymes (that can degrade azo dyes) produced in sulphate reducing bacteria. The enzymes were quantified from the supernatant after sonication.

2.3.4 Localisation of hydrogenase enzyme

Enzyme activity was expressed as a relative percentage in order to allow comparison of the hydrogenase activity of the different cell fractions. The intact pellet fraction was considered as 100% because it represented the activity of the cell in its natural state. The disruption of the cells by sonication resulted in approximately 3-fold increase in hydrogenase activity of the cell free extract (Figure 2.7). This indicates that there was release of membrane-bound (MB) hydrogenase that did not have access to the substrate when the cell was intact, and this together with the periplasmic hydrogenases gave the synergistic activity. The presence and function of MB hydrogenases was demonstrated by Sapra *et al* (2000) who showed that Ni-Fe hydrogenases could function both as cytoplasmic, NAD-reducing enzymes and as part of the conventional MB respiratory chains in ATP synthesis. This was also supported by Heidelberg *et al* (2004) who showed the presence of MB Ni-Fe hydrogenase that took part in ATP generation by electron transfer-coupled phosphorylation. Earlier studies by Jones and Garland (1977)

demonstrated the relative impermeability of the cytoplasmic membrane to charged, non-physiological molecules such as oxidized methyl viologen (MV) and benzyl viologen (BV). They reported that only reduced BV crossed the cytoplasmic membranes of *Escherichia coli* rapidly compared with the diffusion of reduced and oxidized MV which were slow to cross the membrane. This explains the lower activity before cell disruption, in which the cytoplasmic membrane is the rate limiting barrier between enzyme and substrate.

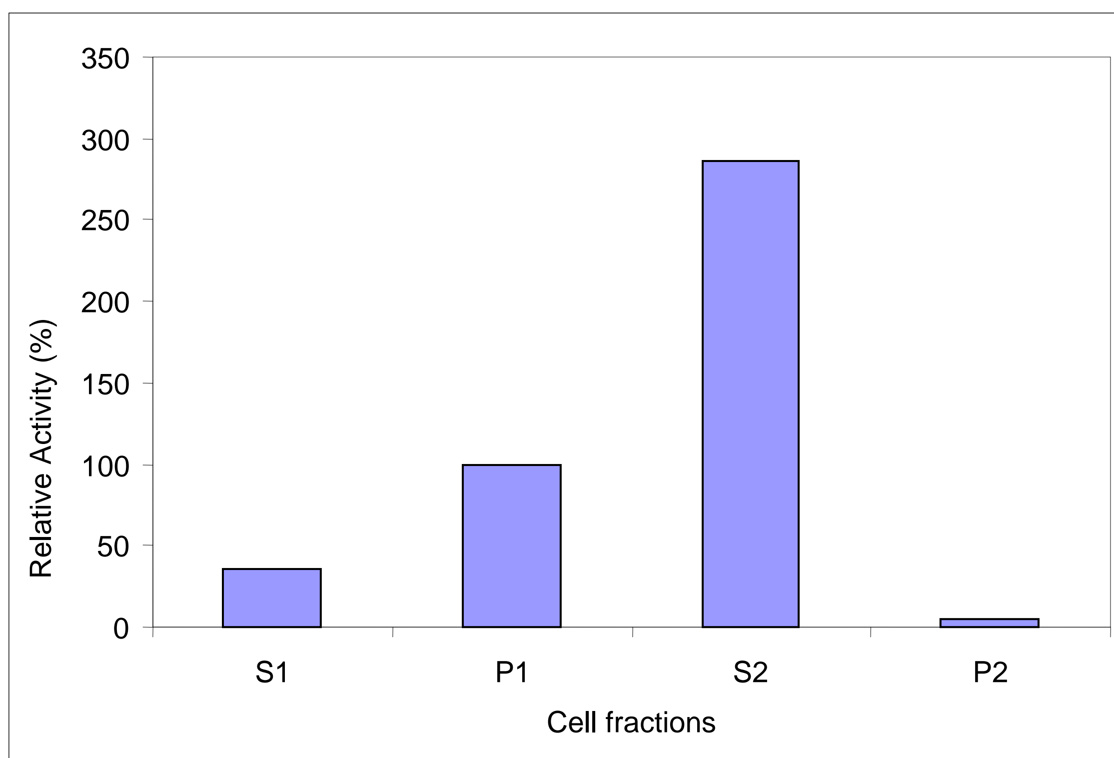


Figure 2.7 Relative activity (%) of hydrogenase enzyme in different cell fractions before and after cell disruption by sonication S1=Supernatant before sonication; P1= Control (intact pellet before sonication); S2= supernatant after sonication; P2= pellet after sonication.

2.3.5 Extraction optimization by sonication

A surface response methodology was used to determine optimum conditions that yielded the highest hydrogenase activity. From the 3-dimensional surface response diagram (not shown), a 2-dimensional cross-section known as a contour map was constructed using the various sonication times and amplitude (Figure 2.8). The best sonication conditions were an amplitude of 10W for 4 minutes ± 10seconds that resulted in a significant increase in

hydrogenase activity after optimization, in comparison with activity obtained in section 2.3.3. Rotman (1956), in his studies on the mechanism of sonic lysis of bacteria, showed that cell disruption occurs in two steps: damage to the structural integrity of the cell followed by the lysis of the cell components to expose the integral components. According to Rotman, the second stage of lysis is an enzymatic process, which was demonstrated by lowering the pH below 6.0; separate heat treatment and inhibiting cell with tetra-ethyl-pyrophosphate (TEPP); there was no lysis of the bacterial cell. This illustrates the importance of other factors such as pH and temperature during sonication. In this experiment, sonication was done at 4ºC and the cells were suspended in Tris-HCl buffer (50 mM, pH 7.6) which has been extensively used in the study of hydrogenase enzymes.

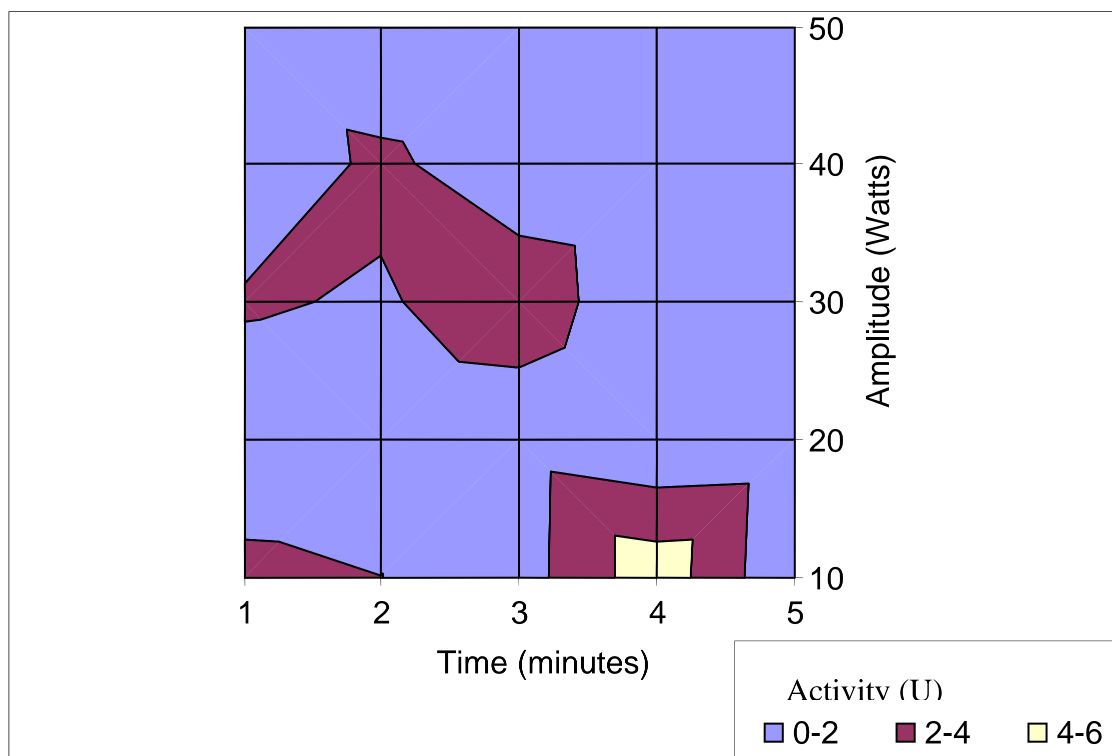


Figure 2.8 Contour map showing the best sonication conditions that yield the highest hydrogenase activity.

2.4 Conclusions

The conclusions that can be drawn from this chapter are:

a) sulphate reducing bacteria are capable of decolourizing azo dyes in agar under anaerobic conditions,
b) laccase, peroxidases and azo reductase are not present in SRB, despite reports to the contrary (Stolz, 2001),
c) hydrogenase enzyme, predominant in SRB, is likely to be the enzyme responsible for degradation of textile dyes,
d) the hydrogenase of interest is located in the cytoplasm and membrane of the SRB, as activity is enhanced after cell integrity is disrupted and,
e) hydrogenase can be optimally extracted by sonicating at 10W for 4 minutes at 30 second intervals.

These findings led to the investigation of the optimal time at which maximum hydrogenase is produced by SRB, and investigating the effect of increasing the enzyme activity through substrate induction. Since the major thrust of this project was to apply the enzymes commercially, the effect of carbon source on enzyme production and degradation of textile dyes was questioned, leading to the experiments conducted in Chapter Three.

3 PRODUCTION AND INDUCTION OF DYE DEGRADING ENZYMES

3.1 Introduction

3.1.1 The role of bacteria in effluent treatment processes

Enzymes produced by bacteria are responsible for the many different biochemical reactions that result in degradation of organic matter. When bacteria are grown in a batch culture, they normally exhibit a typical bacterial growth curve which is characterised by four phases (Figure3.1):

a) Lag phase – cells initially adjust to the new medium after inoculation. At this stage there is no apparent cell division occurring, although cells will be growing in mass due to the synthesis of proteins, RNA, and increasing metabolic activity. It is important to note that the length of the lag phase is dependent on several factors such as bacterial strain, culturing conditions (i.e. temperature, pH and atmospheric pressure), and adaptation of cells to environmental shock after transfer to new medium. The lag phase may take up to 200 hr as demonstrated by Mohanty *et al* (2000) when they cultured sulphate reducing bacteria isolated from a sugar mill effluent.
b) Exponential (log) phase – is characterised by rapid growth, wherein all cells are dividing regularly by binary fission and growth is increasing by geometric progression. Growth rate is dependent on composition of growth medium and incubation conditions. As a result of these conditions and those mentioned in (1), the log phase may vary from 0.16 – 600 hr (Mohanty *et al*., 2000).
c) Stationary phase – as in most batch reactors, cell growth is not continuous. Population growth gradually ceases due to any one of three factors (in any order);
d) Exhaustion of nutrients – accumulation of inhibitory metabolites and exhaustion of space. This stage is also characterised by the production of secondary

metabolites such as antibodies by some bacterial strains. Enzyme production may also occur at this stage depending on the available substrates (Kiran *et al*., 2004). Spore forming bacteria also express the respective genes that result in the sporulation process.

e) Autolysis (death) phase – cells start losing their viability and begin to disintegrate as lysozymes are released into the cytoplasm. Generally, there is geometric decline in cell population.

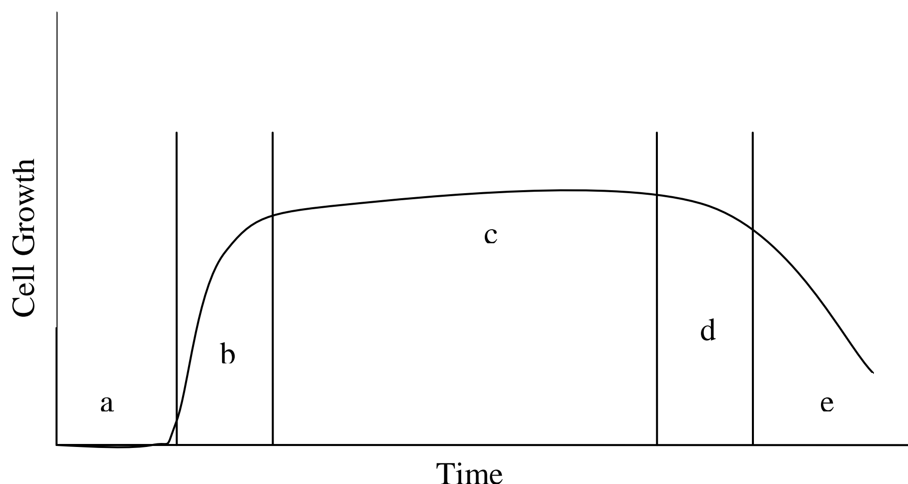


Figure 3.1 Diagrammatic representation of a typical bacterial growth curve. a; lag phase, b; log phase, c; stationary phase, d; exhaustion of nutrients e; autolysis phase.

3.1.2 Production of enzymes by bacteria

As bacteria divide, grow and metabolize, they produce high molecular weight proteins often referred to as enzymes that facilitate the transformation of biochemical substrates. Colonies of bacteria literally serve as factories for enzyme production, although it must be noted that the enzymes can only be produced by actively growing cells grown under specialized conditions so as to harvest the highest percentage of the target catalyst. While humans and other higher animals have a complex digestive micro-flora, bacteria have developed sophisticated mechanisms for the regulation of both catabolic and anabolic pathways (Todar, 2004). Bacteria will not synthesize degradative (catabolic) enzymes unless the substrates for these enzymes are present in the environment, e.g. in the absence of lactose in the media, bacteria will not synthesize lactase enzymes as it is not needed,

however once the substrate becomes available, bacteria will activate the genes for expression of lactase. Similarly, bacteria have developed diverse mechanism for the control of biosynthetic (anabolic) processes. For example if the amino acid tryptophan is readily available in the environment, the bacterium will shut down its own pathway of tryptophan biosynthesis thereby conserving energy (Todar, 2004). In an uncontrolled environment, however, the regulation and control of these metabolic pathways must be reversible since the environment is always changing (Babu and Aravind, 2005).

Wortmann was probably one of the first biochemists to describe the mechanism by which living cells can adapt themselves to the utilization of certain foodstuffs (Wortmann, 1882; Dubos, 1940). In his studies on unidentified bacteria, he observed that the cells produced amylase when grown in media containing starch, whereas the enzyme was not present when the same cells where grown in the absence of starch. In related studies, he also found that yeast cells always produced invertase when grown in the presence and absence of sucrose. Since then, the influence of the composition of the culture medium on enzymatic activity of microbial cultures became the centre of investigation and it was discovered that the production of an enzyme might be greatly stimulated when the substrate is a constituent of the culture medium (Schoenhard and Stafseth, 1953).

Enzymes produced in direct response to the availability of a homologous substrate in the culture medium are called inducible or adaptive enzymes (Dubos, 1940; Todar, 2004). They are only produced as and when they are needed. In the process of induction, the substrate or compound that is structurally similar to the substrate evokes formation of the enzymes and it is often referred to as an inducer (Todar, 2004). On the other hand, enzymes that are always produced by the cells regardless of the composition of the culture medium like those that operate during glycolysis and TCA cycle are generally constitutive and are present in more or less the same concentration in cells at all times.

Enzymatic activity in intact cells is affected by several factors such as age of cell culture, cellular permeability, substrate concentration and product concentration. Therefore it becomes difficult to determine quantitatively, the concentration of a given enzyme when

intact cells are used as an enzyme source. The only effective way of measuring the activity is to disrupt cell integrity and remove conditions where cellular permeability can vitiate the interpretation of results (Borthwick *et al.*, 2005). Based on this, it can be deduced that an increase in enzyme activity does not necessarily mean increased production of enzymes. However, it remains possible that in other cases an increase in enzymatic activity or the appearance of a new enzymatic function may not be the result of production of a new enzyme but rather, mechanisms which result in the activation of enzyme systems already present (Dubos, 1942; Babu and Aravind, 2005). Moreover, it is unlikely in most; if not all cases, that inducible enzymes may take the form of an increased production of the specific enzyme, rather than in the formation of a new enzyme.

While it has been suggested that the formation of inducible enzymes is a result of evolutionary mechanisms (Babu and Aravind, 2005), there is evidence which does not support this hypothesis:

a) Inducible enzymes have been shown to characteristically appear and reach their maximum activity during growth of the first transfer of the culture in the specific medium, but fail to be formed when the culture is transferred into a medium that lacks the specific substrate (Dubos, 1940). It therefore becomes unlikely that an enzyme formed and lost so suddenly could be due to natural selection.
b) It also appears that the enzyme once formed and used up in the reaction becomes useless to the organism in the absence of the substrate. Yudkin (1938) grew *E. coli* in a formate medium (Dubos, 1940). A hydrogenase enzyme was produced to catalyze the reaction (Equation 5). It was then observed that there was a sudden loss in the hydrogenase activity once the organism is transferred into a medium deficient of formic acid.

$$HCOOH = H_2 + CO_2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

The natural selection theory does not support the inductive mechanism of enzymes; rather, enzyme production occurs without increase in cell numbers. Furthermore it was discovered that enzyme production involves synthesis of new protoplasm, during which

most cells undergo a phase of enlargement and elongation prior to cell division (Todar, 2002).

3.1.3 Application of inductive enzymes

Adaptive enzymes exhibit remarkable specificity toward the substrates that initiate their production and have been shown to be able to differentiate monosaccharides from polysaccharides. In light of this, the ability to manipulate microbes into producing desired specific proteins has increasingly attracted a lot of interest resulting in the production of a diversified range of enzyme systems for industrial application. Introduction of new compounds into the environment that may or may not be toxic to the micro-organism results in:

a) overproduction of a protein that neutralizes the effect of the compound,
b) an increase in the efficiency of a transporter that removes the compound from the cell and,
c) an increase in the catalytic efficiency of the enzyme that processes the substrate (Babu and Aravind, 2005).

3.2 Materials and methods

3.2.1 Time course survey

A five litre sulphidogenic bioreactor was setup as previously described in section 2.2.1, Chapter 2 with a starting COD: SO_4^{2-} ratio of 1:1 (2000 mgl^{-1}). Daily samples of 250 ml were withdrawn from the bioreactor and assayed for protein concentration and hydrogenase activity. The bioreactor was degassed daily to maintain an anaerobic environment. Sulphate/ sulphide concentration and pH were measured in triplicate on a daily basis.

3.2.2 Induction of pure SRB with commercial dyes

An actively growing SRB inoculum (750 ml) was inoculated into modified Postgate lactate medium, which served as the carbon source. Four azo dyes, namely Orange II, Reactive Black 5, Amido black 10, Reactive Red 120 and one anthraquinone dye, Reactive Blue 2 (purchased from Sigma, South Africa) were added into the medium

separately to achieve a final concentration of 100 mgl^{-1} of each dye at the start of the five bioreactors that were maintained under standard anaerobic conditions. Daily samples of 250 ml were withdrawn from the bioreactors and cell free extract obtained as previously described (Section 2.2.2, Chapter 2), assayed for protein concentration, hydrogenase activity, pH, sulphide concentration and, decolourisation (at the respective maximum absorbance peak of each dyes with an Ultra Spec 100 spectrophotometer).

3.2.3 Induction of sludge SRB with commercial dyes

Five bioreactors were setup as described in section 3.2.2, Chapter 3. The carbon source was primary sewage sludge from the local municipal and the inoculum was derived from the Environmental Biotechnology Research Unit (EBRU). The sewage biomass was filtered through a cheese cloth to remove debris, the COD of the filtrate was measured, and adjusted to a starting concentration of 2000 mgl^{-1}. Corresponding sulphate levels (2000 mgl^{-1}) were also used. Daily samples were withdrawn and assayed as described in section 3.2.2.

3.2.4 Effect of dye concentration on enzyme induction

This experiment was carried out to investigate the threshold levels of the dye concentration at which bacteria could produce maximal hydrogenases and to simulate the varying concentrations that are found in typical textile effluents. The five dyes were mixed together at a concentration of 100 mgl^{-1} each to achieve a final concentration of 500 mgl^{-1} at the start of the bioreactor while a second bioreactor had 200 mgl^{-1} of each dye leading to a final concentration of 1000 mgl^{-1}. A 15 % (v/v) (750 ml) SRB inoculum was seeded in both bioreactors and standard anaerobic procedures maintained.

3.3 Results and Discussion

3.3.1 Time course survey

A time course survey to determine the highest production of hydrogenase enzyme during the growth of SRB was studied. The highest enzyme activity was observed at day 6 (Figure 3.2) and this corresponds with the mid stationary phase of SRB growth (Rashamuse, 2003; Ngwenya, 2004). It has been reported that enzyme production occurs

in the secondary stage of the stationary phase depending on the availability of substrates (De Lacey *et al*., 2000). During this study with SRB, this stage corresponds with maximal sulphate reduction and sulphide production (Figure 2.3), between day 6 and 9, achieving sulphide concentrations of 354 mgl^{-1}. During this process ATP generation through H_2 cycling occurs in which the sulphate acts as a terminal electron acceptor (Figure 1.6). This corresponds with work done on *D. vulgaris* where it was shown that it derives energy from oxidative phosphorylation by coupling reduction of sulphate to sulphide with oxidation of H_2 (Heidelberg *et al*., 2004). The hydrogen cycling reactions are catalysed membrane bound and cytoplasmic hydrogenases which facilitate oxidation and evolution of H_2.

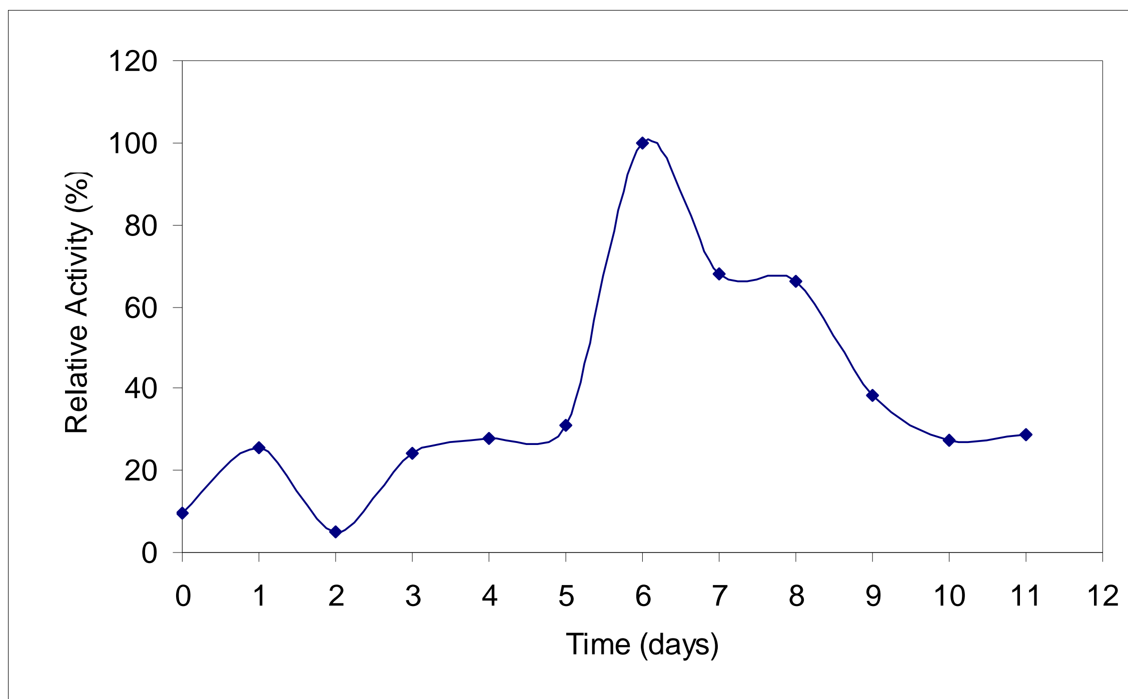


Figure 3.2 Production of hydrogenase enzyme by SRB over time. 100% relative activity = 3.0 U/mg

3.3.2 Production of protein during SRB growth

The protein concentration was determined by the Bradford assay and it gave a representation of the biosynthesis of proteins during bacterial growth. It was observed that during growth of SRB, the protein concentration fluctuated slightly between a maximum of 1.7 mg ml^{-1} and a minimum of 1.1 mg ml^{-1} throughout the time course survey (Figure 3.3).

The highest protein concentration on day three corresponds to the exponential growth phase of the bacteria (Mohanty *et al*., 2000), in which the cells are rapidly growing and there is active synthesis of functional cell proteins (amino acids, transporter molecules, etc), enzymes and RNA resulting in an increase of cell mass (Babu and Aravind, 2005). It can thus be deduced that there is a regulatory mechanism that controls the concentration of protein within bacterial cells at any given time, so that they only contain proteins that are necessary for the immediate use and those not required are not produced. This is supported by Todar (2004) who described bacterial cells as having developed sophisticated mechanisms for the regulation and expression of enzymes. Regulation of protein can be enforced, either by the presence of proteolytic enzymes that degrade proteins not being utilized or through end product inhibition. However, the former is more practical because the Bradford protein test is sensitive towards the amino acids cysteine and arginine and therefore any protein that possesses these two amino acids would be detected, whether it is 'turned' on or off.

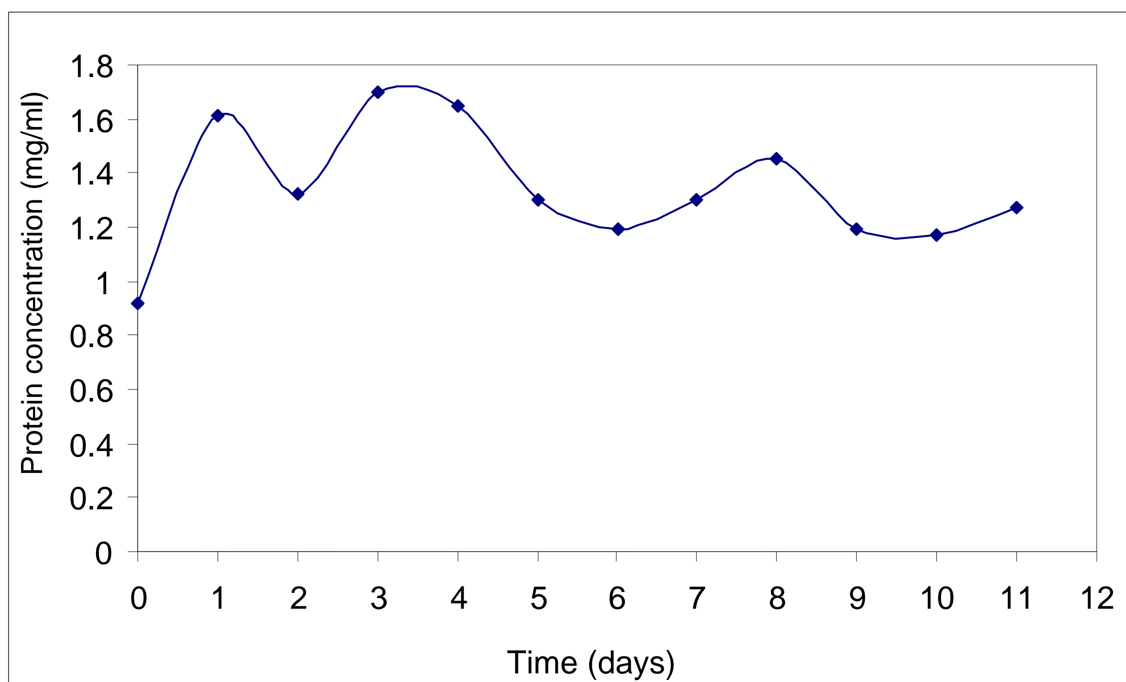


Figure 3.3 Production of protein by SRB over time.

It is interesting to note however, that maximum hydrogenase activity does not correspond to the highest protein concentration. This supports the hypothesis that no new enzymes are formed but that those already existing in the system are 'switched on' or induced to catalyze the conversion of substrate thereby resulting in the observed increased activity (Dubos 1940; Babu and Aravind 2005).

3.3.3 Induction of hydrogenase enzymes with commercial dyes

The aim of inducing production of an enzyme is to harvest the highest amount of enzyme in the shortest time possible. There was induction of hydrogenase with the four azo dyes, but unfortunately there was limited production of hydrogenase in the presence of the anthraquinone dye reactive blue 2 (Figure 3.4). The highest enzyme activity was obtained from induction with Orange II dye which yielded a relative activity of 160 % within 24 h, whilst only 27 % relative activity was obtained from induction with Reactive blue 2. Induction with Amido black 10 was equivalent to the control at 104 %, with same applies for the mixture of all the dyes at 99 % all within 24 h. Reactive black 5 and Reactive red 120 induction levels were quite low as compared to the control; therefore it would not be feasible to utilize these dyes for induction purposes.

The time taken to induce maximal hydrogenase activity was also studied and it was observed that the azo dyes facilitated an increase in enzyme activity within a shorter time period (Figure 3.4). This was quite interesting because the control sample took six days to yield 100 % of the activity whilst, Orange II yielded a much higher activity in only one day. It is also noticed (Figure 3.1), that, in the control at day one, there is only 25 % of the activity whilst, with the induced sample, the activity is at 160 % over the same time period. The same applies for Amido Black 10 and the mixture of the dyes. Although, the overall yield in hydrogenase activity was not as high as the control for reactive black 5 and reactive red 120, it is still evident that there was increased enzyme activity (78 and 45 % respectively) after only one day of incubation. It can therefore be deduced that the presence of azo dyes induced the production or activation of hydrogenase activity.

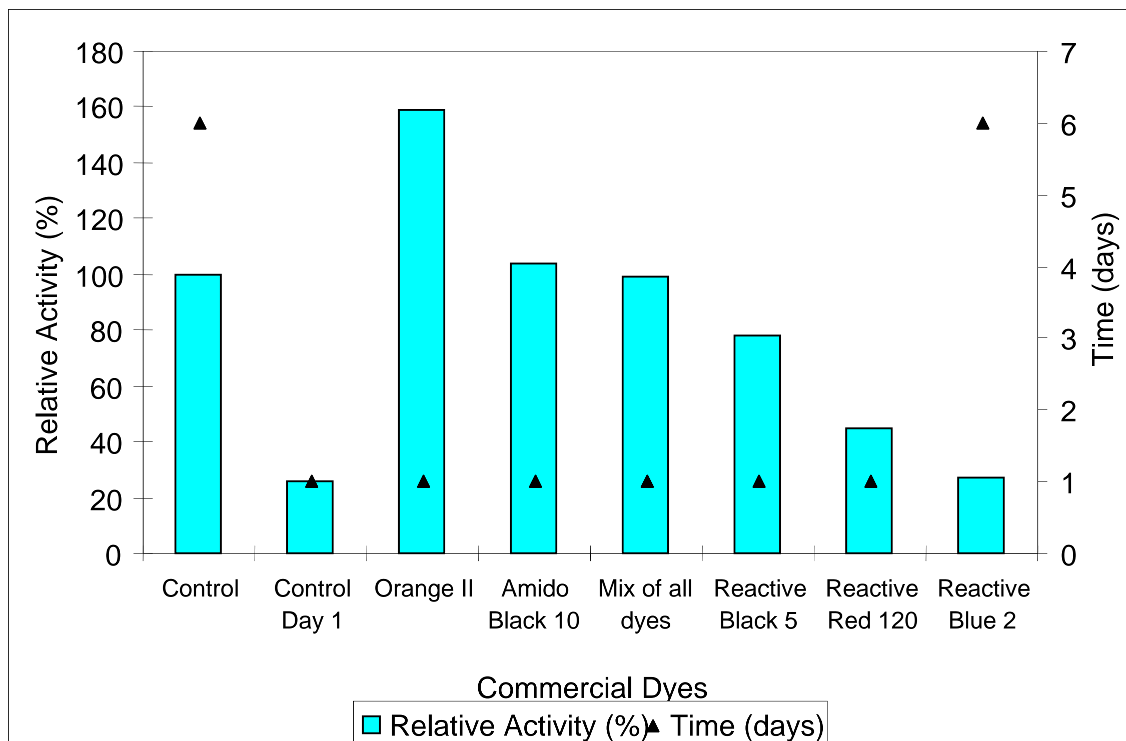


Figure 3.4 Induction of hydrogenase in the presence of commercial dyes. Relative activity (100%) is 3.0 U/mg achieved at day 6. Control 1 represents the maximum activity of hydrogenase in the absence of dye and control 2 represents hydrogenase activity after one day of incubation in the absence of dye as a comparison activity due to induction.

The anthraquinone dye, however, did not induce enzyme activity at all; instead, it took six days for the SRB to produce maximum activity (Figure 3.4), following the same pattern as the control, with an enzyme activity of only 27 % relative activity being produced. It can be speculated that this dye inhibits the production of the hydrogenase enzymes in SRB and therefore would not facilitate the induction of the enzymes required to biodegrade textile effluent.

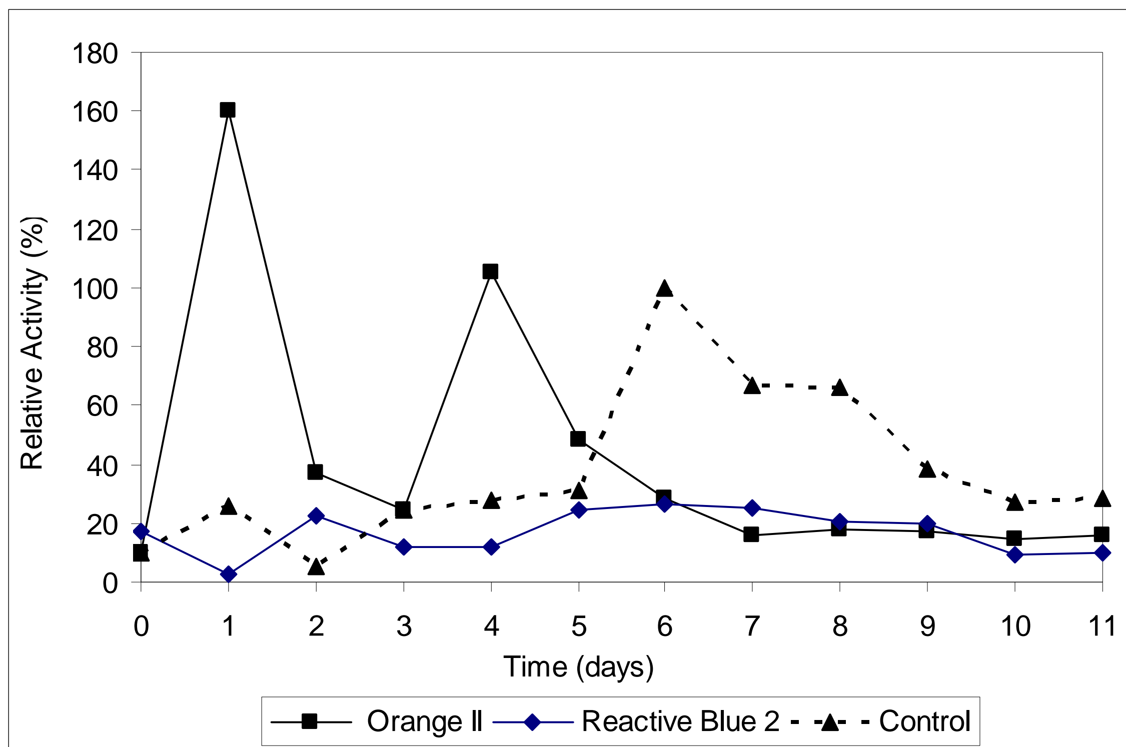


Figure 3.5 Typical time course survey of hydrogenase production in the presence of Orange II and Reactive Blue 2. Relative activity is 4.8 U/mg.

3.3.4 Biodegradation of commercial dyes by hydrogenase enzymes

The induction of enzyme production was accompanied with dye removal or decolourisation. Different dyes had different decolourisation rates, ranging from 92 % for Orange II and 15 % for Reactive Blue 2 (Figure 3.5). It is interesting to note that both the fastest decolourisation rate and highest enzyme production was achieved with Orange II azo dye. On the other hand, the slowest decolourisation rate and least enzyme production were observed with Reactive Blue 2 anthraquinone dye. Previous work on decolourisation of dye containing textile effluent has shown that the structure of the dye chromophore is more important than the dye application class in determining the ease with which decolourisation occurred (Wallace, 2001). The dyes containing mono-, bis- and poly- azo chromophores were reported to show substantial degrees of decolourisation, while dyes with anthraquinone chromophores exhibited a broad variation of results ranging from scarcely degraded (as observed in this study) to substantial degradation (Kremer, 1989; Carliell, 1993). In related studies, the anthraquinone dye Acid Blue 80 which is structurally similar to Reactive Blue 2 only showed 7 %

decolourisation (Brown and Laboureur, 1983a) supports the results obtained in the present study. Interesting observations and perhaps correlating features about anthraquinone dyes are that they are weak and inefficient when applied to fabric, but they tend to be extremely difficult to break down due to their fused aromatic structure (Willmot *et al*., 1998) (Figure 1.3).

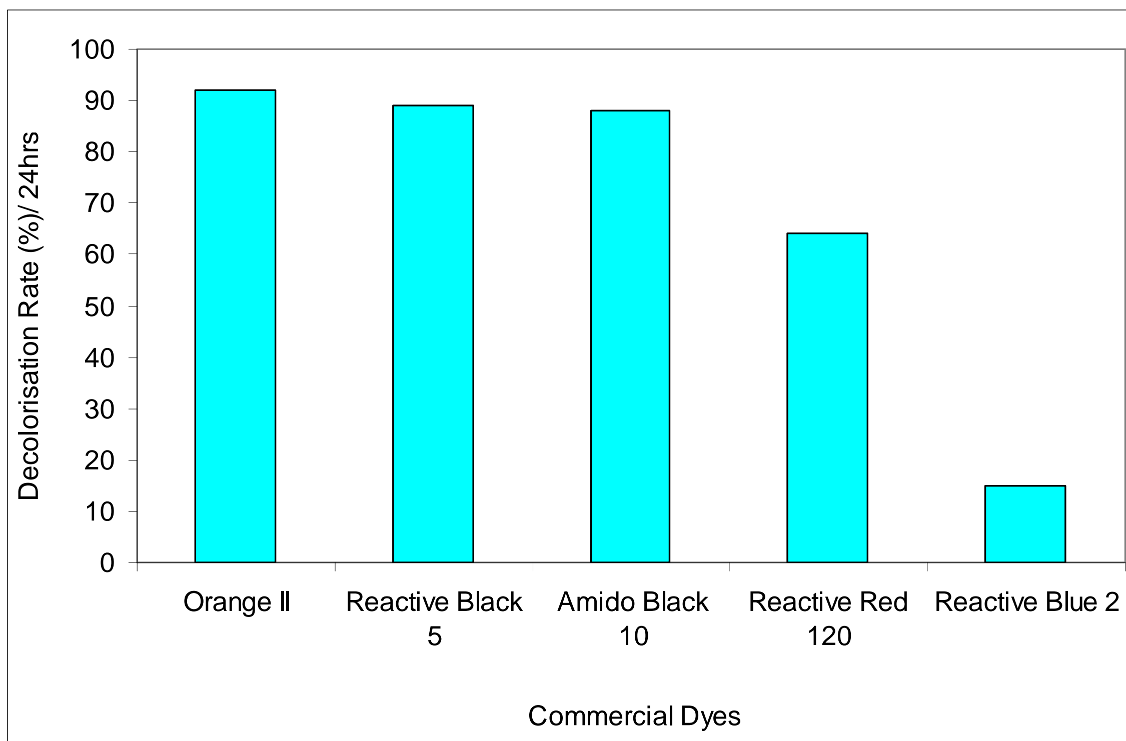


Figure 3.6 Decolourisation rates of commercial dyes by hydrogenases produced by SRB over 24 h.

According to Seshadri and Bishop (1994), the rate limiting step of dye degradation is the permeability of dyes through the cell membrane. Dye permeability through the cell membrane was shown to be a function of the adsorption-desorption equilibrium of the dye at the cell membrane and the food: micro-organism ratio and they postulated that older cells with a reduced supplemental nutrient supply tend to have better degradative capabilities. The reduced decolourisation rate of Reactive Red 120 can be attributed to the complex structure and high molecular weight (Mr = 1469), which impedes the permeability of the dye into the cell and hinders accessibility of the reducing equivalents to the dye chromophore.

3.3.5 Effect of carbon source on decolourisation rate

The range of carbon sources used by SRB as a group is very wide and includes organic acids, alcohols and hydrocarbons. The preferred carbon and energy sources however, are organic acids such as lactate, pyruvate and malate, and alcohols such as ethanol, propanol and butanol (Gibson, 1990). While application of these substrates on a laboratory scale is feasible, up scaling the process onto an industrial site is not economical, and therefore there is a need to investigate a cheaper alternative. In this study, lactate was used in the preliminary experiments in order to eliminate interfering compounds and organisms and to get an understanding of the fundamental principles and mechanisms of dye decolourisation. Once this has been achieved, cheap and more abundant carbon sources such as primary sewage can be investigated.

Decolourisation of Orange II in the presence of lactate resulted in a rapid decolourisation rate as compared to the decolourisation in the presence of sludge biomass (Figure 3.7). With the biomass, an adaptation period was required, which is likely due to the presence of higher molecular weight compounds and the complexity of the medium that made it difficult for the SRB to access the carbon source. In the lactate medium, however, the carbon source is readily available and easily assimilated. These findings are in agreement with the hypothesis that biological azo dye degradation occurs as a result of co-metabolism, when the dyes are introduced as a secondary substrate (Seshadri and Bishop, 1994, Yoo *et al*., 2001). Attempts to utilize the dye as sole carbon and energy sources for their degradation have not been successful under anaerobic conditions. Only simplified azo derivatives were shown to be degraded using a *Pseudomonas* strain (Zimmermann *et al*., 1982) under aerobic conditions. The oxidation of the carbon sources such as lactate (Figure 1.6) facilitates the generation of reducing equivalents that are used to reduce azo linkages. Therefore, although the two carbon sources were added to the bioreactors in the same concentration, the ability to be readily assimilated by SRB differs and as such affects the rate of Orange II azo dye decolourisation. The same concept applies for the other azo dyes (Figures 3.8 – 3.10), though there are several factors that affect the decolourisation rates of these dyes.

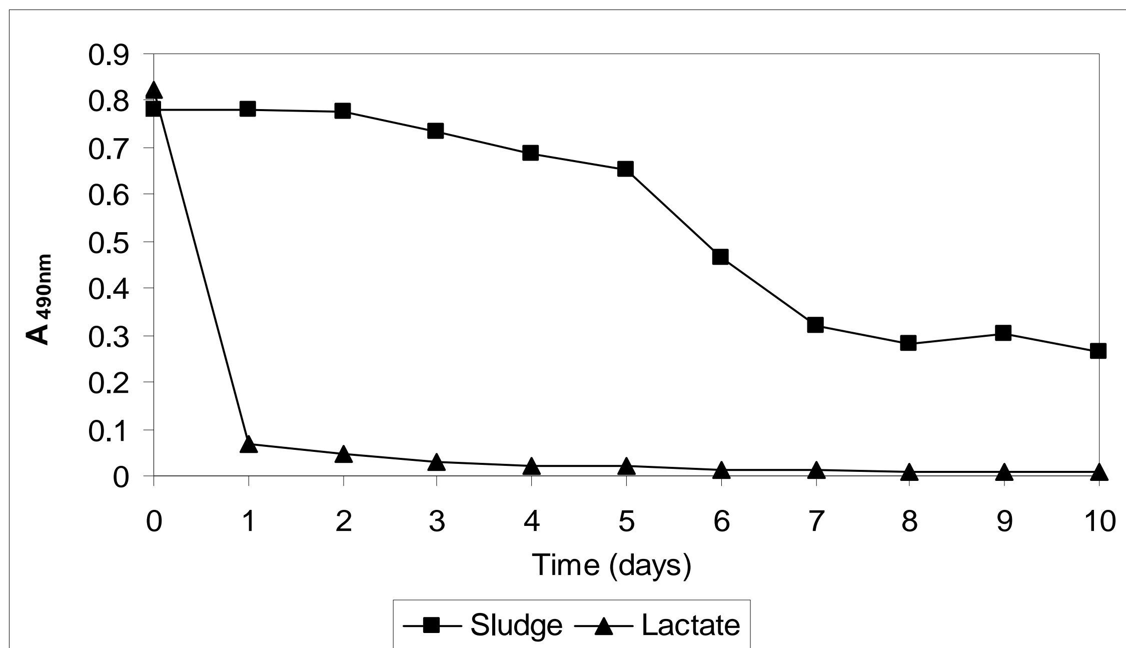


Figure 3.7 Decolourisation of Orange II azo dye by SRB, using lactate or sludge biomass as carbon sources.

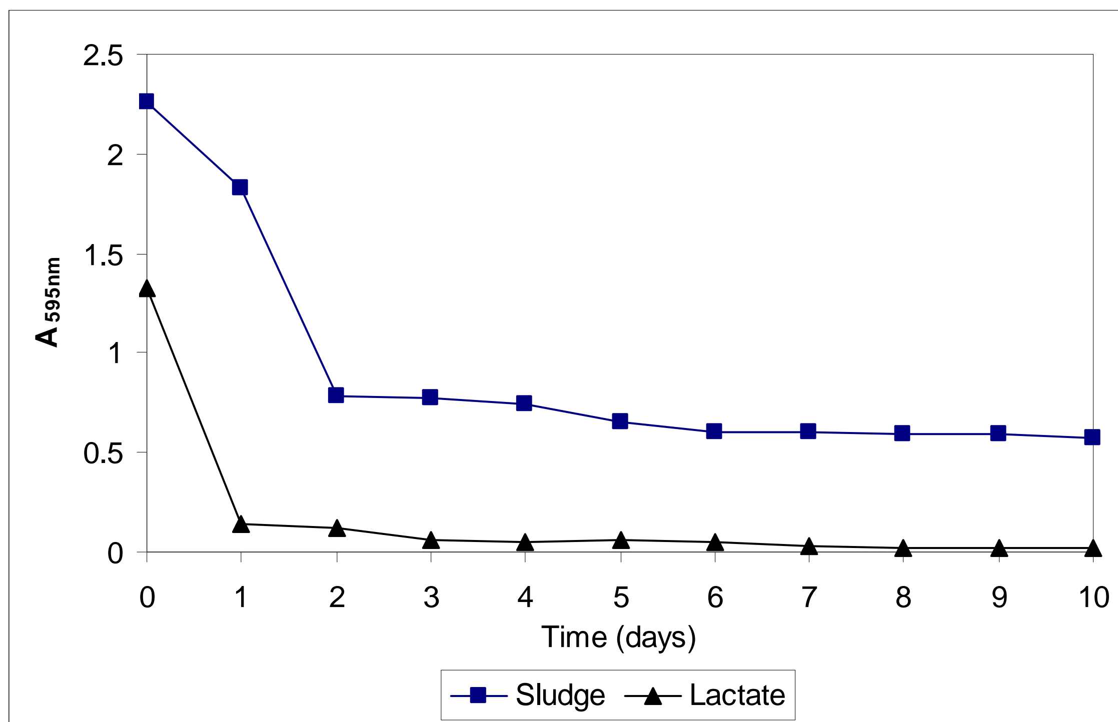


Figure 3.8 Decolourisation of Reactive Black 5 azo dye by SRB, using lactate or sludge biomass as carbon sources.

In the decolourisation of Reactive Black 5, the difference in the starting absorbance values can be attributed to the colour of the sludge biomass which was almost similar to the colour of the azo dye, therefore resulting in considerable interference when colour removal was being measured.

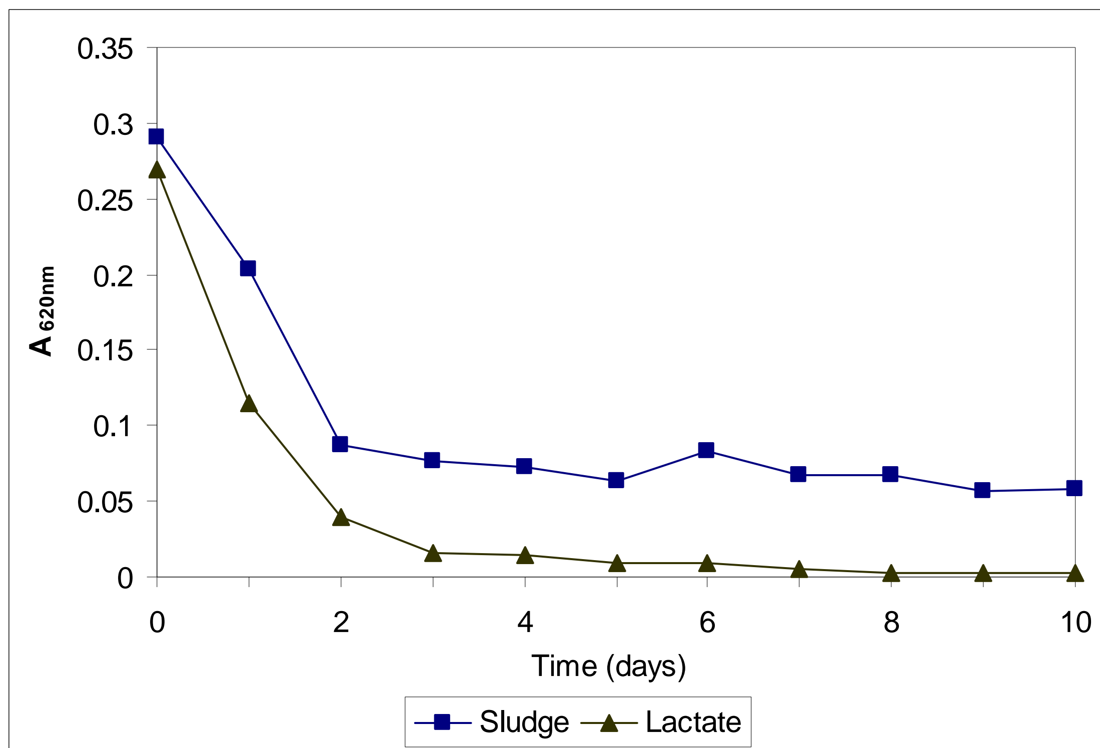


Figure 3.9 Decolourisation of Amido Black 10 azo dye by SRB, using lactate and sludge biomass as carbon sources.

The effectiveness of decolourisation depends on the structure and complexity of each dye as evidenced by the varying decolourisation patterns observed during this study. One common occurrence with all the azo dyes is that, in the presence of sludge biomass the decolourisation is slower, and there is no complete removal of the colour from the medium. The most likely reason for this is the interference of the colour of the sludge biomass which is characteristically black colour.

The slower rates of decolourisation generally observed with Reactive Red 120, Reactive Black 5 and Amido Black 10 are probably as a result of the double azo linkages that make up their structures and will therefore require more reducing equivalents to effect

bond cleavage and subsequent colour removal after disruption of the resonance structures.

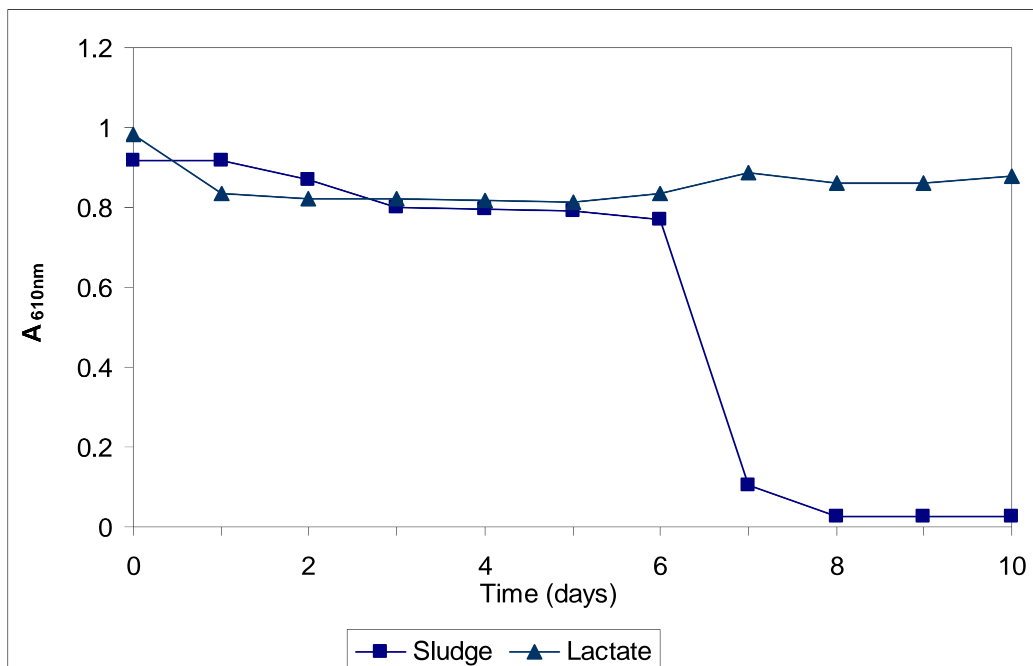


Figure 3.10 Decolourisation of Reactive Red 120 azo dye by SRB, using lactate or sludge biomass as carbon sources.

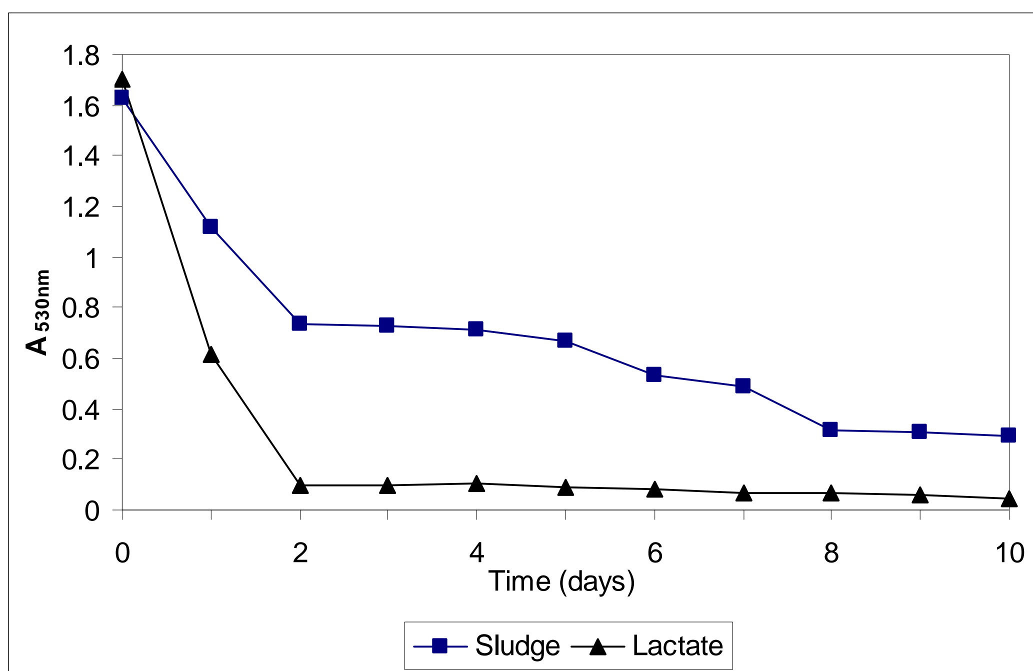


Figure 3.11 Decolourisation of Reactive Blue 2 azo dye by SRB, using lactate or sludge biomass as carbon sources.

Throughout this study, a rather unexpected result was observed with the decolourisation of Reactive Blue 2 by SRB using sludge biomass. After having established that the anthraquinone dye cannot be decolourised when lactate was used as a carbon source, it was observed that after six days of incubation with sludge biomass (Figure 3.10) there was a sudden decolourisation which resulted in complete removal of the dye. The intricate and diverse nature of primary sludge biomass (while not being sterile) may therefore contain other micro-organisms capable of facilitating the aromatic breakdown of the fused anthraquinone ring structure. Carliell (1993) reported the ability of anaerobic sludge culture to degrade anthraquinone dyes.

3.3.6 Effect of dye concentration on enzyme induction and dye degradation

Having determined that the presence and type of carbon source is vital for biodegradation of textile dyes, it became necessary to investigate the threshold levels to which the SRB can be able to optimally decolourise dye containing waste water. Effluent generated from textile dye houses is variable in nature depending on the mode of operation (Robinson *et al*., 2001). If living micro-organisms are to be employed for the removal of colour from textile effluent, the concentration and properties (i.e. pH, temperature and additives) of the influent must be optimal and not toxic to the microbes.

An increase in dye concentration resulted in a decrease in the decolourisation efficiency (Figure 3.12). This correlates with previous studies in which increasing initial concentrations of reactive dyes; Procion Red HE-7B, Drimarene Blue X3LR and Remazol Brilliant Blue R respectively, reduce the rate of decolourisation by as much as 50 % (Carliell, 1993; Ozsy *et al*., 2005). In this study it was observed that removal of Orange II was reduced by 4 % when dye concentration was increased from 100 to 500 mgl^{-1} and a further 36 % when the concentration increased to 1000 mgl^{-1} (Figure 3.12). Increase in concentration of the other dyes yields less decolourisation efficiency as evidenced by the higher loss in rate of degradation for Amido Black 10, Reactive Red 120 and Reactive Black 5 which lose up to 50 % of the decolourisation efficiency. This could be attributed to the structural moiety of the dyes which are characteristically di-azo compared to Orange II which is a mono-azo. They would require more reducing

equivalents to facilitate cleavage of the double azo linkages and as a result there is need for carbon sources which tend to become limiting over time. The generation of aromatic amino metabolites from the breakdown of the dyes may also become toxic to the growth and survival of the SRB, therefore leading to reduced capacity to decolourize the di-azo dyes.

The decolourisation rate of Reactive Blue 2 was not greatly affected by increase in concentration as there was only a decrease in efficiency by 5 % between 100 and 1000 mgl^{-1}. This supports the non-biodegradability of this anthraquinone dye under the present system.

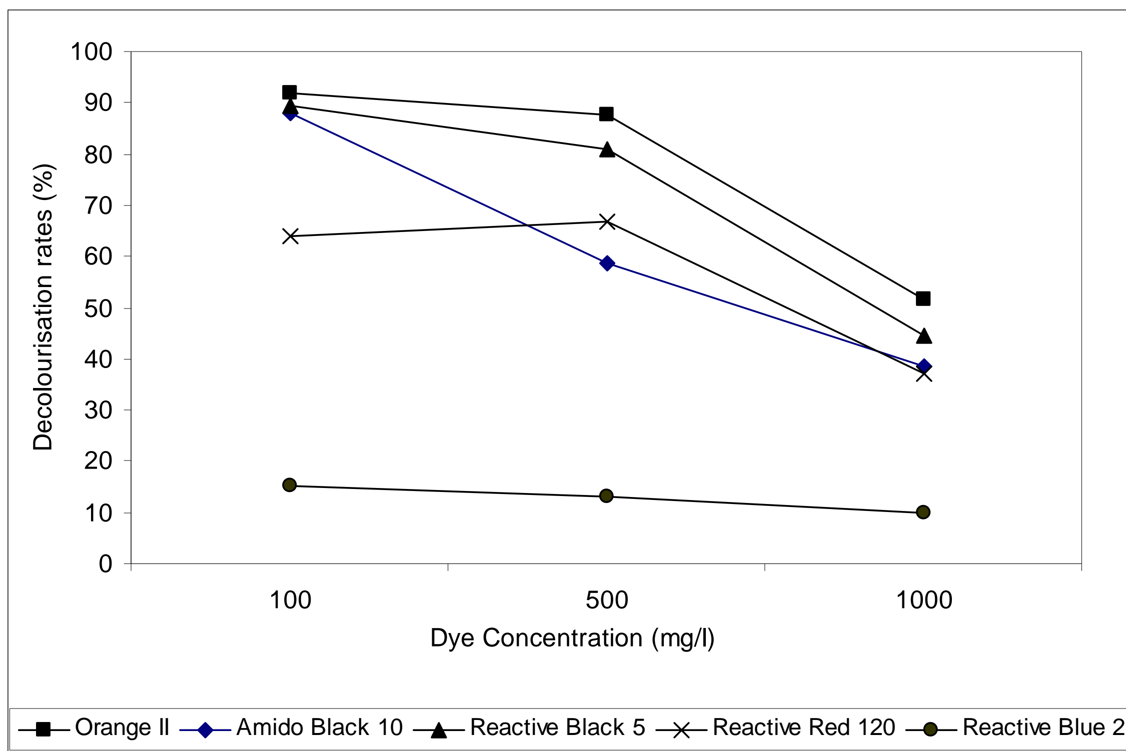


Figure 3.12 Effect of dye concentration dye decolourisation efficiency over 24 h.

The efficiency of dye decolourisation in a mixture of the five dyes as simulated in a typical textile effluent normally made up from a diverse range of dyes was investigated. It was also observed that the decolourisation rates decreased with subsequent increase in dye concentration (Figure 3.13). The highest decolourisation rates were observed after the first 24 h and then declined rapidly thereafter for all the azo dyes. The presence of the anthraquinone dye Reactive Blue 2 did not seem to affect the decolourisation efficiencies

of the azo dyes. This may indicate that the anthraquinone dye does not interfere with the active site of the hydrogenase thereby allowing the enzymes to carry out their normal function, which results in generation of reducing equivalents that facilitate decolourisation of the azo dyes.

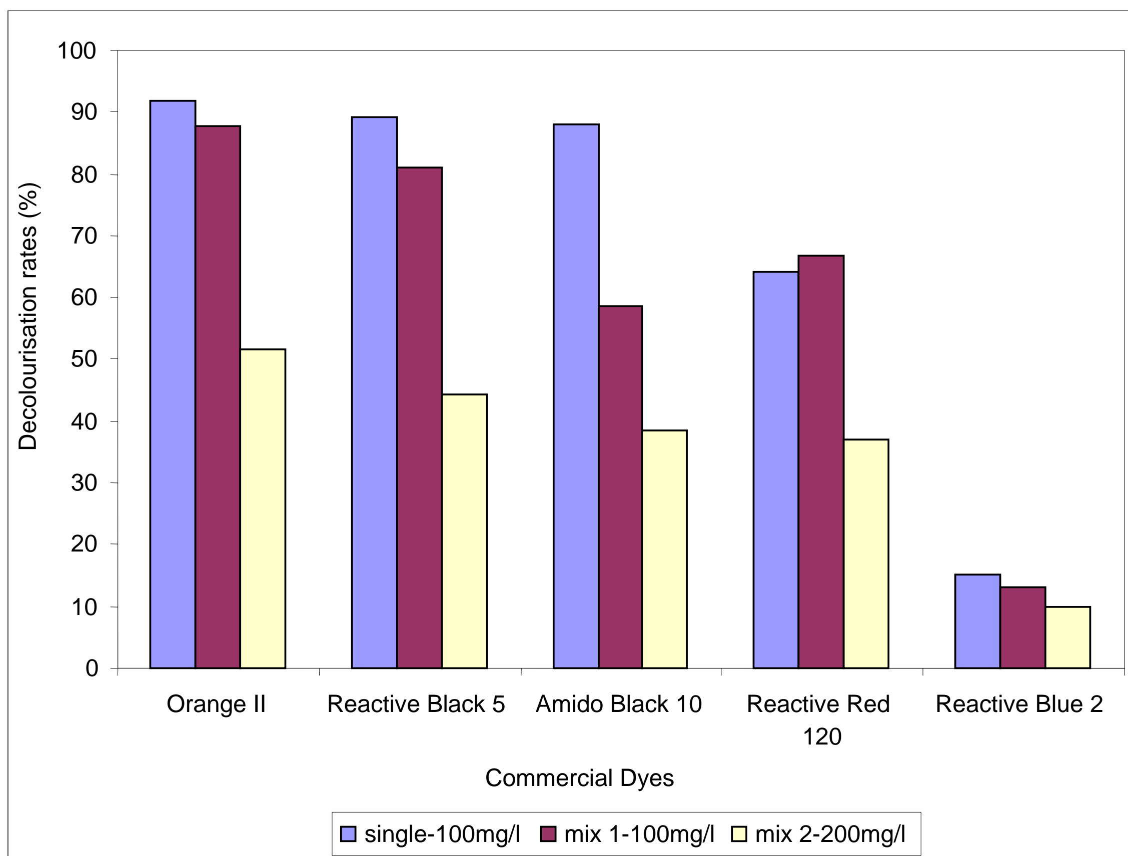


Figure 3.13 Effect of dye mixture concentration on dye degradation.

3.4 Conclusions

Therefore the conclusions that can be drawn from this chapter are:

a) the highest amount of hydrogenase enzyme is produced at day six of the bioreactor in the absence of dyes,

b) hydrogenase activity is increased when SRB are grown in the presence of azo dyes, but is not increased in the presence of anthraquinone dye Reactive Blue 2 and,

c) the mono azo dye Orange II produces the highest amount of enzyme after day one of incubation, while the other di-azo dyes increase or induce production in the

shortest time but do not result in a higher enzyme activity than the control. Reactive Blue 2 appears to inhibit hydrogenase activity.

d) Orange II has the highest decolourisation rates, while Reactive Blue 2 has the slowest,
e) Increase in dye concentration results in reduction of decolourisation rates, therefore the concentration of dyes in an effluent being biodegraded by SRB should be kept between 100 – 500 mgl^{-1} to achieve maximum decolourisation rates and high turnover rates,
f) Lactate yields higher decolourisation rates than sewage sludge due to the bioavailability of the former, as compared to the latter which are made up of a complex mixture of polymeric compounds (Carliell *et al*., 1994). Based on cost implications, however, further investigation of sewage sludge as a carbon source for SRB will be investigated in chapter five.

Once hydrogenase was found to be the enzyme responsible for the degradation of azo dyes, the next phase of this study was to try and isolate, purify and characterize the enzyme in chapter four.

4 PURIFICATION AND PARTIAL CHARACTERISATION OF HYDROGENASE

4.1 Introduction

Protein purification is one the most fundamental steps in understanding the mechanism and characteristics of enzymology. While hydrogenase enzymes have been found in many micro-organisms including algae, the first hydrogenase was described by Stephenson and Stickland (1931) as a bacterial enzyme that catalysed the reversible oxidation of H_2 (Equation 3, page 35). This led to the discovery of more hydrogenases in a number of anaerobic and aerobic prokaryotes. The physiological functions of most prokaryotic hydrogenases are to oxidize H_2, or to reduce electron acceptors and produce H_2 and consequently will maintain the intracellular pH and redox potentials at suitable levels (Ueno *et al*., 1999).

The methods used in the purification and characterisation of enzymes should be reliable, rapid, efficient, and readily applicable during the purification steps (Willson, 1999). The purification of a protein is normally based on the structural and functional properties of the protein and its intended use, and this greatly influences the purification steps that are used. By definition, a protein is pure when it contains only a single protein species, however, in practice, it is more or less impossible to achieve 100 % purity (Wilson and Walker, 1994). Fortunately contamination levels between 5 – 10% of other proteins may be permissible. This is an important point, since each purification step inevitably involves the loss of the target protein. As a result an extra (and unnecessary) purification step that increases the purity of the target protein, say 90 to 98 % may result in insufficient protein for further work. Therefore, it is important to determine purification steps that ensure that purity is of a high quality while retaining enough sample for future intended studies. The purification steps used in this study will be briefly described so as to gain an understanding of their principles.

4.1.1 Enzyme purification

4.1.1.1 Concentration of cell free extract

The main aim of concentrating proteins during purification is to reduce the volume of the aqueous medium, thereby minimizing protein losses by non-specific adsorption to container walls or column matrices. Small volumes of concentrated protein are easier to work with for subsequent purification procedures (Roe, 2001). There are three main concentration methods that have been widely used in the preparation of proteins.

4.1.1.1.1 Ammonium sulphate precipitation

This technique is referred to as 'salting out' and is one of the most common methods for precipitating proteins. The 'salting out' effect operates when high concentrations of salt are gradually added into a protein containing aqueous medium, causing the proteins to aggregate and precipitate out of solution. Salting out is also dependent on the hydrophobic nature of the surface of the protein. In most proteins hydrophobic groups are found within the interior though in some cases they are found on the exterior surface (Harris, 2001). The precipitated protein is usually not denatured and activity is normally recovered upon re-dissolving. Ammonium sulphate is the salt of choice as it combines characteristics such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Bollag, 1996).

4.1.1.1.2 Freeze drying

Also referred to as lyophilisation, involves removal of water from a frozen sample by sublimation, leaving a dry powdered enzyme extract which is much more stable than the aqueous extract (Roe, 2001). Freeze drying is not selective for salts and these may affect activity of the enzyme, although this may be corrected by dialysing before freeze drying. Disadvantages of this technique are that it takes a relatively long time which may result in loss of enzyme activity, and that the sublimation process may cause considerable loss in enzyme activity due to the sudden change in the state of the medium.

4.1.1.1.3 Polyethylene Glycol (PEG)

This is the most commonly used organic polymer. Precipitation by the PEG is one of the simplest and most rapid method of concentrating solutions as it does not require

specialized apparatus. The mechanism of precipitation involves addition of water miscible organic polymers which absorb water from the protein extract. The molecular weight of the polymer should be greater than 4000; normal polymers used are 6000 and 20000. This technique is advantageous in that it does not interfere with subsequent purification steps and it does not affect enzyme activity. It is inert and, unlike ammonium sulphate, tends to stabilize proteins (Bollag, 1996).

4.1.2 Size exclusion chromatography

Size differences between proteins can be exploited in size exclusion chromatography. The basic principle is that molecules are partitioned between solvent and stationary phase of defined porosity. The separation process is carried out using a porous gel matrix (in bead form) packed in a column and surrounded by solvent. Molecules that are too large to pass through the gel are excluded and hence elute first from the column. On the other hand, smaller molecules within the fractionation range of the gel, penetrate the pores, and will appear as the last components in the chromatogram. Apart from separation based on size, the extent of separation also depends on their shape, as elongated proteins (due to their higher hydration radius) pass through faster than compact globular proteins of the same molecular weight. Therefore, the separation mechanism of size exclusion chromatography involves both molecular shape and mass of the molecules, and thus it can be used to determine molecular shape in solution and consequently as a monitor for denaturation (Wilson and Walker, 1994).

4.1.3 Ion exchange chromatography

This is the most widely used form of chromatography which relies on the attraction between oppositely charged particles in proteins and amino acids (Wilson and Walker, 1994). These molecules contain ionisable groups which carry a net positive or negative charge which is thus exploited in their separation. Ion exchange separations are carried out in columns made up of charged groups that are covalently attached to a support matrix. There are two types of ion exchangers, namely cation and anion exchangers. Cationic exchangers possess negatively charged groups which attract positively charged cations. These exchangers are also called acidic ion exchangers because their negative charges result in ionisation of acidic groups. Anion exchangers contain positively charged groups that attract negatively charged anion

groups and they normally result from the association of protons with basic groups and as such are also referred to as basic ion exchangers (Wilson and Walker, 1994).

The mechanism of ion exchange chromatography is made up of five distinct steps:

a) rapid diffusion of the ion to exchange surface in homogenous solutions,
b) diffusion of the ion through the matrix structure of the exchanger to the exchange site which is highly dependent on the degree of cross linkage of the exchanger and the concentration of the solution. This is thought to be the rate limiting step of ion exchange chromatography,
c) the next step is the exchange of ions at the exchange site, which occurs instantaneously at equilibrium. The more highly charged the ionised molecule to be exchanged, the tighter it binds and the less readily it is displaced by other ions,
d) the exchanged ions diffuse through the exchanger to the surface, and
e) the final step is the selective desorption by the eluent and diffusion of the molecule into the external eluent. This is achieved by changes in the pH and/ or ionic concentration or by affinity elution (i.e. in cases where an ion has greater affinity for the exchanger than that bound onto the matrix when it was introduced into the system (Wilson and Walker, 1994).

Over and above these characteristics, size exclusion chromatography may be combined with ion exchange chromatography. In this study, Sephacryl S-200 separates protein molecules by size, as well as ion exchange of charges on the protein molecules. The influence of salt ions enables the elution of charged protein molecules within a sample over a concentration gradient (Harris, 2001).

4.1.4 Enzyme characterisation

Potential application of enzymes as catalysts for biotechnological processes necessitates their characterisation so as to achieve their maximum activity. Physiological properties of enzymes that influence activity include stability to temperature change, pH, catalytic properties, size (molecular weight), charge (electrophoretic mobility), and binding partners (substrates and co-factors) (Willson, 1999).

4.1.4.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

This is the most widely used method for analysing protein mixtures qualitatively and is particularly useful for monitoring protein purification. Since the method is based on the separation of proteins according to size, it can therefore be used to determine the relative molecular mass of proteins. SDS-PAGE can be used to determine the purity of the protein and give an assessment of the purification steps.

The method starts with boiling of target protein sample in buffer containing β-mercaptoethanol and SDS (CH_3-$(CH_2)_{10}$-$CH_2OSO^-_3Na^+$) which is an anionic detergent. The mercaptoethanol disrupts the tertiary structure of the protein by cleaving the disulphide bridges, while the SDS binds strongly to and denatures the protein. This results in the opening up of the protein into a rod – shaped structure with a series of negatively charged SDS molecules of constant charge density per unit mass, and this facilitates electrophoretic mobility which is independent of amino acid composition (Wilson and Walker, 1994).

The samples are loaded onto a stacking gel which serves the purpose of concentrating the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilizing differences in ionic strength and pH between the electrophoresis buffer and the stacking gel, a phenomenon known as isotachophoresis. When an electric current is passed, the proteins migrate through the gel and they interact with the ampholytes (electrostatic interaction). At some point along the gel, each protein encounters its isoelectric point; and the migration of the protein stops. This results in each protein species concentrating at that point thereby forming a very sharp band (Voet and Voet, 1995). The distance migrated by the protein is measured, a plot of $\log_{10}$ polypeptide molecular weight versus the relative mobility (R_f) is used to calculate the relative molecular mass of the target protein (Hames and Rickwood, 1981).

4.2 Materials and methods

4.2.1 Purification methods

4.2.1.1 Culture

Cells were cultured in a five-litre bioreactor containing modified Postgate lactate medium (Appendix A) and harvested as previously described (Sections 2.2.1 and 2.2.2).

4.2.1.2 Hydrogenase activity and protein determination

Hydrogenase activity was assayed spectrophotometrically, by monitoring the reduction of methyl viologen with gaseous H_2 as previously described (Section 2.2.4.5, Chapter 2). Protein was quantified by the Bradford method, using BSA as a standard as previously described (Appendix B).

4.2.1.3 Preparation of cell free extract

The SRB cells were harvested by centrifugation (7000 rpm, JA-10 rotor, Beckman, USA, 15 minutes, 4ºC). The pellet (11 g wet weight) was washed twice with ddH_2O and then re-suspended in Tris-HCl buffer (pH 7.6, 50 mM, 100 ml). Cell integrity was disrupted by sonication at an amplitude of 10W at 30 second intervals for 4 minutes. The lysate was centrifuged at 10000 rpm, JA-20 rotor, 25 minutes, 4 ºC) to remove membrane associated material and other cell debris. The supernatant was then assayed for protein and hydrogenase activity and designated CFE1.

4.2.2 Concentration of cell free extract

The concentration procedures reported here are those that showed the best results from several attempts made in this study. The CFE1 was divided into two parts (50 ml each), one part was concentrated by freeze drying and the other with poly ethylene glycol 20 000.

4.2.2.1 Poly ethylene glycol (PEG 20 000) concentration

The CFE1 (50 ml) was sealed in dialysis tubing (Pierce, Rockford, Illinois, USA), which was then placed in a beaker and covered completely with a dry matrix of PEG

20 000. The sample was left to stand (4 °C, 2h) as the water was removed from the cell free extract until the desired volume (5 ml) was obtained. The concentrated sample was assayed for protein concentration and hydrogenase activity.

4.2.2.2 Concentration by freeze drying

The sample (50 ml) was poured into a freeze drying flask and frozen by gently swirling the flask in liquid nitrogen for 10 minutes. The sample was then transferred onto a freeze dryer and freeze dried at – 60 ° C overnight. The powdered extract was re-suspended in a minimal amount of dH_2O and assayed for protein and hydrogenase activity.

4.2.2.3 Dialysis

Both concentrated samples were dialysed overnight in 10 times sample volume Tris-HCl buffer (pH 7.6, 10 mM) to remove any contaminating salts. The samples were assayed for protein and hydrogenase activity.

4.2.2.4 Size exclusion and ion exchange chromatography on Sephacryl S-200

The concentrated samples (5 ml) each from PEG and freeze drying were applied separately onto a sephacryl S-200 (Sigma, South Africa) column (3 cm x 30 cm). The column was pre-washed with buffer Tris-HCl buffer (pH 7.6, 50 mM) and equilibrated until A_{280} of elute had reached baseline. The sample was applied and eluted using the same buffer. An ion exchange gradient established by using 0.3 M NaCl was eluted at the same time with the buffer. Fractions (4.5 ml) eluted at 2.2 $mlmin^{-1}$, were assayed for protein concentration. Those that contained protein were assayed for hydrogenase activity, pooled together, dialysed then freeze dried.

4.2.2.5 SDS-PAGE analysis

The effectiveness of the purification procedures was determined by running SDS-PAGE on samples exhibiting hydrogenase activity. Samples from each purification step (20 µl) and a standard molecular weight maker (15 – 150 kDa, Merck, South Africa) were electrophoresed on 10 % SDS-PAGE at 120 V. The gels were stained with coomassie brilliant blue R-250 staining solution (Appendix C1), then destained in methanol: acetic acid: water (1:1:8 v/v/v) destaining solution (Appendix C1). The

molecular weight of the partially purified hydrogenase was determined using a standard curve of log molecular weight versus distance migrated (Appendix C2).

4.2.3 Characterisation methods

4.2.3.1 pH profile

The optimum pH for the hydrogenase was established by re-suspending the partially purified enzyme in different pH buffers, sodium acetate (pH 4 – 5.5, 50 mM); sodium phosphate buffer (pH 6 – 6.5, 50 mM); Tris-HCl buffer (pH 7 – 9, 50 mM) and bicarbonate buffer (9.5 – 11, 50 mM). Hydrogenase activity was determined in each of the samples at the different pH levels.

4.2.3.2 Temperature profile

The temperature optimum of the partially purified hydrogenase enzyme was determined over a temperature range of 10 – 70 °C. The reaction mixture was prepared as previously described (Section 2.2.4.5, Chapter 2). The reaction was started by addition of hydrogenase suspension pre-activated with gaseous H_2 at the different temperatures.

4.2.3.3 Thermal stability profile

The optimum pH and temperature were used to determine the optimal thermal stability of the hydrogenase enzyme. Hydrogenase activity at time zero was considered as 100 % relative activity, and was measured at 10 min intervals for 90 minutes.

4.2.3.4 Kinetic parameters (V_{max} and K_M)

The kinetic properties were determined by varying the substrate (methyl viologen) concentration between the ranges 0 – 10 mM. Hydrogenase activity was determined at each substrate concentration.

4.2.3.5 Effect of aromatic amines on hydrogenase activity

It is a strong probability that aromatic amines generated from the reductive cleavage of azo dyes accumulate and become toxic to enzyme activity and SRB growth.

Standard aromatic amines namely, sulphanilic acid and aniline (Merck, South Africa) which are the breakdown metabolites from azo dye Orange II in concentrations ranging from 10 – 100 mM were added into the reaction mixture. Hydrogenase activity was carried out as previously described (Section 2.2.4.5, Chapter 2).

4.3 Results and Discussion

4.3.1 Purification of hydrogenase

4.3.1.1 Concentration of enzyme extract

The effect of the three concentration methods on hydrogenase activity was investigated. Several attempts to concentrate the hydrogenase with ammonium sulphate precipitation resulted in complete loss of enzyme activity (results not shown), and consequently the other two protocols (Freeze drying and PEG) were used throughout the purification. Subsequent purification steps after concentrating the enzyme were dialysis, size exclusion / ionic exchange chromatography. Hydrogenase is a very sensitive enzyme, which can rapidly lose its activity in the presence of oxygen (Kamachi *et al*., 1995). While extreme caution was taken during the purification steps, loss of activity due to exposure to air cannot be ruled out. The purification of hydrogenases in previous work from our research group has also been hampered by the presence of oxygen (Rashamuse, 2003; Ngwenya, 2004). Exposure was limited by extending the pre-activation time of the enzyme with molecular H_2 under strict anaerobic conditions.

4.3.1.1.1 First purification strategy

A summary of the hydrogenase purification with freeze drying, dialysis and Sephacryl S-200 size exclusion-ion exchange chromatography is shown in Table 4.1. The first purification step of freeze drying increased the specific activity from 0.82 to 1.11 Umg^{-1} as it managed to remove the water that constituted the aqueous sample as well as contaminating proteins as seen by the decrease in protein concentration from 331.8 to 133.5 mg.

Table 4.1: Purification table for hydrogenase using freeze drying (FDE) as a concentration step. F17 = Fraction no. 17 and F73 = Fraction 73.

Sample	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery %	Purification Fold
Crude	200	331.8	272	0.82	100	1
FDE	50	133.5	148	1.11	54.4	1.35
Dialysis	50	126.5	150.5	1.19	55.3	1.45
S-200HR F17	22.5	38.7	99.23	2.56	36.5	3.13
S-200HR F73	27	5.05	8.208	1.63	5.5	1.47

The fold purification increased slightly indicating that the target protein had been slightly 'cleaned up'. The dialysis step removed the salts from the buffer and those that had been freeze dried, and brought about a slight increase in the hydrogenase activity as seen in the purification fold and the percentage recovery, from 54.4 to 55.3 %. This most probable reason for this slight increase in activity could have been attributed to removal of the salts from enzyme sample and may have disrupted the optimal pH and ionic strength of the buffer. The freeze dried extract was then loaded onto a Sephacryl S-200 size exclusion-ion exchange column. The elution profile is shown in Figure 4.1. Two protein peaks were observed at fractions 17 and 73 with corresponding hydrogenase activity, after eluting the sample with a NaCl salt gradient from 0 – 0.3 M. This suggested the presence of two types of hydrogenase with different molecular size and ionic charge. There was a significant change in fold purification from 1.45 to 3.13 from the previous purification step with fraction 17, whilst there was no noticeable change in fold purification with fraction 73.

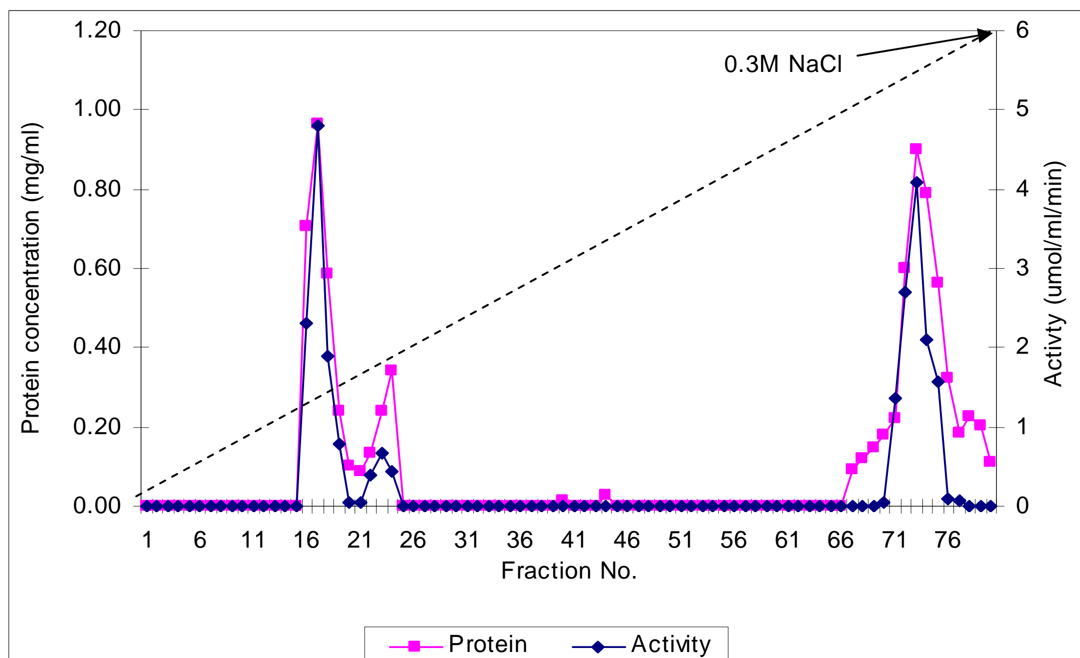


Figure 4.1 Sephacryl S-200 size exclusion-ion exchange chromatography after freeze drying. Column dimension (3 x 30 cm); Flow rate: 2.2 mlmin^{-1}. The hydrogenase activity was eluted by gradient addition of NaCl (0 – 0.3M) with Tris-HCl buffer (pH 7.6, 50 mM).

4.3.1.1.2 Second purification strategy

The cell free extract harvested from SRB was also concentrated using PEG. Unlike with the freeze dried extract, the first purification with PEG, resulted in loss of specific activity from 0.82 to 0.75 Umg^{-1} (Table 4.2). Contrary to what to literature suggests, that it stabilizes the enzyme activity (Harris, 2001), in this case there was a slight decrease in fold purification from 1 to 0.91. Activity may have been lost by the increase in viscosity caused by the withdrawal of water molecules from the cell free extract. It was later recovered after the dialysis step. During dialysis there is removal of salts and ions (that may limit the activity of the enzyme) through osmosis from a highly concentrated solution (enzyme extract) to the dilute solution (buffer). Therefore it can be suggested that the dialysis step reversed the effects of the concentration step giving a specific activity of 0.88 Umg^{-1}. This is also supported by the purification fold which increased from 0.91 to 1.08 Umg^{-1}. On further purification with Sephacryl S-200, there was a significant increase in specific activity from 0.88 to 2.49 Umg^{-1}. While there was significant drop in the yield as a result of the chromatography step, there was an overall increase in fold purification thus indicating that this purification was successful.

Table 4.2: Purification table of hydrogenase using PEG as a concentration step

Sample	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)	Purification Fold
Crude	120	199.08	163.2	0.82	100	1
PEG	50	189.5	141.4	0.75	86.6	0.91
Dialysis	10	34.8	30.7	0.88	18.8	1.08
Sephacryl 200HR	36	21.13	52.56	2.49	32.2	3.03

An interesting observation that was noted between the two purification strategies was the appearance of two peaks with the freeze dried extract (Figure 4.1) and only one peak with the PEG concentrated extract (Figure 4.2). Initially it was presumed that two types of hydrogenases had been partially purified (Freeze dried extract), but the second peak at fraction 73 was not observed in the second concentration step. Several attempts to repeat the PEG did not yield a second peak and this led to the assumption that the second peak that was found with the freeze drying extract could be the same enzyme which eluted at different times due to the ionic strength of the charged amino acids in the structure. Confirmatory studies were performed with SDS-PAGE to determine the molecular weight of the hydrogenase enzyme.

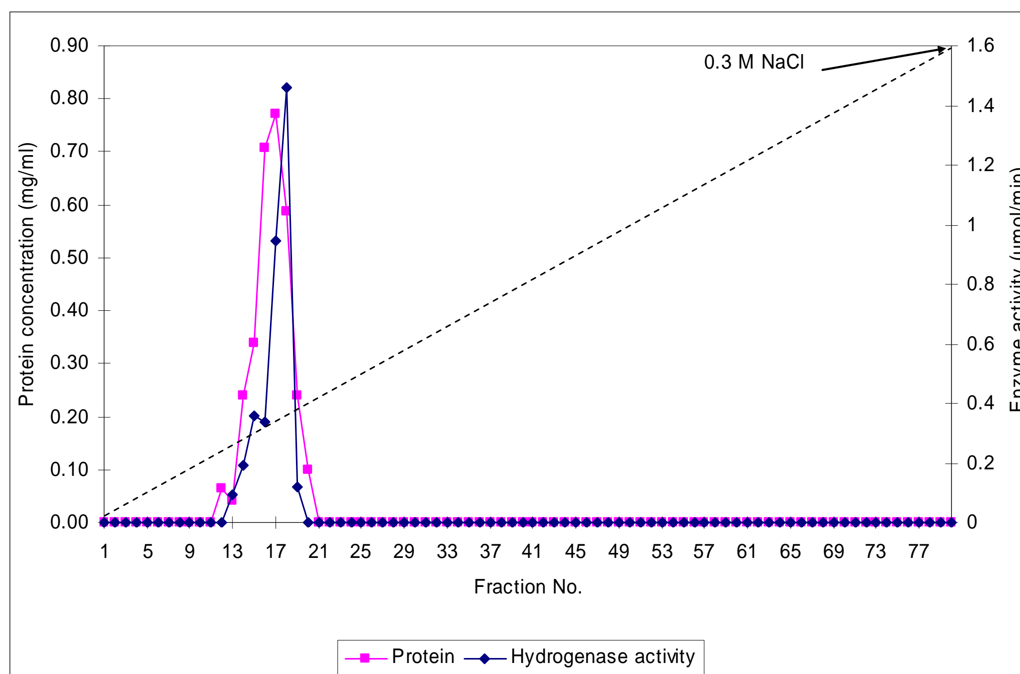


Figure 4.2 Sephacryl S-200 size exclusion-ion exchange chromatography after PEG. Column dimension (3 x 30 cm); Flow rate: 2.2 mlmin^{-1}. The hydrogenase activity was eluted by gradient addition of NaCl (0 – 0.3M) with Tris- HCl buffer (pH 7.6, 50 mM).

4.3.1.2 SDS-PAGE molecular weight analysis

Polyacrylamide gel electrophoresis (PAGE) is one of the most effective methods that is capable of separating molecules on the basis of their physical differences both in molecular size and net charge (Hames and Rickwood, 1981). The results obtained showed that the size exclusion chromatography step was successful with the PEG dried extract as observed by the single band in lane 2 but was not successful for the freeze dried extract in lane 3 where were several bands the presence of proteins with different molecular weights are indicated (Figure 4.3). The appearance of the bands on the freeze dried extract in the PAGE analysis also explains the appearance of more than one peak in the chromatogram (Figure 4.1). The molecular weight of the protein band exhibiting hydrogenase activity was estimated at 38.5 kDa. The hydrogenase appears to be monomeric, and its molecular mass is in the same order of magnitude as that of larger subunit of hydrogenase found in *Desulfovibrio desulfuricans* (Nicolet *et al*., 2000). The likelihood that the hydrogenase purified in this study could belong to the iron only class is due to the fact that the majority of enzymes from this class are monomeric, with molecular weights ranging from 42 – 57 (Ueno *et al*., 1999; Fontecilla-Camps *et al*., 2002). While Ni-Fe hydrogenases are localized within the cell membrane, and because of their function, they tend to be dimeric in nature with molecular weights ranging from 48 – 89 kDa (Winter *et al*., 2005). Therefore the speculation that hydrogenase purified in this study could be a Fe only hydrogenase and not a nickel containing hydrogenase remains a reasonable argument.

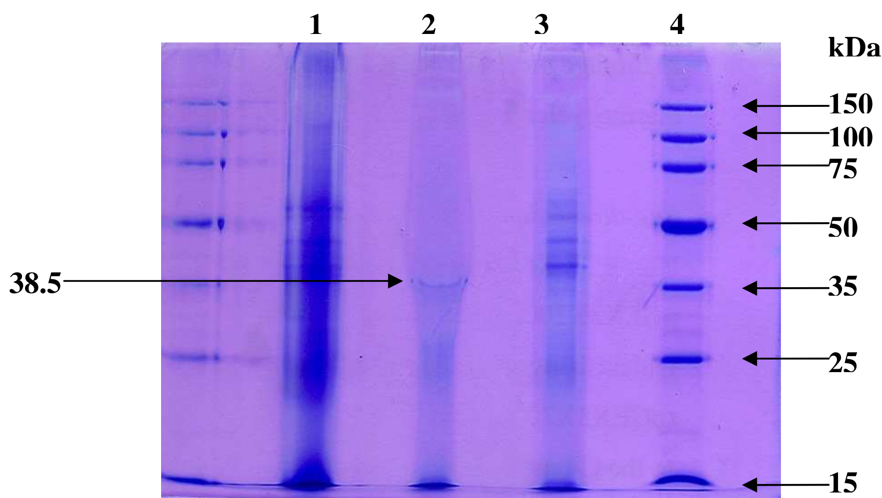


Figure 4.3 10 % SDS-PAGE analysis of purified hydrogenase. Lane 1: Crude extract. Lane 2: PEG concentrated sample after Sephacryl S-200 size exclusion-ionic exchange chromatography. Lane 3: Freeze dried extract after Sephacryl S-200 size exclusion-ionic exchange chromatography. Lane 4: Molecular weight markers.

Literature also suggests that the molecular weights of hydrogenases in SRB alone are diverse and quite dependent on the particular culture of the bacteria as well as the culturing conditions (Kamachi *et al*., 1994; Winter *et al*., 2005). The hydrogenase purified from this study was derived from a mixed consortium of SRB, as a result it is difficult to speculate which organism produced it, because the hydrogenase tends to be uniformly distributed in the sulphate reducing species.

4.3.2 Characterisation studies

4.3.2.1 pH optimum profile

Most enzymes normally exhibit a strong dependence of activity on the pH of the medium (Clark and Switzer, 1977), therefore it is important to optimize the pH of a particular enzyme (hydrogenase in this case). Since hydrogenase has been found in a wide range of organisms including bacteria, fungi, and algae, and in different environmental conditions the pH profile was investigated over a range of 4 – 10, using different buffers coherent with the pH level. An optimum pH of 7.5 for hydrogenase was observed (Figure 4.4) and this in accordance with the optimal pH for growth of SRB which has been reported to be in the range 7.0 – 8.0 (Zehender, 1988). Enzymes have been known to operate within the pH of the organism that they are produced in and as a result the hydrogenase purified in this study had an optimal pH that corresponded with the conditions under which the SRB were grown (Figure 2.4).

Previous studies on hydrogenase characterisation have also found the optimal pH to be in the same range (7.0 – 8.0) (Rashamuse, 2003; Ngwenya, 2004), therefore it can be deduced that hydrogenase enzymes function optimally in a slightly alkaline environment and it can be postulated that pH dependence of hydrogenase reactions is the consequence of the changing degrees of ionization of functional groups in the enzyme as well as the substrate (Clark and Switzer, 1977). This is in view of the reversible redox reactions carried out by the hydrogenase enzymes.

4.3.2.2 Temperature profile

A maximum hydrogenase activity was observed at 40 °C, which is within the growth temperature of mesophilic SRB. The temperature curve follows a characteristic bell shape in which at lower temperatures, the activity is slower but gradually increases as temperature increases (Figure 4.5). This is typical of enzyme kinetics in which rate of enzymatic reaction increases as temperature increases, this being achieved by increase in the rate at which enzyme and substrate 'collide' or become in contact with one another (Walker, 2000). The downside to this reaction, however, is that at higher temperatures (above 50 °C) there is disruption of the quaternary structure of the protein resulting in inactivation and subsequent denaturation of the enzyme. It must be noted, however, that SRB are also thermophilic and have been isolated from environments that have temperatures as high as 70 °C, and so it points to the vast diversity and ubiquity of these organisms.

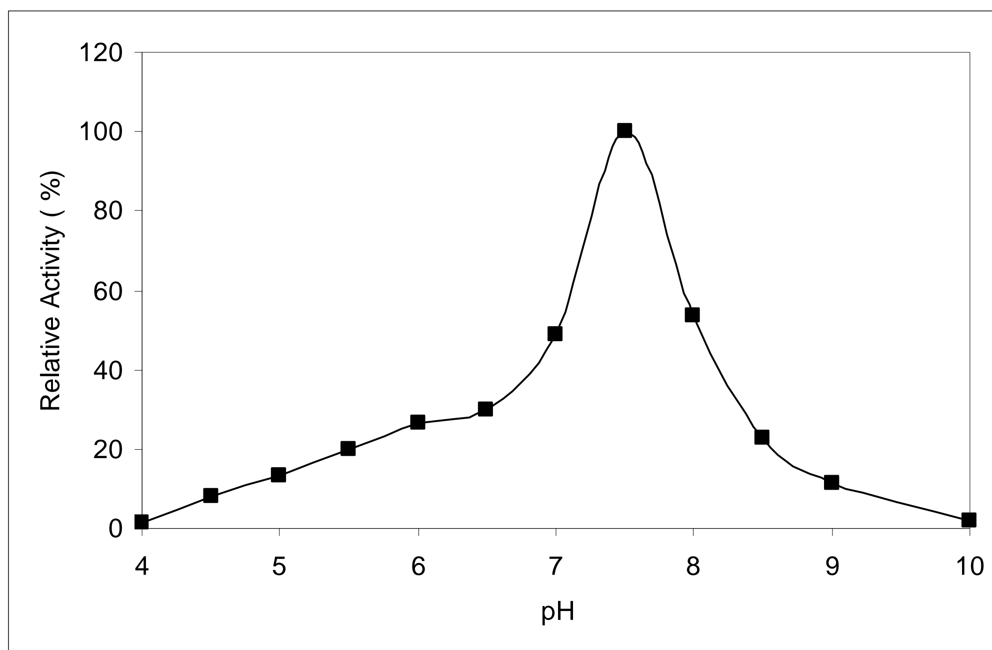


Figure 4.4 pH profile on hydrogenase activity.

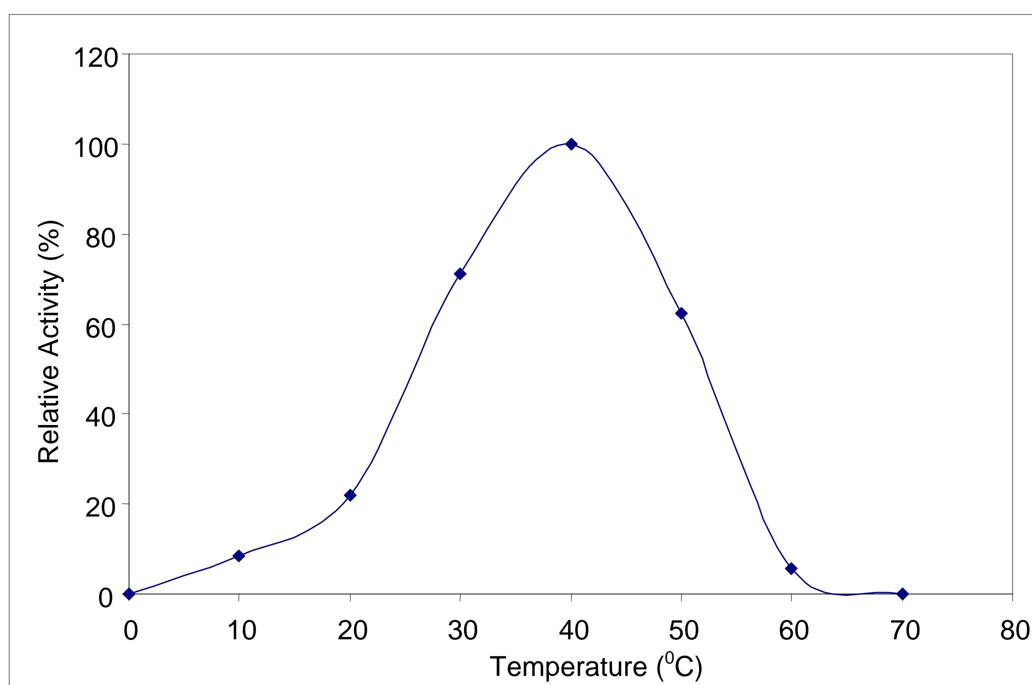


Figure 4.5 Temperature profile of hydrogenase enzyme.

4.3.2.3 Thermal stability of hydrogenase

The thermal stability of hydrogenase was investigated at optimum pH (7.5) and temperature (40°C). Minimal activity loss was observed during the first 10 minutes

and thereafter, activity steadily decreased as incubation time increased (Figure 4.6). The evident loss in activity observed could have been caused by the loss of protective matrices, which normally protect the hydrogenase enzyme *in vivo* as consequence of the isolation and purification steps carried during the course of the study (Rashamuse, 2003). These, together with external factors such as pH and temperature could result in progressive denaturation of the enzyme by altering the structural integrity of the hydrogenase. After 40 minutes, 70 % of the activity had been lost after which very little change in the activity occurred. The hydrogenase purified in the current study had a half life of 32 mins ($t_{1/2}$ = 32).

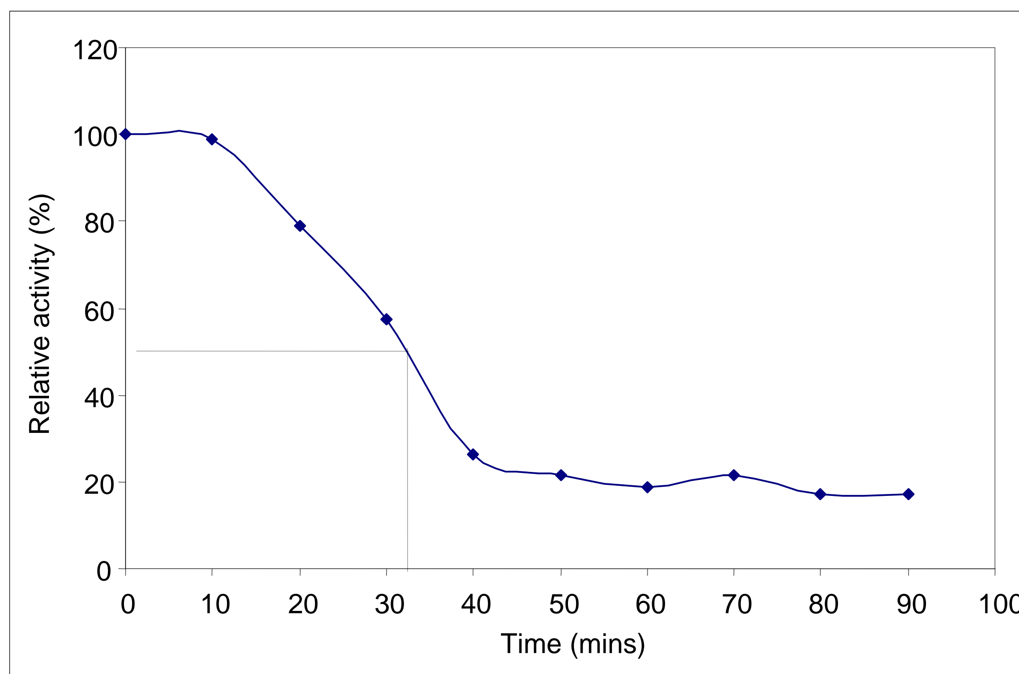


Figure 4.6 Thermal stability of hydrogenase enzyme.

Hydrogenase enzymes are highly sensitive to oxygen and as such all studies involving these enzymes are carried out under strict anaerobic conditions (Kamachi *et al*., 2000). Total anaerobic conditions cannot be achieved and therefore the lack of thermal stability to some extent can be attributed to the exposure to oxygen. The poor thermal stability observed in this study is a major drawback towards the biotechnological application of these enzymes in the bioremediation of textile effluent. Furthermore, there is no evidence of any commercially available hydrogenase enzymes, therefore reflecting the extreme difficulties encountered in purification of this enzyme. These findings suggest it may be wise to use whole cells for the bioremediation of textile waste water instead of purified enzyme extract.

4.3.2.4 Kinetic parameters

An investigation into the effect of substrate concentration on the activity of hydrogenase was investigated by measuring activity over a range of methyl viologen concentrations (0 – 10 mM). A typical increase in substrate concentration which results in a proportional increase in activity until substrate saturation was achieved thus following first order reaction kinetics (Figure 4.7).

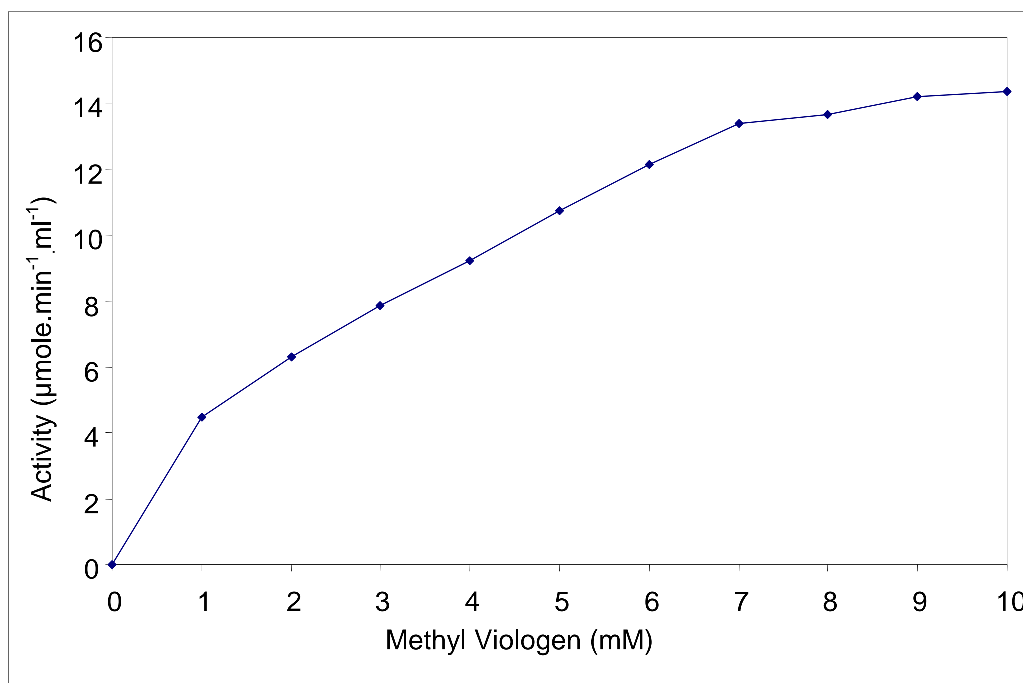


Figure 4.7 Dependence of hydrogenase enzyme activity on methyl viologen concentration.

Hydrogenase activity of the enzyme purified in this study was rather low, with a maximum activity (V_{max}) of 21.18 µmol.min^{-1} ml^{-1} being attained (Figure 4.8), while a Michaelis constant (K_M) value of 4.57 mM was recorded (Figure 4.8). These findings indicate that the purified hydrogenase has a low affinity for the substrate (methyl viologen) meaning that they are capable of catalysing a broad range of substrates and is therefore, appropriate for use in catalysing the breakdown of wide range of waste dyes generated during textile dyeing and processing.

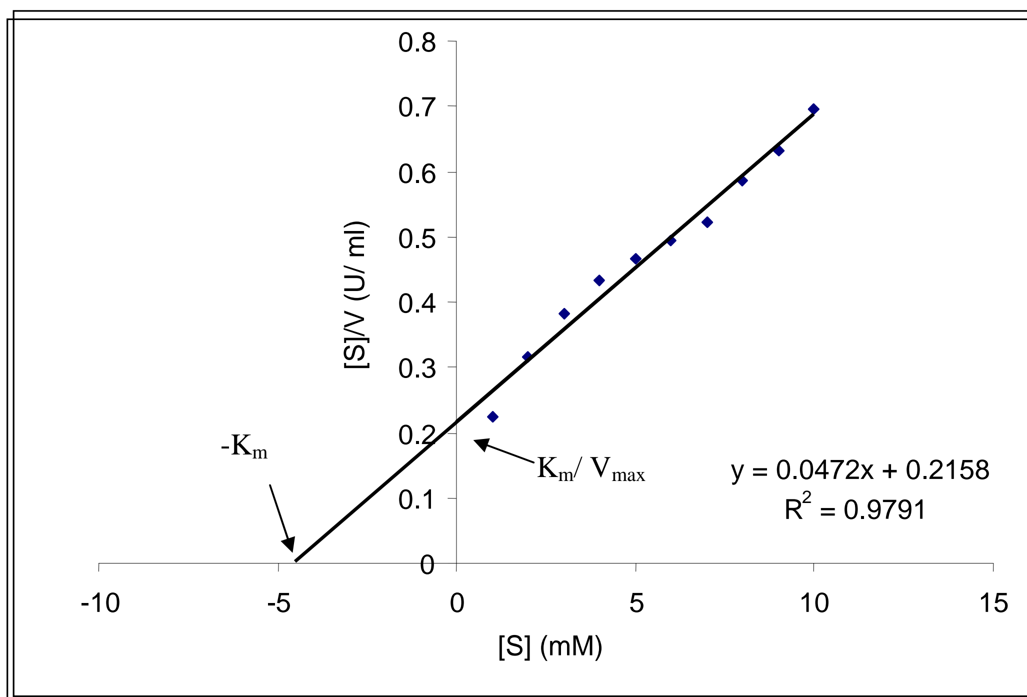


Figure 4.8 Hanes-Woolf plot of hydrogenase enzyme activity versus methyl viologen concentration

4.3.2.5 Effect of aromatic amines on hydrogenase activity

During the induction studies it was found that hydrogenase activity declined rapidly as decolourisation occurred, therefore it became necessary to investigate the effect of aromatic metabolites produced after reductive cleavage of the azo dye Orange II on hydrogenase activity. It was observed that addition of 10 - 50 mM of aniline (metabolite of orange II azo dye) into the reaction mixture stopped the reaction immediately thus suggesting the inhibition of the enzyme.

4.4 Conclusions

From the purification and characterisation studies it can therefore be concluded that:

a) the molecular weight of the purified hydrogenase was determined to be 38.5 kDa, which is in the same magnitude as Ni-Fe hydrogenase previously purified from *Desulfovibrio desulfuricans* (Nicolet *et al*., 2000),
b) the purified hydrogenase showed optimal temperature and pH of 40 ºC and 7.5 respectively,
c) the enzyme showed a poor thermal stability over time with a $t_{½}$ = 32 minutes
d) The kinetic parameters V_{max} and K_m were determined to be 21.18 U ml^{-1} and 4.57 mM respectively.

5 FBIOREMEDIATION OF TEXTILE DYES AND INDUSTRIAL EFFLUENTS

5.1 Introduction

Over the last two decades considerable amount of research has been reported on the use of micro-organisms as bioremediation agents in the treatment of dye-containing waste water (Ramalho *et al*., 2004). Although these dyes contribute a minor fraction to the usually high load of dissolved organic matter, they tend to be highly visible and must be removed before effluent that complies with government environmental legislation can be discharged into the ecosystem. The textile dyeing and finishing industry uses a wide variety of dyestuffs with world annual production amounting to more than 7 x 10^5 tonnes (Khehra *et al*., 2005). The downside, however, is that they generally experience difficulty in meeting waste water discharge limits, particularly with regard to dissolved solids, ionic salts, pH, COD, colour and occasionally heavy metals depending on the dye house (Chen *et al*., 2004). While these factors are crucial components to be considered in dye effluent treatment, coloured industrial effluent is the most obvious indicator of water pollution and the discharge of this highly coloured synthetic dye effluent is aesthetically displeasing (Chang and Lin, 2001; Khehra *et al*., 2005).

Traditional-analytical methods for measuring water colour are calibrated against a yellow-brown standard (Hazen units), and though this measures the colour of natural water on the basis of dissolved organic acid content, it is unrelated to the colour spectrum and is therefore unreliable (Carliell, 1993). A better method was developed by the American Dye Manufactures Institute (ADMI), which is independent of hue and uses the absorbance colour spectrum and can therefore be related to colour imparted by dyes. These colour measurements, however, still have disadvantages such as; interfering suspended solids that have to be removed and insoluble colour bodies that contribute to the overall colour of the effluent. It is also difficult to correlate analytical colour measurements with visual perception and therefore makes it difficult to come to a

decision as to the acceptable colour to allow discharge into the receiving bodies. In practise, the zero colour standard may be modified so that a compromise may be reached in which the impact of the coloured effluent on the receiving water body is acceptable to all existing and potential downstream users (Carliell, 1993).

The waste water characteristics from a dye house are highly variable from day to day, depending on the type of dye and fabric and concentration of chemicals used. Treatment of such waste waters is, therefore, essential but difficult. Physico-chemical treatment methods which include electrochemical oxidation and sorption are effective as tertiary treatments but are not economically viable as they result in the generation of secondary pollutants that require further treatment (Kumar *et al*., 2005). This has led to extensive research for development of cheaper alternatives such as biological treatment. Many of the world's textile manufacturers are equipped with their own waste water treatment plants which usually combine biological processes with physical – chemical process (Coste, 1996).

Many micro-organisms belonging to different taxonomic groups have been reported to decolourise azo dyes and thus their application in the treatment of the downstream azo dye containing effluents has been investigated (Khehra *et al*., 2005; Van der Zee and Villaverde, 2005). Unfortunately the industrial application of pure cultures in the bioremediation of textile effluent has not been successful due to the heterogeneity of the components in the effluents depending on production schedule. The treatment systems having mixed microbial populations are more effective due to the concerted metabolic activities of the microbial community (Sharma *et al*., 2005). In the anaerobic microbial degradation of azo dyes, decolourisation is the first step which results from cleavage of the highly electrophilic azo bond to yield aromatic amines that cannot be further mineralized under these conditions (Rajaguru *et al*., 2000). Therefore, an ideal industrial application of microbial treatment should contain micro-organisms capable of functioning under anaerobic/ anoxic and aerobic conditions in order to achieve complete degradation.

In view of these facts, this study concentrated on the anaerobic phase of textile effluent treatment since a local textile manufacturing company (Da Gama Textiles, King William's Town, South Africa) was experiencing problems with colour removal from their effluent. As far as meeting the municipal effluent discharge regulations on other elements, their effluent quality was acceptable for discharge indicating that their Effluent Treatment Plant (ETP) was functional.

5.2 Materials and methods

5.2.1 Analysis of industrial dyes, rinse water and effluent

The samples used in this study were obtained from Da Gama Textiles (King Williams's town, South Africa), and they included two samples of reactive dyes, a sample of rinse water from the dye house and effluent discharge. The contents of these samples were unknown and therefore tests to characterize them were performed before bioremediation with SRB. An absorbance scan using Thermospectronic Aquamate (Aquamate, England) was carried out on all samples in order to determine the different absorbance maxima of the dyes in the mixture. Samples were measured for pH and COD.

5.2.2 Bioremediation studies of industrial reactive dyes

Batch reactors were setup for each sample as previously described (Section 2.2.1, Chapter 2) with the SRB carbon source as primary sewage as previously described (Section 3.2.3, Chapter 3). The starting concentration of the mixture of reactive dyes was diluted to 2000 mgl^{-1} and the pH was adjusted to 7.5 at the start of the bioreactor. Duplicate samples were withdrawn and assayed for decrease in absorbance and COD concentration.

5.2.3 Bioremediation studies on rinse water and effluent

Two bioreactors were set up as described in section 5.2.2. The starting concentration was determined from the preliminary analysis in section 5.2.1 as it was within the range acceptable for growth with SRB. Duplicate samples were also withdrawn and assayed for decolourisation and COD concentration.

5.2.4 Bioremediation of synthetic dyes

Five bioreactors were setup for each of the five authentic dyes in which the primary carbon source was primary sewage sludge as previously described (Section 3.2.3, Chapter 3). The starting concentration of the mixture of reactive dyes was diluted to 2000 mgl^{-1} and the pH was adjusted to 7.5 at the start of the bioreactor. Duplicate samples were withdrawn and assayed for decrease in absorbance and COD concentration.

5.3 Results and Discussion

5.3.1 Characterisation of industrial effluents

Analysis and characterisation of the samples was done so as to gain an insight into the constituents of the sample. In the textile industry the diversity and magnitude of dyes and colours required is vast. As a result, not all dyes are supplied as single units, rather the dye technologists have devised codes that specify a range of dyes (Da Gama Textiles, personal communication, 2005). This reduces the labour and time involved when single dyes are used and makes up for inefficient mixing of several dyes to achieve a particular hue on the part of the dyeing specialist. The downside of this, however, is that those dyes that are not used up during processing (due to inefficiencies in the operation or process), are discharged into the environment.

5.3.1.1 Spectral scan studies

Absorbance peaks were observed at 215, 260, 290, 400, 450, 490, 510, 515, 600 and 625 nm (Figure 5.1 – 5.3). This is indicative of the presence of different colour absorbing compounds in the reaction mixture. Rys and Zollinger (1972) reported that ‘dyes are characterised by their capacity to absorb the energy of a particular part of the electromagnetic radiation which is visible to the human eye’. Absorbance peaks observed in the range 215 – 260 nm are likely to be representative of aromatic chromophores from within the dye. Peaks in the range 400 – 490 nm are representative of yellowish green, yellow, orange and red, and those in the range 500 – 600 nm are indicative of purple, violet and blue (Table 1.2, Chapter 1, Trotman, 1990). Therefore it can be deduced that the mixture of reactive dyes obtained from Da Gama is quite diverse.

The vat print rinse sample was reported (Da Gama Textiles, personal communication, 2005) to contain insoluble vat dyes and reactive dyes that did not adsorb onto the fabric during processing and are therefore washed out during the rinse stage. The spectral absorbance scan (Figure 5.2) on the vat print rinse samples does not show dominant peaks in the visible range of spectrum, thus implying that most of the dyes applied during fixation are adsorbed onto the target fabric. When examined by the human eye, the sample is lightly coloured as compared to the darkly coloured mixture of reactive dyes. In the ultra-violet range of the spectrum, there is a distinct peak between 280 – 300 nm, indicative of the presence of aromatic compounds within the sample. The most probable origin of these could be as a result of chemical cleavage of the coloured dyes to yield colourless aromatic compounds.

The spectral scan of the textile effluent (Figure 5.3) generated during textile processing and dyeing showed peaks at 450, 510 and 600 nm in the visible range and 215, 230, 275 and 340 nm in the UV range. This is typical as the level of dyeing efficiency in most dye house operations is poor, with up to 40 % of the dyes being discharged as effluent (Stolz, 2001; Pearce *et al*., 2003; Moreira *et al*., 2004). The effluent used in this study was quite dilute (Da Gama Textiles, personal communication, 2005) and the aesthetic features were of light blue colour. This sample was taken prior to entry into a biological effluent treatment plant, and as such, the concentration and colour of the effluent had to be adjusted to suit the growth conditions of the micro-organisms.

It becomes questionable as to the reason for undertaking this current investigation if Da Gama Textiles could treat their own effluent via their Effluent Treatment Plant (ETP). Their major concern, however, was removal of the colour from the effluent which was not possible with their ETP system. The present study has shown the capacity to completely remove colour from commercial dyes and synthetic textile effluent (Chapter Three) and consequently it is reasonable to expect complete bioremediation of Da Gama Textiles effluent with our biosulphidogenic reactor.

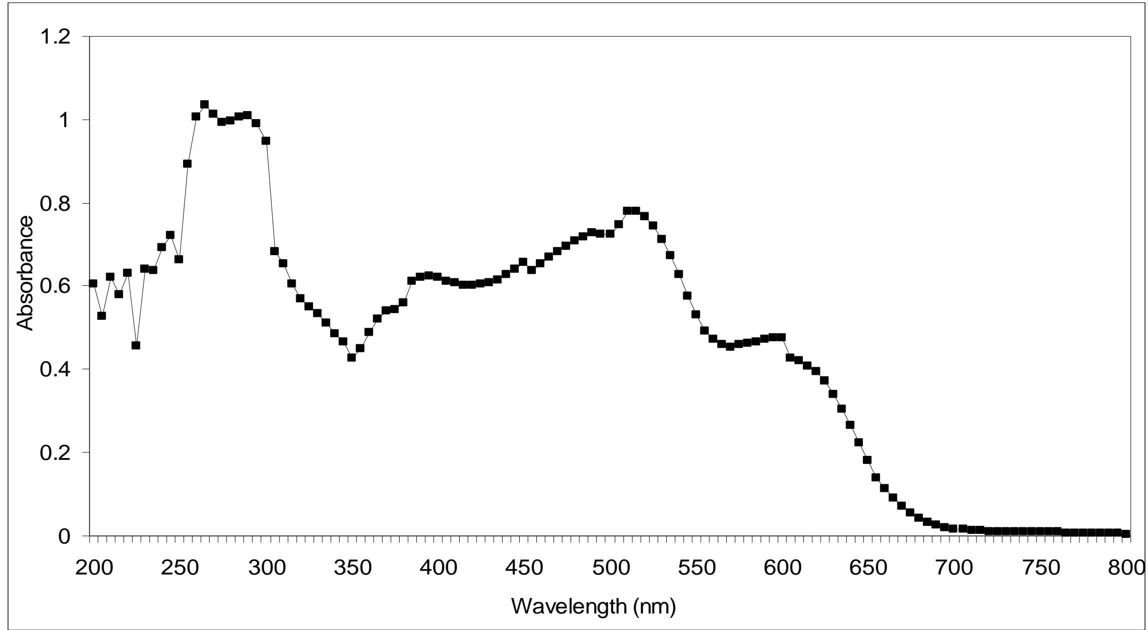


Figure 5.1 UV/Vis scan between 200 – 800 nm of a mixture of reactive dyes used for dyeing textile fibres at Da Gama textiles. The scan is similar to the mixture of reactive dyes in the presence of silicate salts and sodium hydroxide.

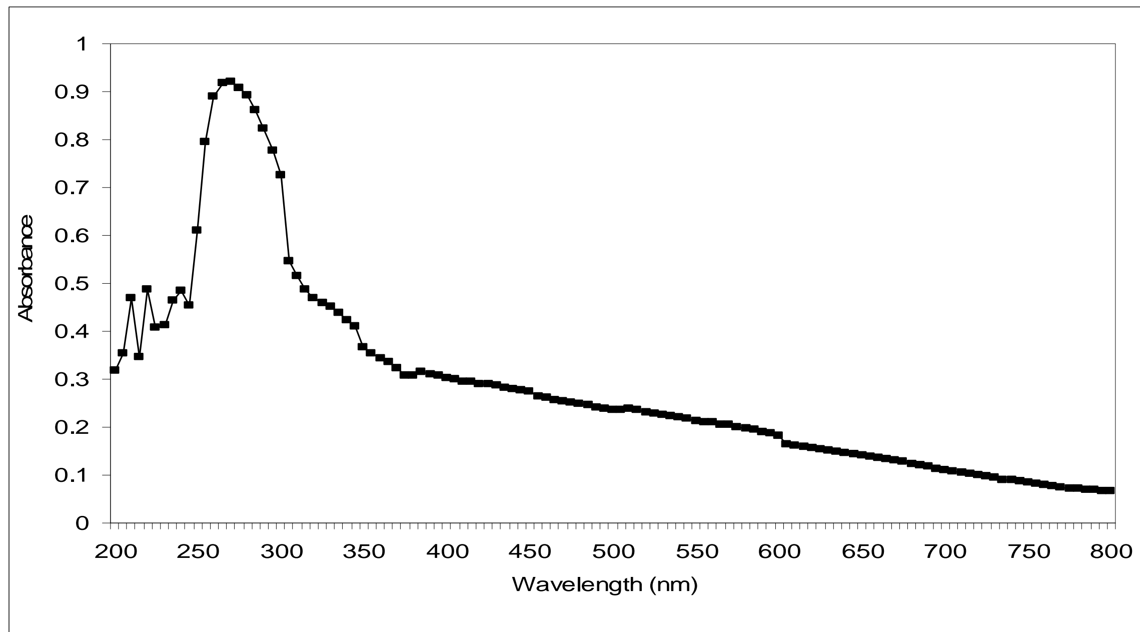


Figure 5.2 UV/ Vis scan between 200 – 800 nm of the vat print rinse from textile processing.

The concentration of the dyes and effluent was determined by measuring the COD to give an estimate of the chemical load of the samples. It was observed that the dye mixture

without alkali had the highest concentration of 178 260 mgl^{-1} COD. Addition of silicate salts and sodium hydroxide into the dye mixture resulted in dilution of the dye (Figure 5.4). Sodium silicates are generally used to enhance solubility of dyes. During the process of bleaching, hydrogen peroxide is used as a bleaching agent, but the presence of common metals such as iron, copper and manganese in the water catalytically decomposes the peroxide solutions thereby necessitating addition of more of the oxidizing agent which effectively translates to cost. The addition of silicate salts blocks the catalytic effect of these metal ions and in turn stabilizes the peroxides (PQ Corporation, 2003).

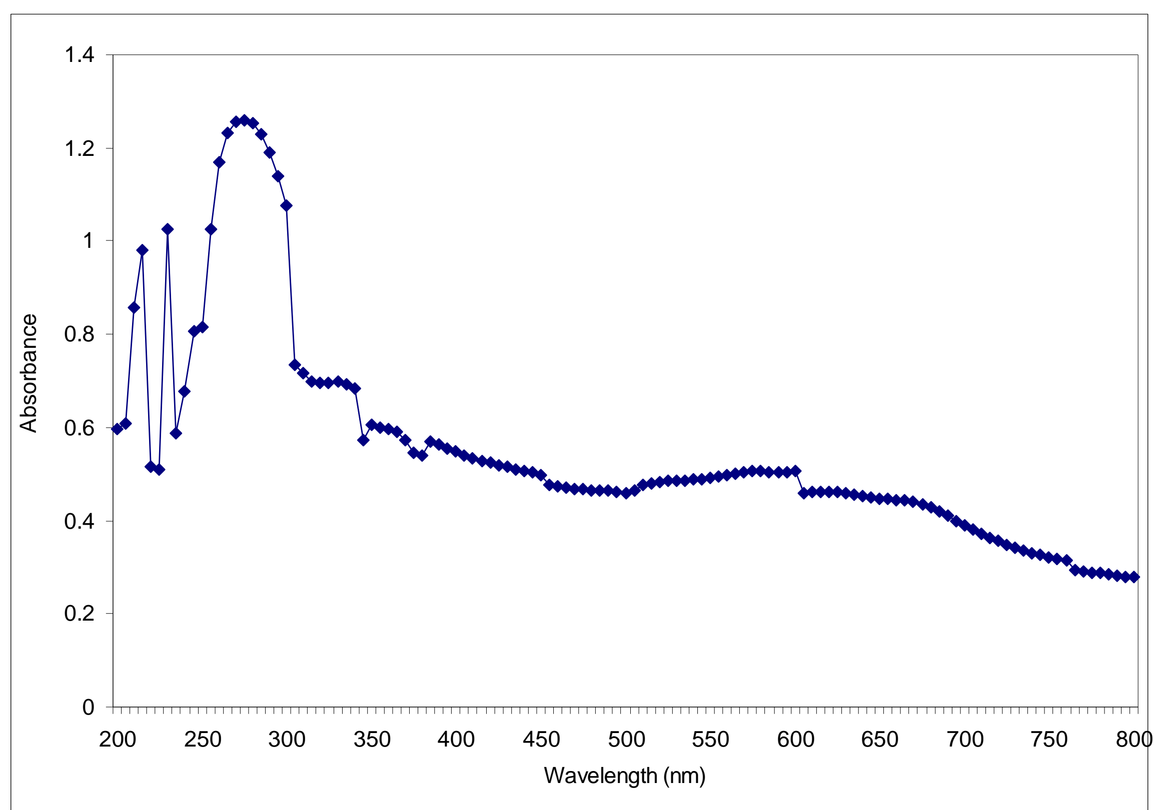


Figure 5.3 UV/ Vis scan between 200 – 800 nm of the waste effluent generated after the dyeing processing.

Alkaline conditions enhance the fixation of dyes onto fabric by providing hydroxyl ions which increase the binding affinity (Trotman, 1990). This explains why there is addition of these additives prior to textile dyeing. It remains to be answered what consequences these additives have on downstream bioremediation of the effluent. While it is apparent that an increased pH is necessary for application of the dyes, biological treatment

demands mild alkaline conditions for optimal bioreactor performance, leading to the slight neutralization done on the reactive dye mixtures.

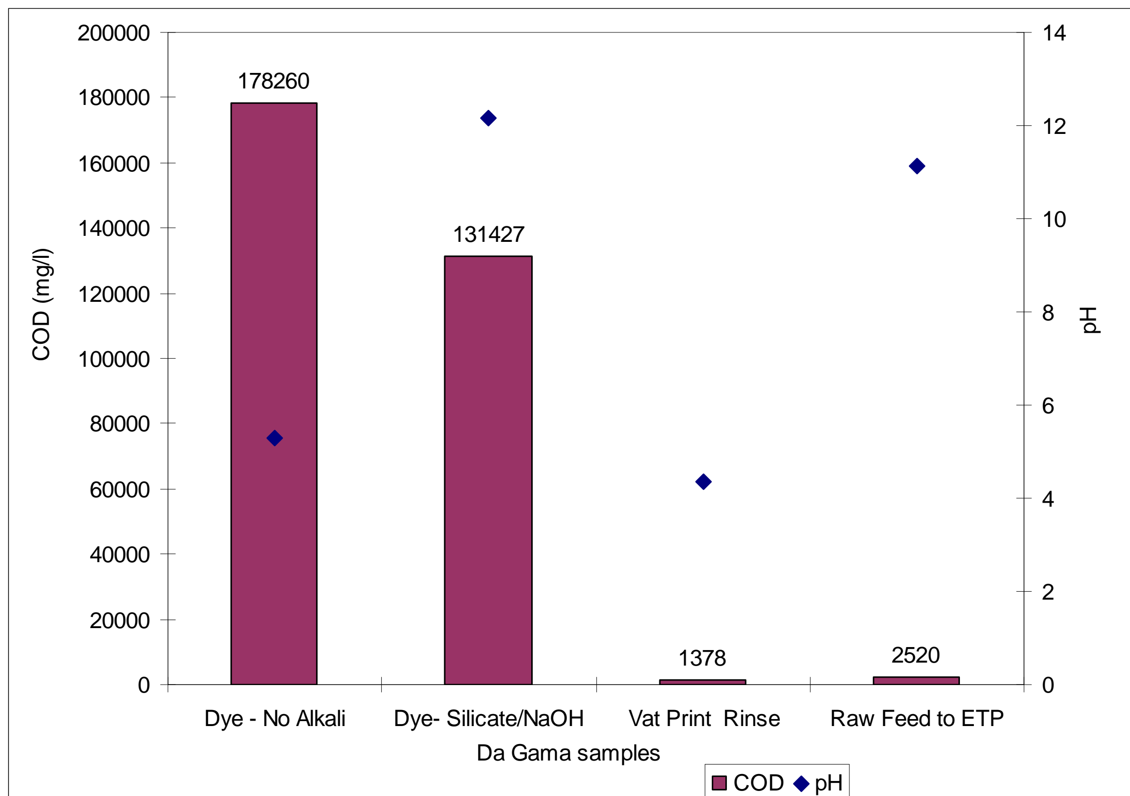


Figure 5.4 The concentration of the dyes and the effluents were measured by determining the COD. The pH of the dyes and effluents was also measured.

5.3.2 Decolourisation of authentic dyes by sludge SRB

The results obtained from the azo dye degradation by pure 'lactate' cultured SRB provided a basis for the application of sludge cultured SRB on the degradation of azo dyes. While the culture from the local municipal sewage works predominantly constituted sulphate reducing bacteria, the presence of other bacteria such as methanogens and *Sphingomonas* species (Van der Zee, 2002) cannot be ruled out and may have contributed to the degradation process.

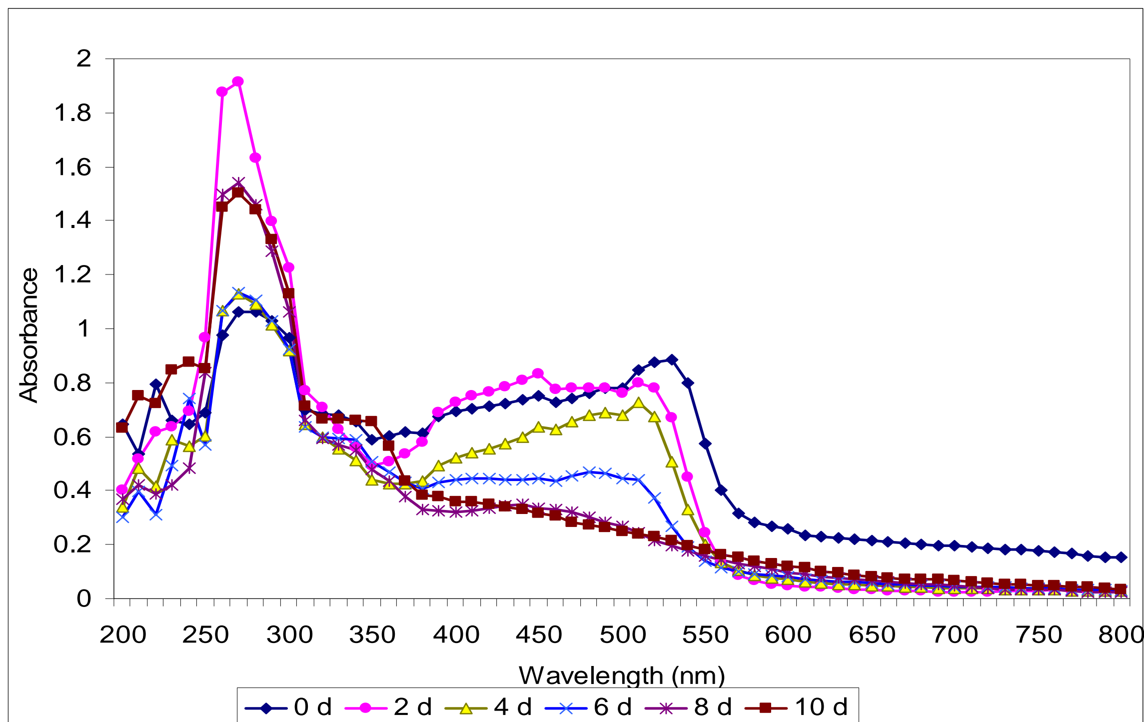


Figure 5.5 Decolourisation of Orange II by SRB from the Grahamstown municipal sewage works.

Incubation of the azo dye Orange II with sludge SRB resulted in 60 % removal of colour after eight days. Unlike with the pure SRB culture studies, an interesting observation was made during these studies in which a shift in the maximum absorbance peak (490 nm to 530 nm) of the dye was noticed at the beginning of the experiments. A spectral absorbance scan over time revealed a shift in the maximum absorbance peak from 530 to 490 nm after day six (Figure 5.5). A probable explanation of this deviation could be the interference of the sludge culture and biomass which is characteristically black, and therefore as the degradation progressed there was a gradual depletion in the colour of the dye as well as the biomass.

It was also noticed (Figure 5.5) that after two days of incubation, there was a sharp increase in the UV range of the spectrum (260 – 290 nm), which indicated the accumulation of aromatic compounds in the medium as a result of azo degradation. The peak at 290 nm then decreased after six days of incubation. After eight days, there was another gradual increase in the peak again, which coincides with maximal rate of Orange II decolourisation. It is possible to assume that the decrease in the UV absorbance range

could have been a result of breakdown of the aromatic compounds by the mixed consortia of micro-organisms. There is, however, an overall increase in the amount of aromatic compounds absorbing in the UV range after 60 % of the colour has been removed from the medium which suggests transformation of the azo dye into its constituent aromatic compounds.

The degradation of the other azo dyes followed the same trend with 70 % of the Reactive Black 5 being removed after the first 24 h (Figure 5.6), reaching a maximum of 74 % after 10 days of incubation. A gradual decrease in the UV absorbance also indicates further degradation of aromatic compounds.

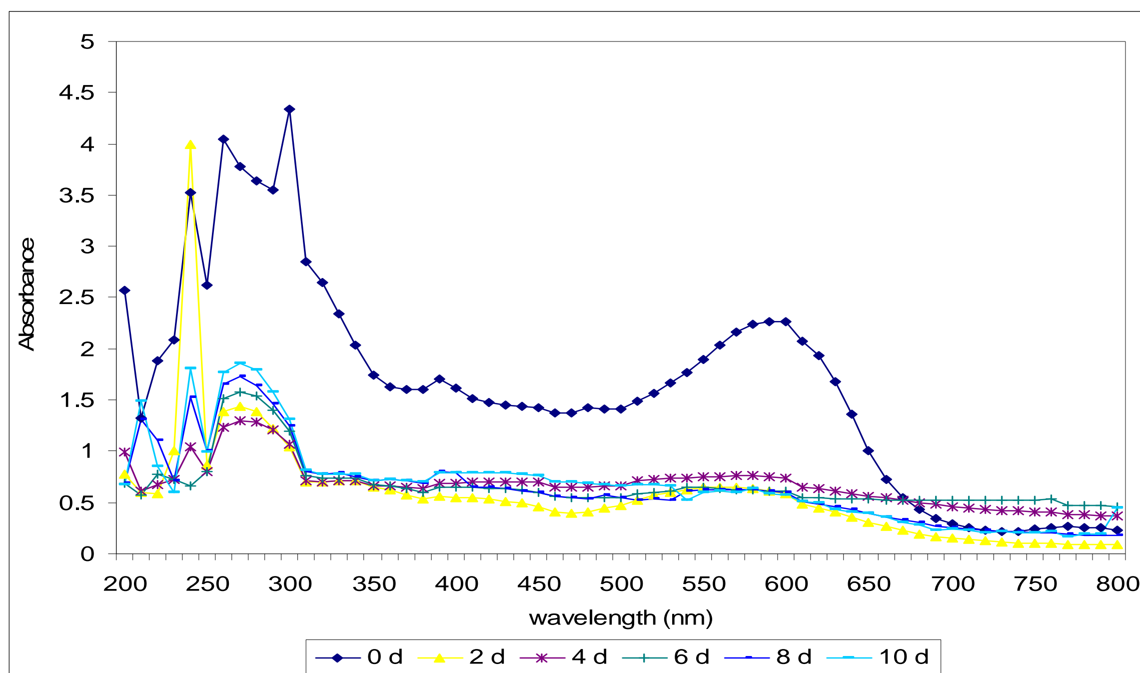


Figure 5.6 Decolourisation of Reactive Black 5 by SRB from the Grahamstown municipal sewage works.

With Reactive Red 120 there was a gradual decrease in colour over time (Figure 5.7) with a maximum decolourisation rate of 49 % being achieved after 10 days. The aesthetic properties of the bioreactor at the end of the incubation showed that the colour had almost been completely removed from the medium (Figure 5.8).

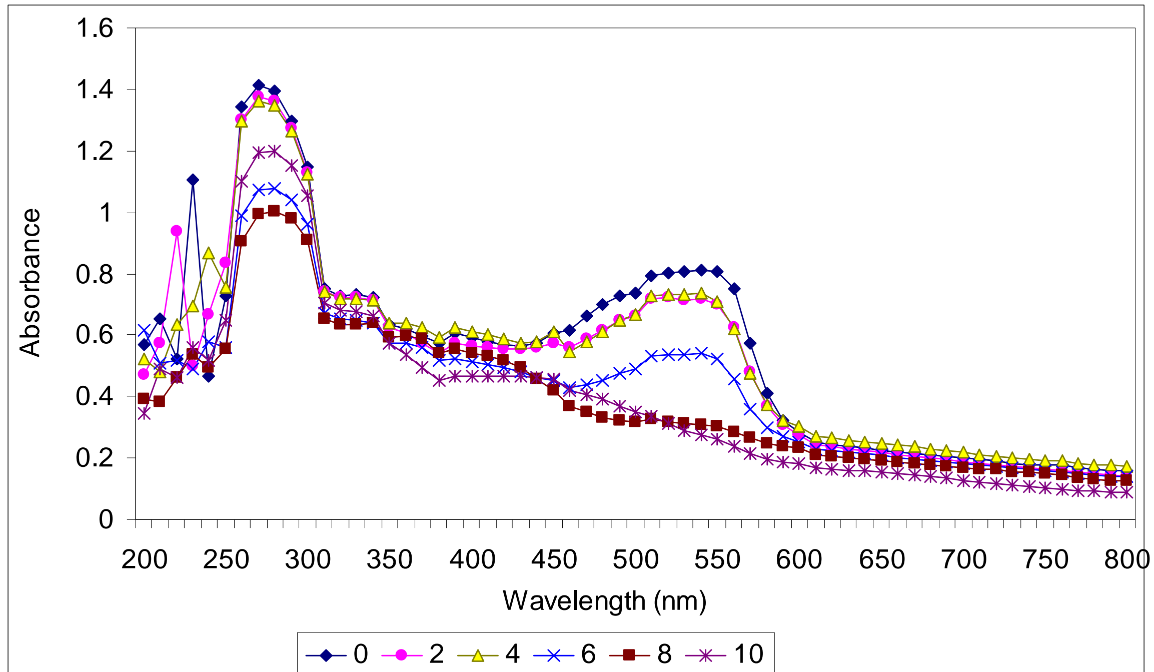


Figure 5.7 Decolourisation of Reactive Red 120 by SRB from the Grahamstown municipal sewage works.

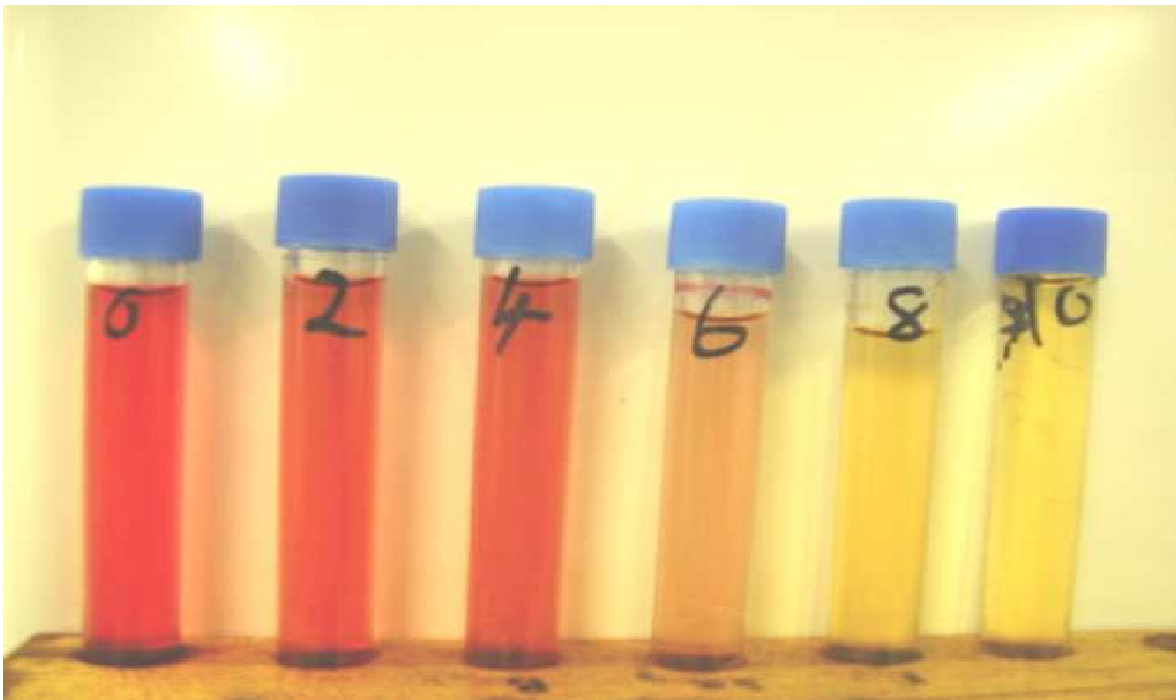


Figure 5.8 Progressive removal of Reactive Red 120 from sludge medium by SRB from the Grahamstown municipal sewage works. The numbers represent days of incubation.

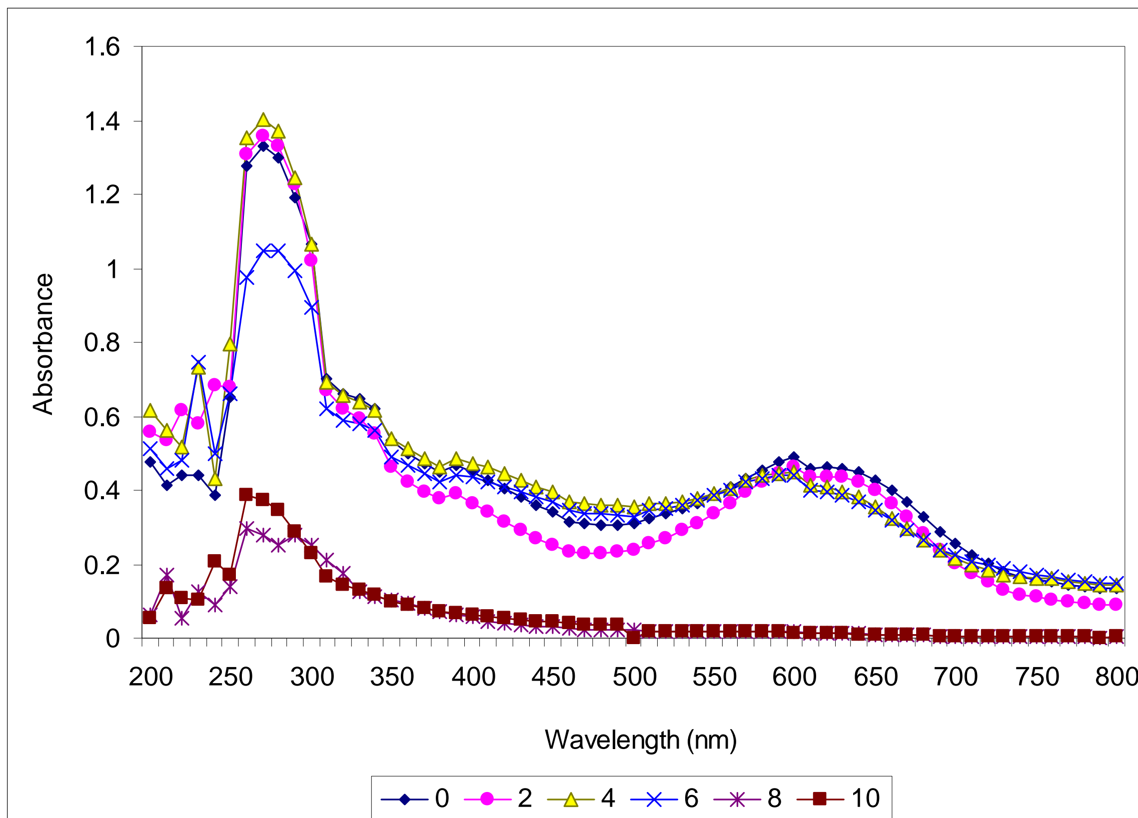


Figure 5.9 Decolourisation of Reactive Blue 2 by SRB from the Grahamstown municipal sewage works.

The decolourisation rate of the anthraquinone dye Reactive Blue 2 was initially quite slow with only 10 % of the dye being removed after six days of incubation. It however accelerated and increased to 96 % after day eight (Figure 5.9) and this was most likely due to adaptation of SRB and other micro-organisms present in the bioreactor, consequently leading to the degradation of the fused ring characteristic of anthraquinone dyes and complete removal of the blue colour (Figure 5.10).

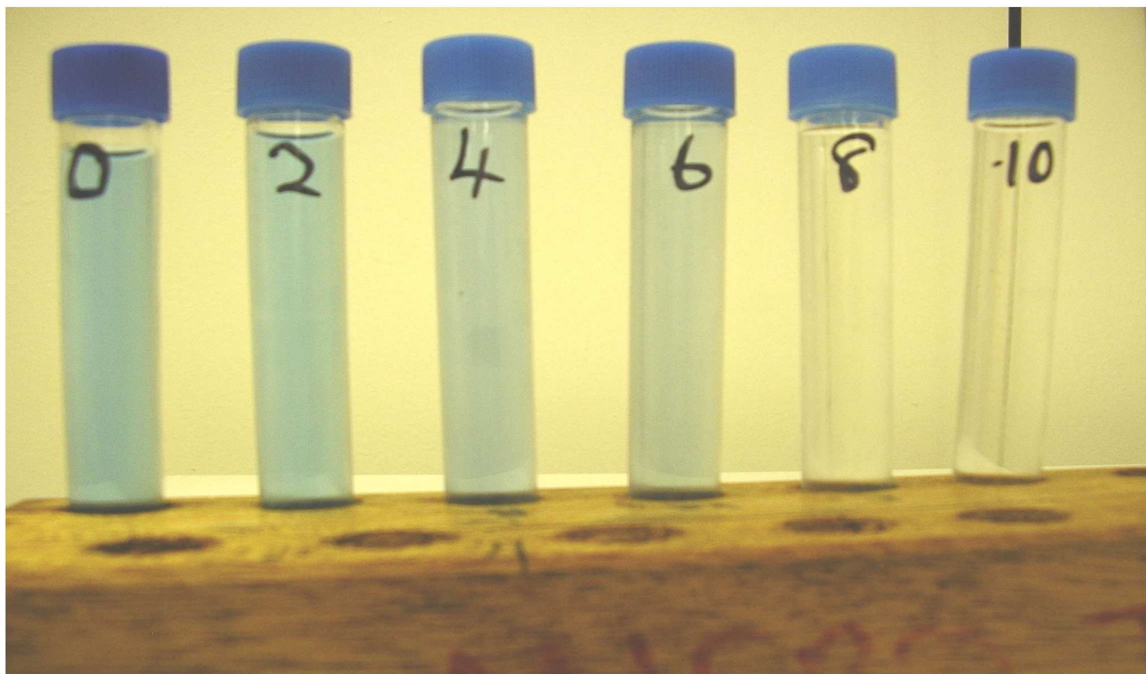


Figure 5.10 Progressive decolourisation of Reactive Blue 2 from sludge medium by SRB from the Grahamstown municipal sewage works. The numbers represent days of incubation.

5.3.3 Decolourisation of industrial textiles and effluents

The successful decolourisation of synthetic azo dyes by sludge cultured SRB led to pilot trials on industrial textile dyes and the respective effluent generated after processing. A decolourisation rate of 93 % was achieved in 24 h for the mixture of reactive dyes (Figure 5.11) and the rate is similar to that observed with the synthetic azo dye Orange II (Section 3.3.4, Chapter 3). The addition of salts and alkali did, however, affect the rate of colour removal from the bioreactors as observed by a decrease in the rate to 72 %. Since the presence of the alkali was not significant as the pH had been neutralized before starting the bioreactors, the presence of the silicate salts must have attributed to the decreased efficiency. This could have been due to the distortion of the ionic charges on the enzymes catalyzing the breakdown of the reactive dyes. The hydrogenase enzyme that was partially purified in this study was shown to be similar to Fe-hydrogenase through SDS-PAGE (Section 4.3.1.2, Chapter 4,), and since it has been reported (PQ Corporation, 2003) that the effect of silicate salts in dye mixtures is to block the effect of ions such as iron, it is probable that these silicates affected the structural moiety of the hydrogenases. The silicates did not completely inhibit the enzyme action because their concentration

had been significantly diluted when the COD was adjusted to meet the requirements for incubation with the bacteria and also the incubation time was minimal (24h). The use of whole cells as opposed to cell free extracts in industrial applications offers the advantage that cells can protect or buffer the enzymes against enzyme inhibiting or denaturing compounds as observed in this study.

Decolourisation rates of the effluent samples were low at 52% and 41% for vat print rinse and ETP samples respectively. This can be explained by the observation that colour in these samples was quite minimal as demonstrated by the spectral scan studies (section 5.3.1.1). The decolourisation thus observed was derived from the residual dyes remaining in the effluent which are not aesthetically significant but can still be detected. It was observed that there was no colour remaining after incubation of the effluents with SRB whole cells.

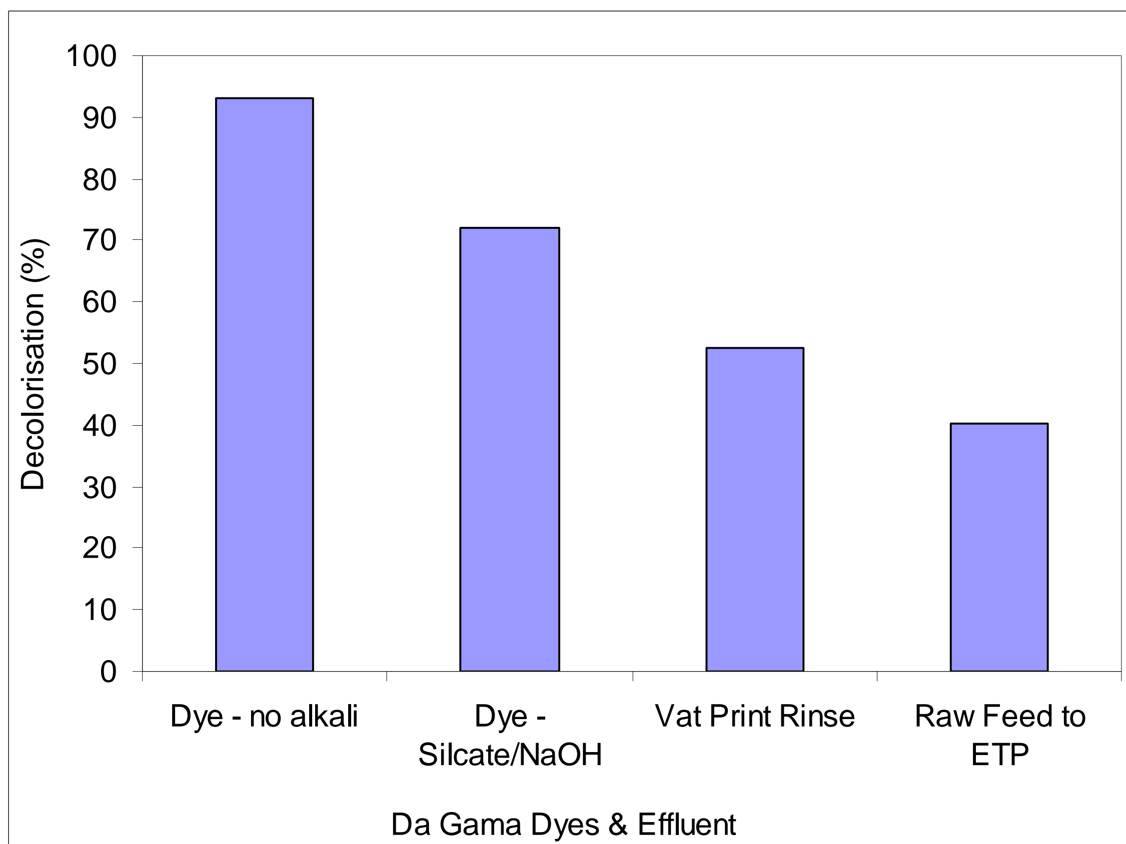


Figure 5.11 Decolourisation rates of Da Gama dyes and effluents by sulphate reducing bacteria after 24 h.

5.3.4 Degradation of aromatic amines

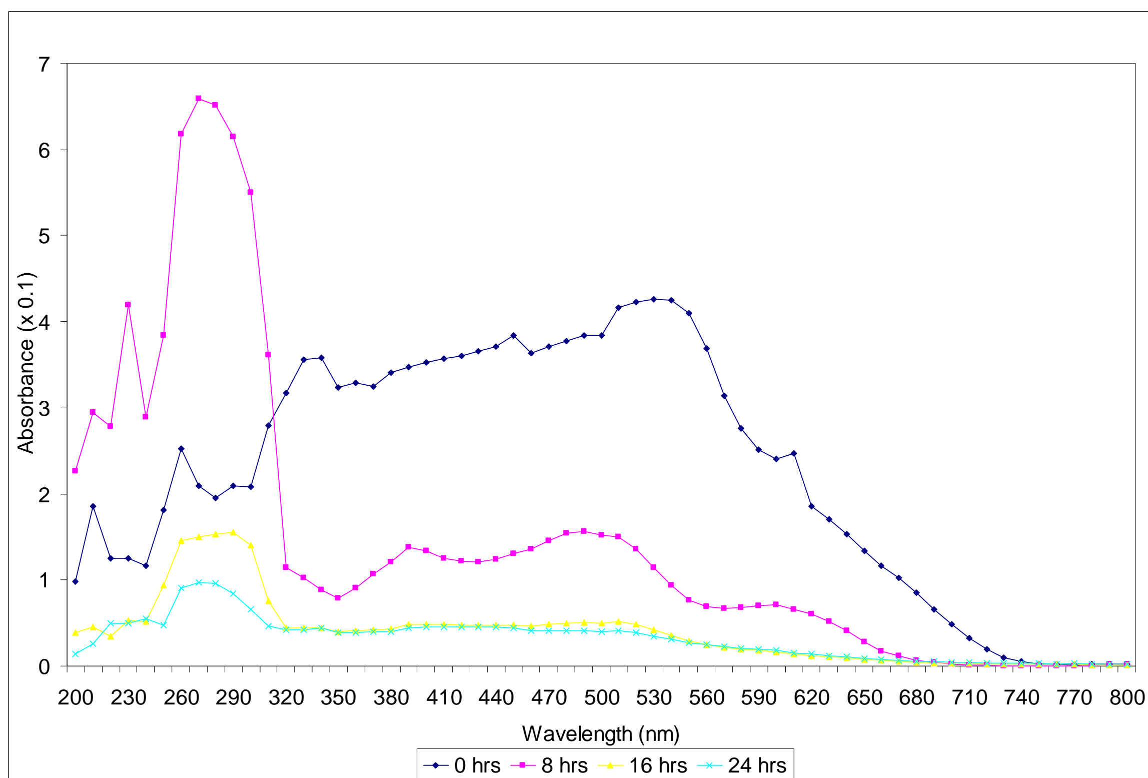


Figure 5.12 Absorbance scan of Da Gama reactive dye degradation by sulphate reducing bacteria.

The degradation of azo dyes is normally characterised by the formation of colourless aromatic amines (Ambrosio and Campos-Takaki, 2004). An increase in the absorbance peak in the UV spectrum in the range 260 – 290 nm was observed in all bioreactors, suggesting that the dyes were being broken down into their constitutive amines. It was observed that these peaks decrease during incubation of the reactive dye mixtures with SRB whole cells. While it has been reported that degradation of aromatic amines readily occurs under aerobic conditions (Peace *et al*., 2003; Sponza and Isik, 2003), an interesting observation was noted during the degradation of Da Gama reactive dyes (Figure 5.12), as the aromatic amines were mineralized, as indicated by the decrease in absorbance in the UV range (260 – 290 nm). It may be argued that the amines underwent auto oxidation, which is highly unlikely as our bioreactors were operated under strict anaerobic conditions. Exposure of the bioreactor to air may have resulted in reverse

oxidation of the aromatic amines, to give coloured compounds not similar to the original colour of the dye. In this case there was no colour formation observed when the bioreactors were stopped and exposed to oxygen. Therefore, it can be concluded that the sewage sludge cultured SRB-'mixed' consortia were able to completely mineralize the reactive dyes from Da Gama textiles (Figure 5.12). This was not observed when pure 'lactate' SRB was incubated with the same azo dye (Section 3.3.5, Chapter 3) and therefore suggests the presence of a synergistic mechanism, in which the dyes are cleaved by the SRB and breakdown of the aromatic amines formed is facilitated by the other micro-organisms present in the culture. Furthermore as a result of the breakdown of the aromatic compounds, the bioreactor is capable of being active for a prolonged period (up to 10 days). This aspect will be investigated by future research.

5.4 Conclusions

Investigation of the ability of sulphate reducing bacteria to bioremediate textile industrial effluent led to the following conclusions:

a) sulphate reducing bacteria are capable of degrading synthetic, industrial textile dyes and their effluents using the sewage sludge as a primary carbon source,
b) bioremediation of textile industrial dyes by SRB results in the breakdown of sewage sludge thus effectively treating one effluent using one process, and
c) there is evidence of complete breakdown of aromatic compounds derived from the dyes under anaerobic conditions.

6 GENERAL DISCUSSION AND CONCLUSION

The combined effect of the increasing population growth and industrial development has inevitably led to an increased volume of industrial based pollutants which have a serious impact on the environment. While, the advent of technology has brought about simplified and modern products onto the market, the downstream effects of these products tend to pose hazardous effects on both the users and the environment. Textile production results in production of potentially carcinogenic and harmful waste water, which if untreated are discharged into the ecosystem either by seepage into aquifers to underground water bodies or into established mainstream water bodies. Traditional treatment methods have not only managed to remove the effluent from the water, but have rather aggravated the problem by introducing secondary effluent through chemicals used in the 'effluent treatment'. This has led man to turn back to Mother Nature to try, where application of micro-organisms in the treatment of various industrial wastes including dye containing effluent has been investigated for the last two decades (Asamudo *et al*., 2005).

In light of this on going research, anaerobic bacteria have found favour in the treatment of textile effluent, mainly due to their robust nature diversified range of carbon sources, with particular reference to SRB which produce sulphide that normally inhibits the proliferation of pathogenic bacteria and can therefore be utilized on a large scale. This current study investigated the potential of SRB to produce enzymes that can degrade textile effluent. Azo dyes are the most commonly used colouring compounds (Pearce *et al*., 2003) and they were thus used in this study to investigate the ability of SRB to degrade them under anaerobic conditions. It can be concluded that SRB can breakdown azo compounds under anaerobic conditions, and hydrogenase enzyme found in the cytoplasm was responsible for this cleavage of the azo bond. While hydrogenases have not been reported to breakdown azo textile dyes, the anaerobic degradation of textile dyes

has been reported to be catalysed by non-specific reductases found in the cytoplasm of bacteria (Stolz, 2001; Van der Zee, 2002). Sonication at low amplitude (10W) for four minutes proved to be the best extraction method for hydrogenase from the SRB.

After identifying and optimizing the extraction of hydrogenase, a time course survey was carried out to determine optimal time at which highest enzyme production occurs, and this was found to occur after six days. Following this, substrate induction using commercial dyes was investigated and it was found that the presence of these dyes in the bioreactor increased the relative hydrogenase activity by 140% in 24h. Not all the dyes induced hydrogenase activity, however, the anthraquinone dye Reactive Blue 2 did not induce enzyme activity even after 10 days of incubation. This was likely due to the structural moiety of the dye which did not stimulate the production of more enzymes. It was shown that the mono azo dye Orange II resulted in the highest enzyme production while the di-azo Reactive Black 5, Amido Black 10 and, Reactive Red 120 resulted in 80 % increased enzyme activity in 24h. Corresponding decolourisation rates were also observed in the same order as the decolourisation rates of the mono azo dye was higher than that of the di-azo dyes. The threshold levels of the dye concentration were determined to be in the range 100 – 500 mgl^{-1}; higher concentrations resulted in limited decolourisation of the dyes (decolourisation was probably due to adsorption by the dead SRB cells). Lactate as a primary carbon source resulted in higher rate of decolourisation when compared to primary sewage sludge.

After the induction trials it was necessary to purify and characterise the hydrogenase enzymes and two strategies were employed. The first involved concentration of the enzyme sample by freeze drying and resulted in two fractions (one at fraction no. 17 and the other at no. 73) containing hydrogenase activity with a specific activity of 2.56 and 1.626 $U.mg.protein^{-1}$ with a fold purification of 3.13 and 1.47. The second strategy used PEG for concentrating the enzyme sample, this however revealed only one fraction that eluted at no. 17 and it had a specific activity of 2.49 and a purification fold of 3.03. Analysis of the fractions from both strategies by a 10 % SDS-PAGE showed a distinct band with a molecular size of 38.5kDa which is in the same magnitude as other

hydrogenases purified from SRB. Characterisation studies further showed that hydrogenase operates optimally at a pH of 7.5 and temperature of 40° C and poor thermal stability over time ($t_{1/2}$ life = 32 minutes). Kinetic parameters K_m and V_{max} for methyl viologen were found to be 4.57 mM and 21.18 U ml^{-1} respectively.

The overall objective of the present study was to effectively bioremediate textile effluent using a purified hydrogenase extract, however, trials conducted using both the crude and purified hydrogenase extracts were not successful. The most likely explanation could be that the redox potentials in the reaction mixture were not favourable enough to facilitate azo bond cleavage. This problem was overcome by making use of whole SRB cells which resulted in complete decolourisation of the azo dyes. It can be concluded that the decolourisation of the azo dyes was a biological reaction because the two controls used, in which one had an autoclaved SRB inoculum while the other did not have any inoculum remained unchanged, thus indicating that the presence of live SRB resulted in breakdown of the dyes.

Analysis of the reactive dye mixtures and their respective effluents after processing enabled the characterisation of these industrial fluids. An absorbance spectrum revealed several maximum absorbance peaks ranging from 215 – 625 nm, thereby revealing the presence of different reactive dyes as specified by the textile company (Da Gama Textiles, King William's Town, South Africa). A spectral absorbance scan of the effluent revealed dominant peaks in the UV range and smaller peaks in the visible range. This gave an indication that the bulk of the dyes had been adsorbed onto the fabric during processing and to some extent it also highlights the efficiency of the dye house. After characterisation of the dyes, and effluents, they were then degraded by SRB under anaerobic conditions. Decolourisation rates of 93 % and 72 % for the dye mixture and the dye mixture plus silicate salts were observed. While the primary function of these salts is to facilitate dye-fabric interaction, their presence, downstream, inhibits the bio-catalytic action of enzymes during effluent treatment and as such would need to be removed or diluted to levels that don't affect bioremediation of the effluent.

The use of primary sewage as a carbon source for the bioremediation of textile effluent offers a cheaper alternative, and was thus investigated in the current study. Successful decolourisation of commercial dyes with sewage sludge as the primary carbon source were investigated with decolourisation rates ranging from 96 – 49 % for Reactive Blue through to Reactive Red 120. This led to the degradation of industrial dyes and their effluents by SRB using primary sewage sludge, and yielded decolourisations rates ranging from 93 – 41% and thus proved that sewage sludge effluent could be used as a carbon source for the bioremediation of industrial textile dyes containing effluent.

Future work involves

a) the chromatographic analysis of the aromatic breakdown products that are formed after breakdown of the azo bond,
b) setup of an aerobic system to effectively degrade the aromatic compounds that are formed during azo dye degradation,
c) trials on other industrial effluents such as pulp and paper, dairy, tannery, brewery using SRB and primary sewage sludge as a carbon source,
d) establish the enzymatic mechanism for the breakdown of aromatic amines, and
e) establish a quantitative evaluation of the process.

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APPENDICES

Appendix A: Modified Postgate B preparation

Solution A: Add 6 ml Sodium lactate (60% solution w/w), 4.5g Na_2SO_4, 1.0g NH_4Cl, 1.0g Yeast extract, 0.5g KH_2PO_4, 0.1g Sodium citrate.$2H_2O$, 0.06g $CaCl_2.6H_2O$ and 0.06g $MgSO_4.7H_2O$ and dissolve all components in 990 ml of distilled/deionised water.
Solution B: Dissolve 1.0g ascorbic acid and 1.0g sodium thioglycholate in 100 ml of distilled/deionised water.

Final preparation of media: The pH of solution A was adjusted to pH 7.5 with 5M NaOH solution. The solutions (A and B) were then autoclaved separately for 15 minutes at 15 psi -121°C. To make 1L of medium, 10 ml solution B was then added to 990ml solution A under a UV hood.

Appendix B: Method of protein determination - Bradford assay (Bradford, 1976)

B1: Protein stock solution (2mg/ml)

Dissolve 0.02g of Bovine serum albumin (BSA) in 10 ml of ddH_2O.

B2: Protein standard curve

Different protein concentrations were prepared from the BSA stock solution (2mg/ml) as shown in Table A1. A protein standard curve was generated by reading the absorbance of these different concentrations at 595nm (Figure I). For the test sample, 250µl Bradford reagent was added to 5 µl diluted sample and the absorbance read at 595nm.

Table B1: Preparation of protein standard curve

BSA Stock solution (40µg/ml) (µl)	ddH_2O (µl)	Bradford reagent (µl)	Protein concentration (µg.ml^{-1})
0	5	245	0
1	4	245	8
2	3	245	16
3	2	245	24
4	1	245	32
5	0	245	40

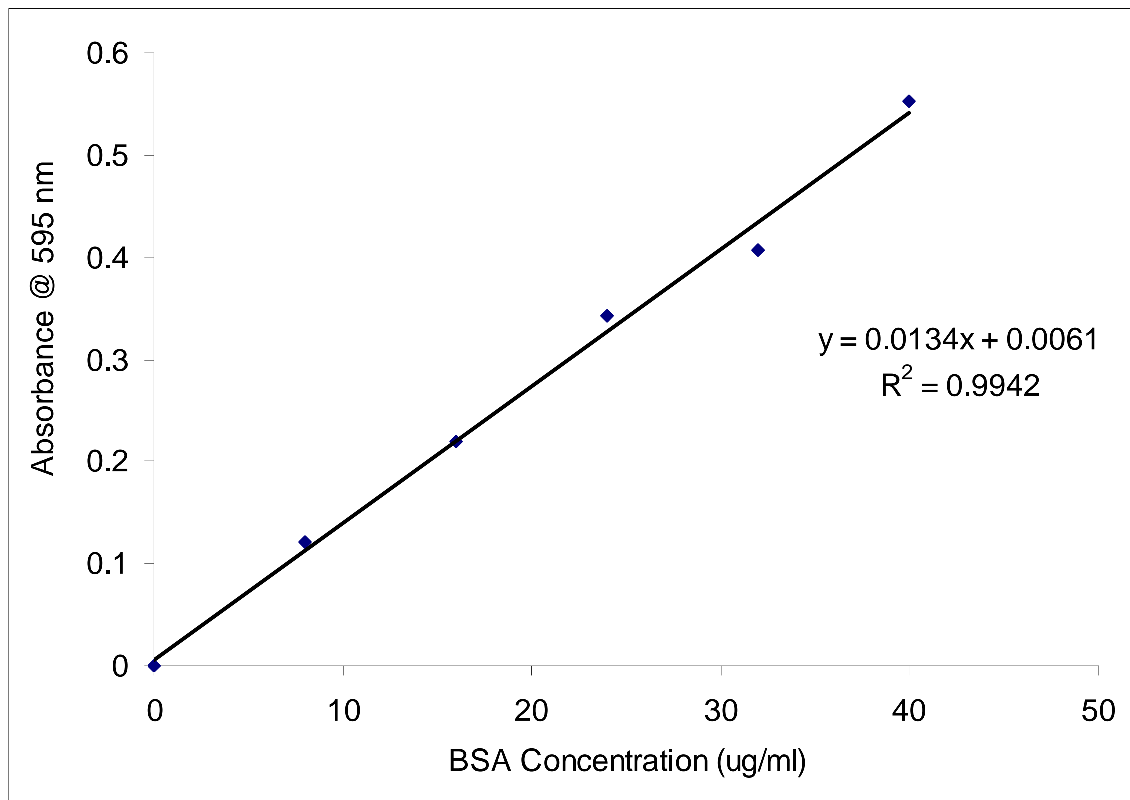

Figure I Protein Standard Curve.

Appendix C: Methods used for purification and partial characterization of hydrogenase enzyme produced by sulphate reducing bacteria

C1 SDS-PAGE

Solution A: Acryl amide stock solution (30% acryl amide, 0.8% bis-acryl amide) – weigh 29.2g acryl amide and 0.8g bis-acryl amide, and then dissolve in 100ml de-ionised water and stir until acryl amide powder is completely dissolved. Store in the dark at 4 °C (30 days maximum).

Solution B: 4x Separating Gel buffer – add 75ml of 2M Tris-HCl buffer (pH 8.8) and 4ml of 10%SDS and then make up the volume to 100ml with deionised water.

Solution C: 4X Stacking Gel buffer – add 50ml of 1M Tris-HCl buffer (pH 6.8) and 4ml 10% SDS, and make up the volume to 100ml with deionised water.

Solution D: 10% Ammonium per sulphate solution (APS)-dissolve 0.5g ammonium persulfate in 5ml deionised water.

Solution E: Electrophoresis buffer – add together 3g Tris base, 14.4g glycine and 1g SDS. Dissolve all three in 1 litre of deionised water.

Solution F: 5× Sample buffer – add together 0.6ml 1M Tris-HCl buffer (pH 6.8), 5ml 50% glycerol, 2ml 10% SDS, 0.5ml 2-mercaptoethanol, 1ml 1% bromophenol blue, and make up the volume to 10ml with deionised water.

Solution G: Coomassie blue staining solution – dissolve 1.0g Coomassie Blue R250 in a mixture of 450ml methanol, 450ml deionised water and 100ml glacial acetic acid.

Solution H: Coomassie Gel Destain solution – add together 100ml methanol, 100ml glacial acetic acid and 800ml de-ionized water.

Table C1 Recipe for Resolving Gel

Solutions	Volume (µl)
Water	2300
30% Stock Acryl amide	5000
Tris- HCl pH 8.8	2500
10% SDS	100
10% Ammonium per Sulphate	100
TEMED	10

Table C2 Recipe for stacking gel

Solutions	Volume (µl)
Water	2700
30% Stock Acryl amide	600
Tris- HCl pH 6.8	500
10% SDS	40
10% Ammonium per Sulphate	40
TEMED	10

C2 Molecular weight determination

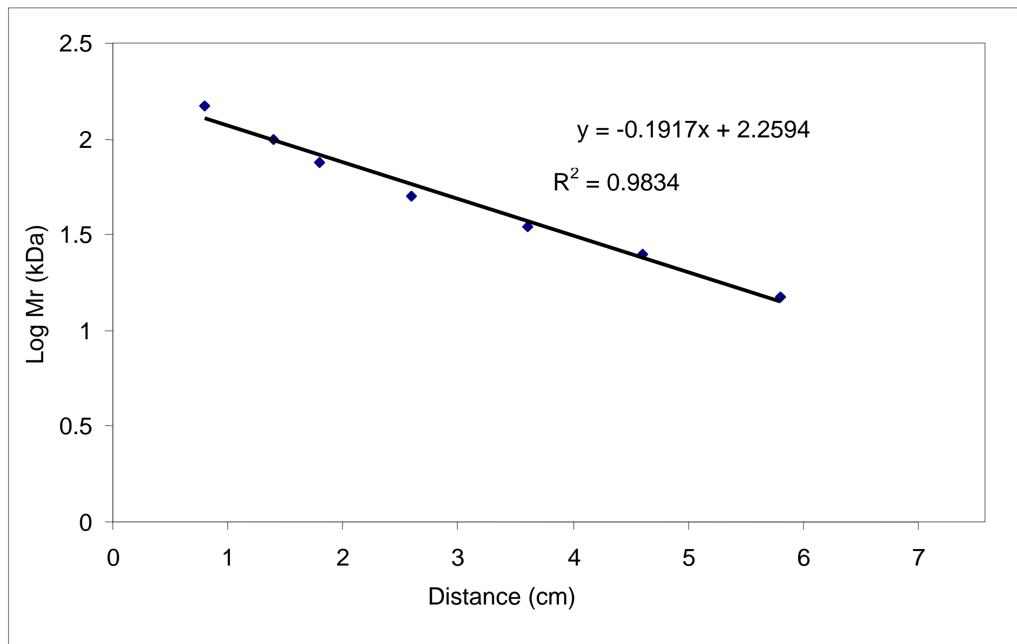

Figure II Calibration curve of the log molecular weight (M_r) markers. The molecular weight markers used consisted of seven precisely sized recombinant proteins of 15 000, 25 000, 35 000, 50 000, 75 000, 100 000, 150 000 daltons (Perfect Protein ™ Markers, 15 – 150 kDa, Novagen, Germany)