EXECUTIVE SUMMARY

Background

A previous Water Research Commission Project (WRC Report No: 1170/1/04) into the enzymology of solubilisation of municipal sewage sludge (Rhodes University BioSURE Process®) identified the involvement of a plethora of hydrolase enzymes such as phosphatases, sulphatases, proteases, lipases, endoglucanases and glucosidases (Pletschke *et al.*, 2004; Whiteley *et al.*, 2002; Enongene, *et al.*, 2003; Ngesi, *et al.*, 2002) isolated from a biosulphidogenic reactor. Furthermore it was found that these enzymes could be used, *in situ*, to bioremediate effluents from acid mine drainage, tanneries and abattoirs. It is the intention of the current research to exploit this idea further and undertake a thorough investigation to show that hydrogenase enzymes, also found within the biosulphidogenic reactor, could be used to bioremediate industrial waste effluent from the textile dye industry. Azo dyes are the most commonly used colouring compounds (Pearce *et al.*, 2003) and they were therefore used in this study to investigate the ability of sulphate reducing bacteria (SRB) and associated cytoplasmic hydrogenase enzymes to degrade them under anaerobic conditions. Several advantages of using such a system are forthcoming:

1) Although the enzymes from the sulphidogenic bioreactors are produced under anaerobic conditions, they are perfectly able to work within an aerobic environment.

2) Using enzymes in a sulphidogenic environment supports that the sulphide produced would be strongly inhibitory to the survival and proliferation of pathogens.

3) Biological (enzymatic) processes have an added advantage over traditional chemical/physical methods as they are regarded as "clean and green".

4) The high cost of industrial enzymes is prohibitively expensive and so the use of these enzymes, from the BioSURE Process®, is a cheap alternative and a tool for bioremediation.

The novelty of our approach allows for the generation of the essential enzymes within a sulphidogenic bioreactor with a simultaneous *in situ* 'one-pot' bioremediation of industrial effluent from the textile industry.

Hypothesis and Objectives

Hypothesis

Enzymes generated within a sulphidogenic bioreactor were capable of totally decolouring and degrading textile dyes, both from an authentic commercial source and from the influents and effluents of a textile industry.

Objectives

1. To set up a laboratory scale biosulphidogenic reactor to generate the enzymes under study.

2. To extract various enzymes (α-glucosidase, β-glucosidase, lipase, phosphatase, sulphatase, protease, cellulase, endoglucanases, hydrogenase, azoreductase) from the reactor.

3. To assay the enzymes and monitor their percent distribution.

4. To test the enzyme mixture on industrial effluents from the textile dye industry.

5. To establish a resource base in the enzymology of and industrial waste water treatment processes and to support cognate research areas in South Africa.

6. To promote student training and corporate technology collaboration to enhance wastewater management in South Africa.

Outline of Approach

The overall initial objective of the present study was to develop a powdered enzyme extract obtained from a biosulphidogenic reactor that would effectively bioremediate industrial effluents such as paper and pulp, tanneries, olive mill, textile dye, petroleum, abattoir, fishing, mining and wine distilling. Commercial enzymes are costly and so it would make this approach fairly favourable and cost effective. Furthermore, ordinary municipal sewage sludge, obtained through the Rhodes University Environmental Biotechnology Research Unit (EBRU) BioSURE Process®, can be used as a prime source of carbon for the sulphate reducing bacteria that exist in the reactor. The idea of a 'cocktail of enzymes' implies a bacterial cell-free powdered preparation of crude enzymes. Though the production and purification of several hydrolase enzymes (glucosidases, proteases, lipases) within this 'cocktail of enzymes' has met with considerable success with respect to the defouling of membranes and the bioremediation of abattoir effluents, trials conducted using both the crude and purified hydrogenase on textile dye industrial effluents extracts were not successful. In the case of the hydrolases, the crude enzyme mixture is self sustaining and their respective reactions can occur without any necessary cofactors. With the textile dye effluent, however, it was necessary to reduce the azo –N=N-bond through the action of the hydrogenase enzyme. Since this initial reduction had not taken place it was felt that either the redox potentials in the reaction mixture were not favourable to facilitate azo bond cleavage or that specific and essential co-factors were absent in the purified samples. This led us to examine the SRB cells as a whole, or rather the SRB cells from within the BioSURE Process® sludge itself. We now report that this work has resulted in a complete decolourisation – and degradation – of the azo dyes from within the textile dye industrial effluent.

In view of the complex nature of both the industrial textile dye effluent and sewage sludge, it was decided to follow a four-level protocol investigation with various control reactions:

1) Use SRB that has been cultured on a 'pure' lactate medium;

2) Use SRB that have been cultured from BioSure Process® sludge;

3) Use five authentic dyes (orange II, amido black 10, reactive black 5, reactive red 120, reactive blue 2) purchased from a commercial source;

4) Use various sources of influent and effluent from a textile dye industry either prior to the dyeing process or after dyeing and before the effluent had passed to the Environmental Treatment Plant.

Results

Following our success with a previous study that involved the enzymology of sludge solubilisation using enzymes from a biosulphidogenic reactor, we decided to study a possibility that hydrogenases and/or azoreductases also present in the reactor could decolour and hence bioremediate industrial effluent from the textile dye industry. Consequently a batch reactor to produce the enzymes was set up, seeded with an inoculum of sulphate reducing bacteria (SRB), sulphate and a cheap source of carbon as sewage sludge from the Rhodes University BioSURE Process®. After stabilising the reactor and ensuring that it was operating maximally (by monitoring sulphate consumption, pH, COD levels, sulphide production), methodology was established for optimum enzyme production and extraction. A time course survey was conducted to determine optimal time at which highest enzyme production occurred and this was found to be at six days. Sonication, of the SRB-Biomass, at low amplitude (10 W) for four minutes proved to be the best disruptive method for releasing the hydrogenases from the SRB.

Induction studies were then undertaken with four commercial azo dyes (Orange II, Reactive Black 5, Amido Black 10 and Reactive Red 120) and one non-azo dye (Reactive Blue 2), to confirm firstly that the growth of sulphate reducing bacteria are not compromised in any way, and secondly that the yield of the enzymes could be increased in a shorter period of time. The presence of the azo dyes in the bioreactor increased the relative hydrogenase activity by up to 140% in 24h except the non-azo anthraquinone dye Reactive Blue 2 that failed to 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% increase in enzyme activity in 24h. Corresponding decolourisation was also observed in the same order as for enzyme induction with the decolourisation of the mono azo dye being higher than that of the di-azo dyes. The threshold level of the dye concentration was determined to be in the range 100-500 $mg.l^{-1}$; 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 sludge from the BioSURE Process®.

Purification and characterisation of the hydrogenase enzymes using PEG to concentrate the enzyme was achieved by chromatography on Sephacryl S-200 and analysis of the fraction by a 10% SDS-PAGE showed a distinct band with a molecular size of 38.5 kDa which is in the same magnitude as other hydrogenases purified from SRB. The hydrogenase operates optimally at a pH of 7.5 and temperature of 40°C but has poor thermal stability with 50% loss in activity in 32 minutes and 70% loss in activity within an hour under optimal conditions. Kinetic parameters K_m and V_{max} for methyl viologen as the substrate were determined.

Absorbance spectra of the industrial textile reactive dye mixtures and their respective effluents 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).

After characterisation of the dyes, and effluents, they were then degraded by SRB under anaerobic conditions. Four industrial textile dye samples were tested – two influents, that consisted of a pre-dye mixture and another that is 'fixed' with caustic soda and silicates and two effluents, consisting of a vat print rinse and a final effluent (after all of the dyeing processes) just prior to passing into the Environmental Treatment Plant (ETP). Decolourisation of 93% and 72% for the influent dye mixture and the dye mixture plus silicate salts, respectively, 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. Successful decolourisation of both commercial dyes and industrial effluents with SRB-BioSURE Process® sludge was achieved with decolourisations ranging from 96-49% over a five day period. The process of decolourisation for each of the dyes can be monitored by a decrease in absorbance at the λ_{max} of the inherent chromophore. This is supported by a reduction of the azo link into two colourless aromatic amine compounds. At the same time as there is a decrease in absorbance of the dye in the visible region (480-610 nm) there is an increase in the absorbance at 280 nm, reflecting an increase in concentration of single aromatic amines. With an extended period of time, there was a subsequent decrease in the absorbance at 280 nm indicating that the aromatic amines had been degraded further, perhaps by some other unknown factors, into CO_2, H_2O and NH_3. Both of the influent and effluent samples followed similar trends to the authentic dyes in that:

1) There was decolourisation of the dye(s), monitored by a decrease of absorbance in the visible region (480-610 nm).

2) There was an increase in absorbance at 280 nm due to an increase in aromatic amines.

3) There was subsequent decrease in absorbance at 280 nm due to a total breakdown of these aromatic compounds.

As a control measure, the effect of a 'pure' culture of SRB (using lactate medium as a carbon source) on both authentic dyes and on the various influents and effluents from the textile industry was studied. The time taken to degrade the dyes using SRB from BioSURE Process® sludge in the sulphidogenic bioreactor was much longer than if the SRB were used from a 'pure' culture.

It is interesting to reiterate that with 'pure' SRB from a culture on lactate medium there was very little breakdown of the single aromatic compounds as the absorbance at 280 nm remained fairly significant. This was evident with both authentic dyes and industrial samples. With SRB from the BioSURE Process® sludge there was complete degradation and a subsequent removal of the aromatic compounds absorbing at 280 nm. It supports other factors, within the sulphidogenic reactor, that may be responsible for complete degradation. It is hypothesised that an anaerobic degradation of the dyes into their constituent aromatic amines followed by an aerobic degradation into CO_2, H_2O and NH_3. With the 'pure' SRB system this doesn't happen.

Though each bioreactor is different on any particular day and consequently the yield and activity of the hydrogenase enzyme varies, the amount of sludge required to completely decolour a specific volume of textile effluent can be estimated. For example if the enzyme activity per ml of sulphidogenic sludge is estimated at 2200 $\mu mol.min^{-1}$ then 1 kg of sludge (1000 ml) would decolour 2.2 mols of azo dye (770 grams Orange II) in one minute.

Recommendation

Purified hydrolase enzymes, extracted from a sulphidogenic bioreactor can be concentrated into a dried powdered cocktail preparation, using established concentration techniques. Though this powdered extract was suitable to bioremediate certain abattoir effluents and acid mine drainage they failed to decolour and degrade dyes from the textile industry. It is the recommendation from this project that in order to completely decolour and degrade the azo dyes from an industrial waste effluent a dried powdered extract of SRB-BioSURE Process® sludge from a biosulphidogenic reactor, including all of the necessary enzymes and cofactors *in situ* be used.