BIOREMEDIATION OF A BLEACH PLANT EFFLUENT FROM THE PULP AND PAPER INDUSTRY

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CONTENTS

Abbreviations

APHA	American Public Health Association
AOX	Adsorbable organic halogen
AS	Activated sludge reactor
BGIT	Bacterial growth inhibition test
BOD	Biological oxygen demand
C	Chlorine stage of bleach plant
CU	Colour unit
CV	*Coriolus versicolor*
COD	Chemical oxygen demand
D	Chlorine dioxide stage of bleach plant
2,4-DCP	2,4-dichlorophenol
DSVI	Diluted sludge volume index
E	Alkaline extraction stage of bleach plant
Eo	Alkaline extraction (in oxygen) stage of bleach plant
GPC	Gel permeation chromatography
HRT	Hydraulic retention time
F/M	Food to Microorganism ratio
M_w	Molecular mass
PCU	Platinum cobalt unit
Q	Activated sludge reactor flow speed
Q_s	Activated sludge reactor return sludge flow rate

Q_w	Sludge-residence time
RBC	Rotating biological contactor reactor
RM7	*Rhizomucor pusillus*
SVI	Sludge volume index

CHAPTER I

INTRODUCTION AND LITERATURE STUDY

1. INTRODUCTION

The pulp and paper industry is one of the major consumers of water (Wang *et al*. 1992; Kallas and Munter 1994). Pulp and paper industrial wastewaters usually contain halogenated organic materials, because general use is made of chlorine containing compounds as bleaching agents during pulp and paper manufacture (Nagarathnamma and Bajpai 1999). Kraft pulping is the most common commercial chemical delignification procedure (Addleman and Archibald 1993). Chlorination is generally the first stage in kraft pulping and during this treatment phase chlorinated organic compounds are produced. The chlorinated substances are extracted with dilute alkali from the pulp in the subsequent extraction phase (Prasad and Joyce 1991). Chloro-organic compounds tends to persist in nature because of their inherent recalcitrance; they are often toxic to aquatic live; many are genotoxic and have the potential to migrate widely throughout the ecosystem, ultimately accumulating in the fatty tissues of organisms (Suntio *et al.* 1988). Legislation has been introduced to limit the levels of these toxic compounds in effluents (Sierka and Bryant 1994, Thompson *et al*. 2001). Additionally, during bleaching treatments chromophoric, highly oxidised, polymeric lignin/chlorolignin derivatives are formed that give rise to a dark colourisation in the effluent (Livernoche *et al*. 1983; Bergbauer *et al*. 1991). The colour poses an aesthetic problem and contributes to the biological oxygen demand (BOD) (Bajpai and Bajpai 1994). Conventional biological treatments cannot effectively remove the colour and current research efforts are directed into solving this problem (Boman *et al*. 1988; Prasad and Joyce 1991).

The reason traditional methods have failed to remove colour from bleach plant effluents can be attributed to the recalcitrance of chlorolignin (Mehna *et al*. 1995, Bajpai and

Bajpai 1997). Fortunately white-rot fungi have the ability to degrade lignin and chlorinated lignin derivatives effectively (Eriksson 1991; Bajpai and Bajpai 1994). However, lignin cannot serve as the sole carbon source for these fungi because the degradation of lignin is apparently a very energy intensive process (Leisola *et al*. 1983; Boman *et al.* 1988). The specific cultivation conditions necessary for white-rot fungi to degrade lignin (Boman *et al.*1988) have led to the employment of different strategies to overcome these difficulties. Various cultivation methods have been devised. For instance: the patented MyCoR process, MYCOPOR-system (Jaklin-Farcher *et al*. 1992), continuous-flow systems (Prasad and Joyce 1992) and various other immobilisation techniques employing white-rot fungi (Livemoche *et al.* 1983; Kirkpatrick *et al.* 1990). Organisms, such as, soil inhibiting fungi imperfecti (Rodriques *et al.* 1996), algae (Lee *et al*. 1978), and streptomyces strains (Hernández *et al*. 1994), have also been studied for their ability to decolourise chromophoric substances.

White-rot fungi produce a variety of ligninolytic enzymes, including peroxidases and laccases (Lamar 1992) that in all probability play a pivotal role during lignin degradation. Although considerable progress had been made in studying the ligninolytic process the precise mechanism whereby these fungi degrade lignin is still not fully understood (Manzanares *et al*. 1995). Ligninolytic enzymes can convert/biotransform phenolic/polyphenolic substances and this has led to interest in the use of these enzymes to treat wastewaters (Paice and Jurasek 1984; Roy-Arcand and Archibald 1991; Al-Kassim *et al*. 1994; Limura *et al*. 1996). Immobilised biomass and/or enzymes are a new and promising area in bioremediation (Anselmo and Novais 1992). Immobilisation leads to increased stability and allows continuous use of enzymes that would be beneficial for the treatment of wastewaters from bleach plants.

A variety of physical and chemical treatments have been tested for the treatment of bleach plant effluents. These include: adsorption, ion-exchange, ultra-filtration, chemical precipitation (Boman *et al.*1988). Also, chemical treatments of effluent have been investigated by using various oxidizing agents (ozonation, hydrogen peroxide, etc.) (feijoo *et al*. 1995) and furthermore, photo-catalysis, involving irradiation of effluent

(Sierka and Bryant 1994). However, it is generally believed that these methods are too expensive for commercial application (Christov *et al*. 1999). Studies indicate that certain physical/chemical methods have improved cost wise and therefore hold promise for the treatment/pre-treatment of effluent (Kallas and Munter 1994). Many investigations indicated that adsorption of coloured compounds on biomass occurred during cultivation on media containing bleach plant effluent (Livernoche *et al*. 1983; Royer *et al*. 1985; Feijoo *et al*. 1995). Of interest is also the observation that oxidised phenolic compounds exhibited a high affinity for chitosan, a compound found in certain fungal cell walls (Muzzarelli *et al.* 1994). Chitosan exhibits polycationic characteristics at acidic pH levels that could facilitate sorption of anionic substances such as the anionic coloured compounds found in bleach plant effluents. Attention has been focused recently on the use of a chitosan coated hollow-fiber membrane process for the bioremediation of a phenolic containing effluent (Edwards *et al*.1997).

In conclusion, Manzanares *et al*. (1995) observed that the problem involving the colourisation of paper mill effluent has not been solved. This reflects, to a great extend, the technical difficulties encountered when treatment of bleach plant effluent is attempted and the problems of setting up an effective but also an economic process for the bioremediation of paper and pulp effluent.

2. AIM OF STUDY

The main goal of this study is the decolourisation of a bleach plant effluent from the pulp and paper industry. Additionally, the reduction of effluent adsorbable organic halogen (AOX) content in the shortest treatment time possible by application of physico-chemical and biological treatment methods is also envisaged. A further goal was to render the bleach plant effluent non-toxic. To achieve these objectives the following areas were addressed:

1. Chemical analysis and characterisation of bleach plant effluent.

2. Evaluation of physicochemical methods for the treatment of bleach plant effluent.
3. Cultivation of selected microorganisms on effluent containing media using bioreactors and the employment of various biological treatment methods in combination.

3. LITERATURE STUDY

Chemical composition of pulp and paper mill bleach plant effluents

The content of chlorinated substances in effluents are usually measured as total organically bound chlorine (TOCl) or AOX (Eriksson 1993). Chloroorganic material from pulp mill effluent is also generally described in terms of their molecular mass. The molecular mass of these materials has serious implications when biodegradation, toxicity, and decolourisation of effluent are examined (Martin *et al*. 1995; Jokela and Salkinoja-Salonen 1992).

HIGH MOLECULAR MASS COMPOUNDS

More than one-half of the colour load from bleach plant effluent originates from high molecular mass lignin-derived material in the alkaline extraction (E) stage (Paice and Jurasek 1984). Sundman *et al*. (1981) examined the chromophoric rich substances and proposed that it consisted of a repeating unit composed of $C_9 H_{9.01} O_{5.56} Cl_{0.56} [OCH_3]_{0.13.}$

The high molecular mass substances are generally believed to be stable and therefore biologically inert (Jokela and Salkinoja-Salonen 1992). However, Eriksson *et al*. (1985) reported that high molecular mass material in spent liquors from the chlorination and alkali extraction stages of bleachery softwood kraft pulp were chemically unstable under conditions prevailing in receiving water systems. These materials slowly released chlorinated catechols and guaiacols (Eriksson *et al*. 1985). Martin *et al*. (1995) concluded that conflicting results on the origin of aromatic compounds originating from high molecular mass compounds are prevalent. However, literature reveals both adsorption and chemical bonding might account for the presence of some low molecular chloro-aromatics in high molecular mass material. Therefore there seem to be evidence both for

desorption, as well as, chemical decomposition, as sources of chloro-aromatics originating from high molecular mass compounds. The ultimate fate of high molecular mass compounds in effluent, during biological treatment, is also still unknown.

Working with high molecular mass fractions (> 30 000 Dalton), Bergbauer *et al.* (1991) observed that sequential depolymerisation, using *Trametes versicolor*, does not necessarily lead to complete degradation of these polymeric compounds. Furthermore, once degradation of lignin derivatives by *T. versicolor* had reached a certain level, neither additional co-substrates, nor inoculation of the culture filtrate with fresh mycelium, could induce any further degradation. Thus, it seems that the end result of using this fungus could be the accumulation of highly recalcitrant compounds in the ecosystem.

Frequently gel permeation chromatography was employed to determine molecular mass distribution of bleach plant effluents (Bergbauer *et al*. 1991, Hernández *et al.* 1994, Feijoo *et al*. 1995). High molecular mass material from extraction stage bleach plant liquors can furthermore be characterised by ultra-filtration. Polymeric lignin and chlorolignin concentrations in samples can also be determined spectrophotometrically (Wang *et al*. 1992). Additionally, high molecular mass material in bleach plant effluent can be hydrolysed with alkali and the resultant chlorophenolics analysed (Martin *et al*. 1995). These methods are examined below.

Lignin determination

Lignin and chlorolignin concentrations can be determined spectrophotometrically by measuring the absorbency in effluent samples at 280 nm and assuming that the validity of Beers law apply (Wang *et al*. 1992):

$C = A/a * D = A/20*D$

Where C = lignin or chlorolignin concentration in g/l;
A = absorbance at 280 nm
D = dilution factor

a = absorptivity = 20 l/g cm

Molecular mass analysis

Molecular mass distribution was determined by high performance liquid chromatography, using a Superdex 75 HR l0/30 column, at a flow rate of 0.4 ml/min and monitoring at 280 nm. As eluent, 750 mM NaCl and 10% (v/v) methanol was utilised (Feijoo *et al.* 1995). Jokela and Salkinoja-Salonen (1992) employed aqueous and non-aqueous size exclusion chromatography (SEC) and ultra-filtration to determine molecular mass distribution of pulp bleaching organic halogen compounds. Results indicated that the molecular mass distribution of bleached kraft effluent chlorinated material obtained by non-aqueous SEC was more accurate than that obtained in aqueous media unless high dilution was applied. Bergbauer *et al.* (1991) determined molecular size distribution by gel chromatography with a Sephadex G-50 column, using 0.75 M NaCl, pH 7.0, as solvent. Gel permeation chromatography was performed by Hernández *et al.* (1994), after pH adjustments of effluents to pH 7.6. A Sephadex G-l00 column was employed and the absorption of elluent was measured at 280 nm to monitored sample elution.

Alkali digestion.

High molecular mass materials were degraded in serum bottles, sealed with teflon-coated caps, after adjustment of the alkali strength to 2M or 5M. The solutions were pre-heated to the respective temperatures of 50°C or l00°C. Digested samples were immediately quenched on ice and acidified to pH < 2 with slow addition of 18M H_2SO_4. Chlorophenolics were subsequently analysed (Martin *et al.* 1995).

LOW MOLECULAR MASS COMPOUNDS

Compounds with a relative molecular mass lower than l000 g/mol are acutely toxic to aqueous organisms (Roy-Arcand and Archibald 1991). These substances include chlorinated phenols, guaiacols, catechols (Limura *et al.* 1996), as well as, chlorosyringols and chloroaliphatics (Roy-Arcand and Archibald 1991). Suntio *et al.* (1988), in a review covering the nature and properties of chemicals in pulp mill effluents of relative low

molecular mass (< l 000 g/mol), divided these compounds in the following categories: acidic compounds, phenolic compounds and neutral compounds.

Chlorinated acidic compounds present in bleaching effluent are largely aliphatic acids, mainly chloroacetic acid. Although a variety of low molecular mass substances do occur in the effluent streams, most organic chlorine present is associated with high molecular mass material (Smith *et al*. 1994).

Chlorinated phenols in bleach effluents are typical chlorinated lignin degradation products that contain methoxyl groups. The predominant compounds of the neutral fraction are chloroform, chlorodimethylsulfones, and chloroacetones, while minor concentrations of additional chlorinated hydrocarbons, ethers, ketones, aldehydes, lactones and thiophenes have been detected (Smith *et al*. 1994). Low molecular mass compounds are usually extracted from bleach plant effluent by organic solvents, dried, concentrated and subsequently determined by gas chromatography. Internal standards are regularly employed (Smith *et al*. 1994).

Biological treatment methods developed for the remediation of bleach plant effluent

As noted before, none of the traditional biological methods can degrade high molecular mass chromophoric compounds effectively. These methods include aerated lagoons, activated sludge systems and anaerobic cultivation (Boman *et al.* 1988, Archibald *et al*. 1990). Aerated lagoons can reduce TOCl by about 25%, whereas activated sludge systems can remove chlorinated compounds by 40%. However, aeration costs are high and in the case of activated sludge systems, it seems as if a substantial part of the chloro-organics is adsorbed onto the surface of the biomass, which could lead to disposal problems of excess sludge.

Anaerobic treatment have gained popularity in the pulp and paper industry because of low energy consumption and the low production levels of well stabilised excess sludge. Nevertheless, bleach plant effluent are more diluted than desired for a low retention time process and varying effluent composition can lead to toxic shock conditions, detrimental

for the efficient functioning of the anaerobic systems. By combining ultra-filtration of the E-stage effluent and incorporating the permeate with the C/D effluent, followed by treatment in an anaerobic filter, an overall reduction of 62% in chloro-organics was achieved (Boman *et al*. 1988).

However, an effective biotreatment system must lead to the degradation of both high and low molecular mass compounds and white-rot fungi can achieved this effectively. Major draw backs are the need for an easily degradable, inexpensive carbon source and the complicated physiological demands of some of these fungi when degrading lignin (Boman *et al*. 1988). White-rot fungi usually degrade lignin under certain nutrient (nitrogen, sulphate or phosphate) limiting conditions (Bajpai and Bajpai 1997). High dissolved oxygen levels are also essential for ligninolytic activity. Furthermore, the pH level used during cultivation has to be in the range of 3.5 to 5.5 (Garg and Modi 1999). Various parameters and cultivation strategies need to be considered in an effort to develop a successful treatment process. Bioreactors used for bleach plant effluent treatment are discussed below.

CONTINUOUS-FLOW SYSTEMS

Eaton *et al*. (1982) described a system, called a FPL/NCSU MyCoR method and proposed that a fixed film MyCoR reactor could be charged with nutrients that could include primary sludge. Although steam treatment initially might be beneficial, further aseptic procedures would not be required with fungi like *Phanerochaete chrysosporium*. In the MyCoR-process fungi are immobilised on the surface of rotating disks that could be enclosed for additional oxygen supply. The disks are partially submerged during operation. A growth stage is necessary before decolourisation commenced, during this stage nutrient nitrogen was depleted and the fungus becomes ligninolytic. The MyCoR-process reduced colour in an alkaline stage spent liquor by 80% in less than 24 h in the laboratory. An illustration of this type of reactor is presented in Fig. 1.1.

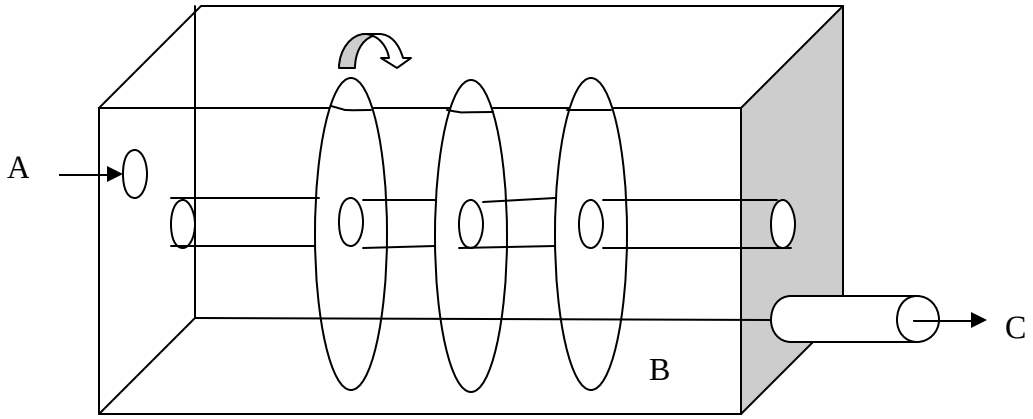

FIG. 1.1: Illustration of a MyCoR type reactor. Legends: A, influent; B, disk containing immobilised fungi; C, effluent.

Some limitations of the MyCoR process seem to be the poor surface to volume ratio since only 40% of the mycelium is in contact with substrate. Moreover, the mycelium is present in a thick layer that could result in deficient oxygen and nutrient supply and a lower general productivity.

Three continuous-flow laboratory-scale reactors were examined (Prasad and Joyce 1991). System I consisted of a set up similar to a conventional oxidation basin with a surface area of 5l cm^2 for fungal cultivation. Fungal mats of *Trichoderma* sp, were transferred to the reactor and decolourisation was carried out without aeration.
System II utilised a vessel in which the height was increased by decreasing the surface area and four baffles were introduced to divide the vessel into compartments. *Trichoderma* packed in wire bags were suspended in the middle of each zone and continuous aeration was supplied.

System III was similar to the MyCoR process in that fungal mats were clasped between circular wires, supported by outer central rings and securely fixed in a metallic frame. The discs were rotated continuously. All these systems could reduce colour by at least

50% for the first 6 days, but system III gave the best results with a greater than 78% total colour removal from extraction-stage effluent as well as COD reduction of 25%. Colour removal in excess of 57% was sustained for 18 days with system III.

Prouty (1990) tested a 10 l bioreactor in which aeration and mixing were achieved with a defuser placed at the side of the bottom of the reactor. The temperature was maintained at 40°C. The author concluded that results from the bench-scale study provided a reasonable basis for the evaluation of an industrial fungal colour removal process and set out a hypothetical continuous-flow process consisting of a mixing tank, aeration basin and clarifier. Effluent would first be adjusted to the proper pH and nutrients added before entering the aeration unit for treatment. The effluent would then go through a sedimentation basin and fungal solids removed would be recycled to the head of the aeration unit or hydrolysed for use as nutrients. Foaming was a serious problem encountered during bench-scale experiments.

In the MYCOPOR-process white-rot fungi immobilised in polyurethane foam was used in a continuously trickling filter reactor to treat bleach plant effluent. Treatment of bleaching effluent from the alkaline stage of a sulphate mill by this process resulted in a maximum decolouration of 80%. This was accompanied by 50% COD reduction and 80% toxicity elimination (microtox) after one passage through a trickling filter of l m length (Jaklin-Farcher *et al*. 1992).

Pallerla and Chambers (1997) studied bleach plant effluent treatment in a fluidised-bed bioreactor containing Ca-alginate-immobilised *T. versicolor* operated in a continuous mode. The effects of various residence times were investigated. About 71% colour removal was attained at a residence time of 16 h. No significant difference was recorded in decolourisation between residence times of 24 h and 16 h however, there was a definite lowering in the bioreactor performance at a residence time of 10 h. The effect of inlet liquor concentration on the bioreactor performance was also investigated over the range of 1 000 CU) up to 1 538 CU. Lowest decolourisation and AOX reductions were observed at an inlet colour value of 1000 CU. Lower liquor concentrations did not

enhance reactor performance. The reactor could decolourise caustic stage effluent by 69 % and reduce AOX of this effluent by 58% at a residence time of 1 d. Steady state data were used to calculate decolourisation rate and reaction rate constants. Results of the continuous cultivation studies indicated a linear relationship between decolourisation rate and bioreactor colour concentration.

Suspended carrier technology has been in use for a number of years. Carrier particles with a density close to that of water are used to minimise the energy required to keep the carriers suspended during treatment. Various types and forms of carrier particles have been reported, including polypropylene mats, polyester sponge-foam cubes, porous plastic foam cubes and ionically modified porous polyurethane granules. It was suggested that carrier particles should be engineered with more surface roughness and with surface charge as well as hydrophilicity to better accommodate microorganisms (Strehler and Welander 1994). COD reductions of 55% were achieved during treatment of bleached kraft effluent at pH 7, 37°C and with hydraulic retention times of longer than 3.5 h in a suspended carrier process. The suspended carrier treatment was also operated at pH 9 and 45°C as well as at pH 7.0 and 50°C with more than 50% reduction in COD in each case at a hydraulic retention time of 4 h. A support matrix composed of particles that are kept in suspension by aeration have major advantages when compared to static biofilm systems for the treatment of kraft mill effluents. The reason being the risk for clogging of the stationary biomass support material with fines and fibers are eliminated (Strehler and Welander 1994).

Neutral sulphite mill wastewater was treated with a moving bed biofilm system during a pilot-plant trial with a 70% COD reduction and 96% BOD_7 elimination that was obtained at a organic load of about 25 kg COD/m^3.d. No clogging was experienced during the pilot-plant run and the toxicity removal was about 98% (Microtox). It was reported that a full-scale treatment plant was under construction at the mill using the moving bed biofilm system developed by the authors (Broche-Due *et al.* 1994).

BATCH TREATMENT OF BLEACH PLANT EFFLUENTS

Royer *et al.* (1985) used *Coriolus versicolor* in the form of mycelial pellets to decolourise lignin-containing kraft E_1-stage effluent. Both adsorption and oxidation generally proceeded best between pH 4 and pH 5 and at temperatures of 25°C to 30°C. Decolourisation was practically non-existent at 40°C, which corresponds to the temperature of the liquor effluent at the E_1-stage. As magnesium ions improved the degradationtion of chromophores, this might indicate that an enzymatic mechanism takes place since magnesium ions are activators of many enzymes. At an initial colour level of 7 000 CU, the mean colour removal proceeded at a rate of 300 CU/g mycelium h. The authors suggested the use of airlift reactors employing the pelleted form of the fungus. This strategy would facilitate recycling and the use of large amounts of fungal biomass.

Mehna *et al.* (1995) were able to achieve a colour reduction of 92% with a COD elimination of 69% using a *T. versicolor* strain in flask cultures, after 7 d. The fungus was used in the form of pellets. Optimal conditions were employed namely: pH 4.5, temperature 30°C, sucrose 7.5 g/l and at an inoculation concentration of 5.0 g/l. Primary sludge and bagasse pith, added as co-substrates, gave 26% and 48% colour reduction, respectively. Decolourisation was optimal with an ammonium nitrate concentration of 1.75 g/l and leveled off beyond this concentration.

Trametes versicolor was cultivated under optimal aeration in a laboratory fermentor with 0.8% glucose plus 12 mM ammonium sulphate and at a controlled pH level of 5.0. This resulted in an 88% reduction in the colour units within 3 d, whereas an 80% reduction was observed in flask cultures after 6 d. This emphasises the importance of culture conditions on the efficiency of decolourisation (Bergbauer *et al.* 1991). *Trichoderma* sp, was initially cultivated in shake flasks and washed before further experiments were conducted under specific conditions to determine optimal pH and carbon sources. Under optimal conditions (pH 4.0, and glucose as carbon source) this fungus decreased the colour of kraft bleach plant effluent by 85% and reduced the COD by 25%, after 3 d incubation in shake flasks. The maximum total decolourisation at pH 4.0 without an additional carbon-source was 68.6% after 3 d cultivation and therefore glucose stimulated

decolourisation. Other carbon sources such as pulp and pith that are abundant and inexpensive increased the decolourisation after 6 d, since they were not metabolised immediately. The results of these studies again emphasised the importance of carrying out the treatments under strictly defined conditions (Prasad and Joyce 1991).

Nararathnamma and Bajpai (1999) evaluated different fungi for bleach plant effluent treatment and selected *Rhizopus oryzae* based on high decolourisation and low sugar requirements during colour removal. Experiments were conducted in shake flasks. During treatment 92 to 95% of the colour, 50% of the COD and 72% of the AOX were removed in 24 h at 25 to 45°C and a pH of 3.5.

Ligninolytic enzymes and their role in decolourisation of bleach plant effluent

The study of ligninolytic enzymes have attracted intense interest as more researchers investigate these catalytic agents of delignification in an effort to understand the mechanisms involved in lignin and lignin derivative removal. The size, recalcitrance, heterogeneity and complexity of lignin demand that its initial degradation be oxidative and nonspecific. Furthermore, that it be mediated by an extracellular mechanism (Kirk and Farrell 1987). Figure 1.2 depicts the complex structure of lignin. White-rot fungi produce a variety of key ligninolytic enzymes composed of lignin peroxidase (LiP), manganese dependent peroxidase (MnP) (Cameron *et al*. 2000), possibly also laccase (Youn *et al*. 1995) and other oxidases (Regalado *et al*. 1999).

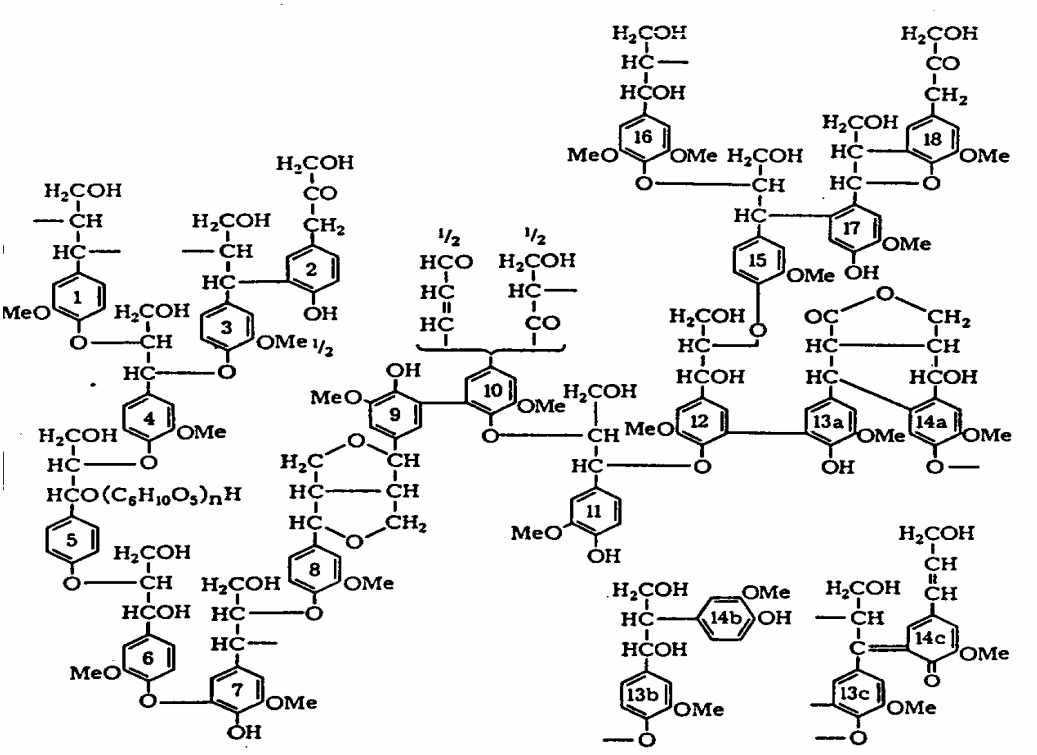

FIG. 1.2: Representation of the molecular composition of lignin (Eriksson 1991).

Confusion was caused when mixtures of peroxidases and/or laccases were studied to demonstrate *in vitro* depolymerisation of high molecular mass lignin. Depolymerisation was usually accompanied by re-polymerisation of the low molecular mass fractions (Lamar 1992, Eriksson 1993). Subsequent work have however established a role for these enzymes since it was shown that depolymerisation of synthetic lignin can be achieved by crude LiP (Hammel and Moen 1991) and a purified MnP from *P. chrysosporium* (Wariishi *et al*. 1991). Further evidence indicated that a mixture of MnP and laccases from *Rigidoporus lignosus* could degrade *Herea* lignin (Galliano *et al*. 1991).

For the treatment of wastewater it is important to recognise that lignin-hydrolysing fungi are able to degrade lignin derived chromophores as well as a variety of aromatic and

chlorinated aromatic, xenobiotic compounds from darkly-coloured pulp bleach plant effluent (Lamar 1992). Current knowledge of ligninolytic enzymes is however insufficient to allow the formulation of an enzyme mixture that prevents the re-polymerisation reactions from taking place in a cell-free state (Eriksson 1993). Therefore processes based on the whole fungus are preferable (Ritter *et al*. 1990).

LIGNIN DEGRADING AND COLOUR REMOVAL SYSTEM OF PHANEROCHAETE CHRYSOSPORIUM

Phanerochaete chrysosporium is one of the well-known and studied white-rot fungi. Figure 1.3 shows the various mechanisms involved in lignin and cellulose degradation. The key extra-cellular ligninolytic enzymes of *P. chrysosporium* apparently are LiP and glyoxal oxidase. The latter oxidises the glyoxal and methyl glyoxal metabolites with the conversion of oxygen to hydrogen peroxide, which activates lignin peroxidase. Lignin peroxidase oxidise non-phenolic aromatic nuclei in lignin by one electron to generate aryl cation radicals, which convert nonenzymatically via many reactions. Many of these reactions involve polymer cleavages, generating both aromatic and aliphatic compounds that are assimilated by the fungus (Kirk *et al*. 1992).

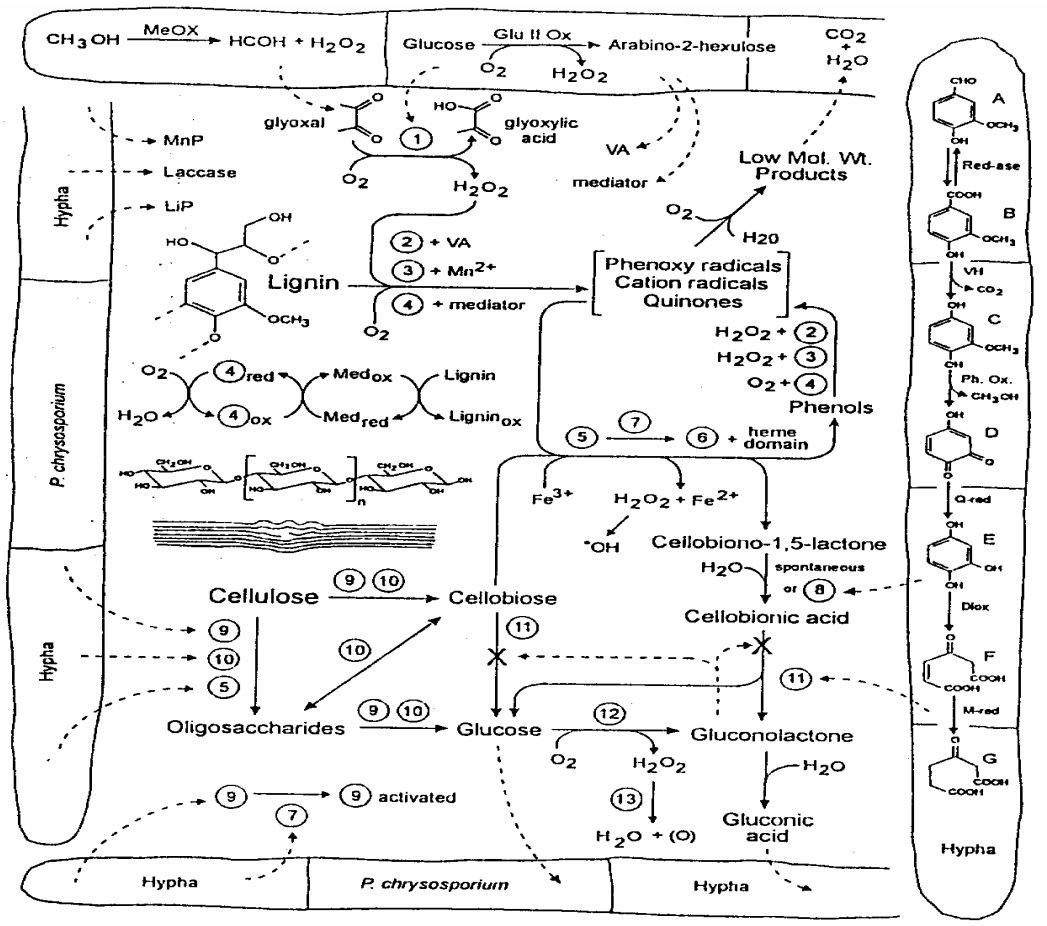

FIG. 1.3: Enzymatic pathways associated with lignin and cellulose degradation by *Phanerochaete chrysosporium*. Nomenclature: (1) glyoxal oxidase, (2) lignin peroxidase, (3) manganese peroxidase, (4) laccase, (5) cellobiose dehydrogenase, (6) cellobiose: quinone oxidoreductase, (7) protease (8) lactonase, (9) endo-1,4-ß-glucanase, (10) exo-1,4-ß-glucanase, (11) 1,4--ß-glucosidase, (12) glucose-1-oxidase, (13) catalase. *Meox* methanol oxidase, *Glu II Ox* glucose-2-oxidase, *VA* veratryl alcohol. Vanillic acid metabolism: *VH* vanillate hydroxylate, *Ph.OX* phenol oxidase, *Q-red* NAD(P)H: quinone oxidoreductase, *Diox* dioxygenase, *M-red* maleylacetate reductase. Metabolic products from lignin degradation: A vanillin, B vanillic acid, C methoxyhydroquinone (MHQ), D hypothetical ortho-quinone (II), E hydroxyquinol, F maleyl acetate, G ß-ketoadipate (Kuhad *et al*. 1997).

Manganese peroxidase produced by *P. chrysosporium* are also involved in lignin degradation. In the presence of H_2O_2, MnP oxidises Mn^{2+} to Mn^{3+}, which in turn can oxidise phenolic species in lignin (Lackner *et al*. 1991). The ligninolytic system also requires manganese, oxalate and veratryl alchohol (Cameron *et al*. 2000). Veratryl alcohol seems to play many roles, including stimulation of the production of the enzymes and electron transfer reactions during ligninolysis.

Two independent investigations have demonstrated that the extra-cellular peroxidases of *P. chrysosporium* are responsible for bleach plant effluent decolourisation. Furthermore, a direct relationship between decolourisation and depolymerization of chlorolignin was indicated, mainly through the action of MnP. Mutants of *P. chrysosporium* lacking the ability to produce MnP and LiP did not show significant decolourisation activity when grown under nitrogen limiting conditions. Also, decolourisation was marginally effected when LiP-activity was suppressed, in contrast to the situation when MnP-activity was inhibited. Direct evidence linking MnP with decolourisation was provided by the *in vitro* depolymerisation of high molecular mass chlorolignin by MnP in the presence of Mn^{+2} and peroxide (Lackner *et al.* 1991).

Presnell *et al.* (1992) indicated that the enzyme profile, as determined by fast protein liquid chromatography and polyacrylamide gel electrophoresis, of *P. chrysosporium* were significantly different when this fungus was cultivated in effluent-free and effluent-containing cultures. Moreover, when various molecular mass fractions were prepared and used in separate cultivations, different protein patterns were obtained for each molecular mass fraction used. Unfortunately the proteins associated with the effluent-containing samples were not studied, but the authors indicated this would be the subject of further research.

An interesting observation using *P. chrysosporium* was apparently that the type of fungal pellets, induced by initial culture conditions, had an influence on enzyme production. Decolourisation and MnP production were apparently only obtained when the fungus grew in the form of a fluffy pelleted material (Jaspers *et al*. 1994). These authors also

indicated the importance of manganese peroxidase in the decolourisation of kraft pulp bleach plant effluent, since *in vitro* studies with purified MnP and LiP showed that only MnP had a decolourising activity. Also, MnP but not LiP activity was detected when *P. chrysosporium* was cultivated on the effluent.

LIGNIN DEGRADING AND COLOUR REMOVAL SYSTEM OF TRAMETES VERSICOLOR

Trametes versicolor decolourise stable high mass chromophoric compounds originating from kraft mill bleacheries (Archibald *et al.* 1990). Decolourisation by *T. versicolor* was unaffected by scavengers of O_2^-, OH^-, H_2O_2 and no extra-cellular peroxidases or hydrogen peroxide could be detected during decoulorisation, although substantial levels of laccase-type phenoloxidase activity was present. Therefore, although cell surface peroxidatic activity could not be ruled out, these results indicated that extra-cellular peroxidases are apparently not crucial for E_1 effluent decolourisation (Archibald *et al*. 1990). Although nitrogen-limiting conditions has been reported as a requirement for decolourisation by ligninolytic cultures of *P. chrysosporium*, this seem not to be the case for *T. versicolor* (Archibald *et al.* 1990, Swamy and Ramsay 1999).

The work of Manzanares *et al*. (1995) revealed that there was a direct relationship between the concentration of effluent from the alkaline cooking of cereal straw and laccase activity of *T. versicolor*. These workers reported manganese dependent peroxidase activity in the decolourisation media when $MnSO_4$ was added, whereas no LiP activity was detected prior to the addition. Additionally, they concluded that the presence of laccase was not a factor by itself that influenced the progression of the decolourisation process by the fungus, since higher laccase activities could not always be linked consistently to high decolourisation activities. These results are collaborated by the findings of Royer *et al*. (1991) who indicated that no correlation could be found between laccase production and the rate at which decolourisation occurred.

Laccase catalyses the abstraction of one electron from phenolic hydroxyl groups and this has previously been shown to polymerise or depolymerise lignin model compounds (Youn *et al*. 1995).

THE INVOLVEMENT OF OTHER ENZYMES IN THE LIGNINOLYTIC PROCESS

Apart from the key ligninolytic enzymes namely: lignin peroxidase, manganese peroxidase and possibly laccase, there is growing evidence for the participation of other important enzymes in the lignin degradation process. Samejima and Eriksson (1992) suggested that cellobiose: quinone oxidoreductase (CBQ) and cellobiose oxidase (CBO) play important parts in the lignin degradation by reducing phenoxy radicals compounds. These radical substances are formed by peroxidases and laccases during lignin degradation and spontaneously re-polymerise in the absence of CBO and CBQ. It has also been shown that when *T. versicolor* laccase is incubated with glucose oxidase the result is an improvement in lignin depolymerisation. This is probably attributable to the action of glucose oxidase in reducing quinones and thereby limiting their re-polymerisation (Szkiarz and Leonowicz 1986). According to Szkiarz and Leonowicz (1986) the same mechanism probably exist in nature where various enzymes released by the fungus may function in concert to transform lignocellulose into effectively utilised carbon sources. Furthermore, Ander *et al*. (1990) also suggested that the rapid reduction and metabolism of quinone compounds might be one mechanism whereby the polymerisation/depolymerisation equilibrium is shifted towards degradation.

An additional role of CBQ may be the supply of cellobionic acid, which could function as a Mn (III)-complexing agent. These complexing agents are required by MnP to oxidise lignin. Moreover, CBQ, while oxidising cellobiose to cellobionic acid, reduces insoluble Mn (IV) to Mn (II), thereby recycling these cations. Mn (II) is also required by MnP during oxidation of lignin, since Mn (II) is converted to Mn (III) by this enzyme. The small size of the organic acid Mn (III)-complexes make them plausible delignification agents as they can easily diffuse in lignocellulosic wall regions where protein-size molecules cannot (Roy *et al.* 1994). It was however proven that vanillic acid decarboxylation by lignin peroxidase, laccase, horseradish peroxidase and manganese

dependent peroxidase were strongly inhibited by CBQ plus cellobiose. Oxidation of veratryl alcohol was also inhibited by CBQ activity (Ander *et al*. 1990).

Hydrogen peroxide is required during lignin degradation (Evans 1985). The work of Daniel *et al.* 1994, suggest a cooperative role between pyranose oxidase (POD) and MnP during white-rot decay since pyranose oxidase was reported to be a major source of hydrogen peroxide. Furthermore, the periplasmic distribution in hyphae and extra-cellular occurrence of POD are consistent with the reported distribution of hydrogen peroxide-dependent MnP. It was also indicated that a mycelial-bound lignolytic enzyme or a hydrogen peroxide-producing system in the cell wall was necessary for dye decolourisation by *T. versicolor* (Swamy and Ramsay 1999).

UTILISATION OF FREE AND IMMOBILIZED ENZYMES FOR BIOREMEDIATION PURPOSES

Tyrosinase has been used to oxidises phenols and aromatic amines from industrial wastewater. A colour change from colourless to dark brown was observed during treatment, but these coloured compounds were easily removed by cationic polymer treatment. Tyrosinase was also immobilised on magnetite with 80% retention of activity. Utilization of immobilised tyrosinase resulted in less coagulant being required to remove coloured enzymatic reaction products, compared with soluble tyrosinase. Furthermore, it was shown that immobilised tyrosinase could treat a large quantity of phenol-containing wastewater compared to soluble tyrosinase (Wada *et al.* 1994). Quinones are obtained when phenols are oxidised by tyrosinases. These oxidised compounds adsorb rapidly and strongly onto chitosan, a cell wall compound of some fungi (Muzzarelli *et al.* 1994).

Al-Kassim *et al*. (1994) studied the removal of phenols by fungal peroxidase from *Coprinus macrorhizus*. The authors concluded that discontinuous addition of the reactants increased the turnover obtained by the fungal peroxidase. Up to 91% removal of phenol could be achieved at equimolar concentrations of H_2O_2 when using peroxidase at a concentration of 0.3 U/ml and employing the discontinuous addition method.

Paice and Jurasek (1984) investigated peroxidase catalysed decolourisation of a bleach plant effluent. At pH 7.6, horseradish peroxidase catalysis reached a maximum level at 40°C in a 4 h assays with l0 mM peroxidase. When compared to the colour removal by *Coriolus versicolor*, the rate of colour removal using peroxide plus peroxidase was initially faster over the first 4 h. However, after 48 h the extent of colour removal was higher with the fungal treatments and the decolourisation of the enzymatic system could not be improved, even if both peroxide and peroxidase were replenished. Feijoo *et al*. (1995) pre-treated kraft mill effluent with mycelial pellets or extra-cellular ligninolytic liquid from *P. chrysosporium* before anaerobic treatment was implemented. Anaerobic degradation of high molecular mass compounds were improved 34% and 27%, respectively, compared to anaerobic digestion without pre-treatment. The authors indicated that the application of ligninolytic enzymes could overcome the problems related with mycelial growth and stability in bioreactor performance.

During the use of the purified enzymes MnP and LiP from *P. chrysosporium*, it was shown that only MnP had a decolourisation activity, but that this was limited to about 25%. Therefore, *in vivo* decolourisation, which attained more than 80%, could depend on the production of other enzyme compounds by this fungus (Jaspers *et al*. 1994).

The ligninolytic enzymes of white-rot fungi can also transform a variety of organic and chloro-organic compounds. For instance, the laccase of *T. versicolor* oxidises anthracene and benzo[a]pyrene (Collins *et al*. 1996), as well as dechlorinates tetrachloroguaiacol (Limura *et al*. 1996). The work of Roy-Arcand and Archbald (1991) indicated that *T. versicolor* laccase rapidly partially dechlorinated a number of toxic poly-chlorinated phenols and guaiacols. *Phanerochaete chrysosporium* and other white-rot fungi are able to degrade a broad range of xenobiotics. This can in general be attributed to the non-specificity of the ligninolytic enzymatic systems of these fungi (Kirk *et al*. 1992).

THE OCCURRENCE OF ADSORPTION ON BIOMASS DURING EFFLUENT TREATMENT

Many researchers report that adsorption of compounds on biomass occurred during treatment of effluent. Boman *et al*. (1988) reported that 40-50% of chlorinated phenols and l0% of the adsorbable chloro-organics are first adsorbed onto the fungal biomass before actual breakdown starts. The authors employed the fungus at 38°C and pH 4.5, and suggested the involvement of phenoloxidase in the polymerisation of chlorinated phenols on the mycelial surface.

Evidence indicated the initial colour decrease (up to 30%, after 3 d), using *P. chrysosporium* to decolourise kraft pulp mill effluent, could possibly be attributed to adsorption of chromogenic compounds, during fungal growth. This was confirmed when colour, initial adsorbed, was completely recovered from the samples by extraction with 0.1 M $Na_2B_4O_7.10H_2O$ (Feijoo *et al*. 1995). Jaspers and Penninckx (1996) reported that depending on the conditions of incubation, pellets of *P. chrysosporium* strongly adsorbed colour and AOX from kraft bleach plant E_1-effluent. Furthermore, it was observed that *P. chrysosporium* removed colour from textile dye by both degradation and by bioadsorption (Bakshi *et al*. 1999).

Royer *et al*. (1985) regarded the decolourisation process by *T. versicolor* of kraft bleach effluent to consist of two parts, namely: adsorption, completed after 24 h, and secondly, subsequent oxidation. In experiments using fungal pellets and ultra-filtered kraft liquor, the lignin compounds were adsorbed after 24 h. Livernoche *et al*. (1983) employed dead or living *C. versicolor* mycelia under anaerobic conditions to treat kraft mill wastewaters. After 3 d a 45% decolourisation were obtained which the authors believed to be due to adsorption of coloured material. In contrast, when living mycelia were used under aerobic conditions, 80% decolourisation was obtained throughout all cycles. About l0% of the total organic halogen (TOX) removal, obtained after treatment of a mixture of first

chlorination stage and first alkaline stage effluent with *Ganoderma lacidum* and *C. versicolor*, was estimated to be due to adsorption (Wang *et al*. 1992).

Hernández *et al.* (1994) used *Streptomyces* strains to decolourise paper mill effluent obtained after semi-chemical alkaline pulping of wheat straw. They estimated that about 20% of the initial colour was lost due to adsorption by the mycelia. Furthermore, only in strains in which previous adsorption have been observed did transformation of the major chromophoric groups occurred in the effluent. Therefore, these results illustrate that adsorption and transformation of coloured compounds was linked and decolourisation required initial adsorption of the colour to the mycelia.

In an interesting variation, biosorption of high-molecular mass organo-chlorines was studied using municipal waste water sludge as sorbent. Biosorption equilibrium was reached in the first 30 min. The maximum removal of high-molecular mass organo-chlorine was 70% at a biomass concentration of 2.0 g volatile suspended solids (VSS)/l or higher. Only 8% of the organo-chlorines desorbed from live biomass, after 24 h, indicating that the process was not readily reversible (Srinivasan and Unwin 1995).

STUDIES USING VARIOUS ADSORBENTS

Fly ash

Fly ash is a waste product of the electric power supply industry, its main component is silica, which acts as the adsorbent for colour elimination. A comparative study by Gupton and Bhattacharya (1985) found that 94% of colour could be removed by fly ash whereas excessive amounts of lime were necessary to remove colour beyond 90%. High molecular mass amine yielded better colour removal than fly ash but at comparatively high costs.

Colour removal with fly ash was not due solely to physical adsorption, as was evident from water extract analysis. Calcium was released during effluent treatment and this

assists colour removal by chemical precipitation. The optimum pH for colour removal was obtained at pH 3.75 with a fly ash content of 0.5 g/l. It was evident that colour removal with acidified fly ash is a function of effluent pH (Gupton and Bhattacharya 1985).

Sell *et al*. (1994) reported that acidified fly ash could effectively remove both colour and COD from caustic kraft bleach plant effluents. Fly ash can be an effective sorbent because of its large surface area per unit volume. The authors examined fly ash from different localities and found that for all the ash types there is a general increase in removal efficiency as the ash is acidified to pH 1. Precipitate formation was observed during some of the ash treatments. It was postulated that this phenomena was due to the presence of Ca-salts since calcium is known to react with humic substances and tannins. COD removal levels as high as 95.4% was achieved when treating kraft caustic mill effluent.

A major operating expense could be the acid required to treat the effluent. It was estimated that at a dosage of 0.5% v/v, a 75 million pound effluent per day plant would need about 188 tons of acid daily at a cost greater than 2% of the value of the pulp. Furthermore, in the event that the effluent would be rendered to acidic for disposal, the pH would have to be raised, which would incur further costs. These factors could have a negative economic impact when fly ash is evaluated as a decolourising agent (Sell *et al*. 1994).

Alum

Fukui *et al*. (1994) treated the C-stage chlorine effluent from a hardwood Kraft mill and achieved an 80% reduction in AOX by using NaOH at pH 12.5 prior to conventional sedimentation with alum. Simply increasing the pH to more than 12 reduced AOX-levels by 62%, while precipitation and flocculation with alum alone reduced COD levels by up to 45%. Apparently alum is converted to $AlOH_3$ which precipitates large organic compounds whereas alkali serves to dechlorinate the effluent.

Inexpensive adsorbent materials

Meyer *et al*. (1992) tested low cost adsorbent materials. These authors found the best results using barbecue charcoal (67%) and rice husks (65%) when treating textile effluent for colour removal. These results are of interest since both barbecue charcoal and rice husks are cheap products that are readily available in South Africa. The question remains however as to what extend these materials can remove colour from pulp and paper effluents?

ADSORPTION MODELS

Adsorption isotherms (models) are studied to measure the adsorption capacity of adsorbent material and to determine the liquid solid equilibrium distribution of a solute (McKay and McConvey 1985). Various models are available to describe adsorption. Some of these are presented below.

The Langmuir model

$X/M = (K_L * C_e)/(1+ a_L * C_e)$

X/M = amount of substance biosorbed per unit weight of adsorbent

a_L = constant

K_L = constant

Ce = equilibrium concentration of biosorbed compound in solution after adsorption (Liversidge *et al.* 1997)

The Langmuir model assumes that the maximum adsorption occurs when a saturated mono-layer of solute molecules is present on the adsorbent surface and the energy of adsorption is constant. Furthermore, that there is no migration of adsorbate molecules in the surface plane (Kapoor and Viraraghavan 1995).

Freundlich model

$Q = K * Ce^{1/f}$

Q = uptake capacity of adsorbent

K = sorption equilibrium constant

F = sorption equilibrium constant

Ce = equilibrium concentration of adsorbate after adsorption has taken place

The Freundlich model was developed for heterogeneous surfaces and was derived on an empirical basis (Kapoor and Viraraghavan 1995).

Brunauer-Emmett-Teller model (BET)

$Ce/(Cs - Ce) = 1/B*Q^o + [(B - 1)/B*Q^o] * (Ce/Cs)$

Cs = saturation concentration of adsorbate

Q^o = amount o adsorbent adsorbed per unit weight of adsorbent for monolayer sorption

B = constant relating to the energy of interaction with the surface

Ce = equilibrium concentration of adsorbate after sorption has taken place

The BET-model was developed for multi-layer adsorption at the adsorbent surface and assumes that a Langmuir isotherm applies to each layer (Kapoor and Viraraghavan 1995).

These adsorption models were originally developed for gas adsorption on surfaces and may not apply to the more complex conditions prevalent in biological systems (Kapoor and Viraraghavan 1995).

Scatchard model

Scatchard (1949) described a model that characterises the attraction of proteins for small molecules and ions. This model has also been used to describe biosorption equilibria. The adsorbate interaction with the binding sites on the cell surface can be described by an association constant K, following the equation:

A+X ? AX

K = [AX]/[A] * [X]

[A] is the adsorbate concentration and [X] is the number of binding sites on the biomass surface.

The graphical representation of the amount of bound adsorbate on biomass divided by the equilibrium adsorbate concentration versus the amount of bound adsorbate, gives a typical Scatchard plot. The equation can be expressed as follows:

MA/Me = K(Xo-MA)

MA = the quantity of adsorbate sorbed on biomass
Me = equilibrium adsorbate concentration
Xo = concentration of potential binding sites
K = equilibrium constant

The slope of the plot MA/Me versus MA, gives -K and the abscissa intercept gives Xo. These plots have also been used to represent the binding of ligands to macro-molecules.

Applications of adsorption models

Kallas and Munter (1994) used parameters such as COD, AOX, lignin + tannin, and platinum cobalt colour (PCU) units to estimate the coefficients in the Freundlich and Langmuir equilibrium equations. Analysis of the isotherm data indicated that of all the adsorbents tested powder activated carbon exhibited the best adsorption capacity.

COAGULATION TREATMENT METHODS FOR THE REMEDIATION OF PULP AND PAPER MILL EFFLUENT

Coagulation refers to a process whereby charges on suspended particles in a liquid are neutralised, while flocculation refers to the agglomeration and capture of the neutralised particles into a large solid network. Most inorganic coagulants require a specific pH for optimum results. The proper selection of chemical dosages and feed concentrations is critical for effective treatment. An important parameter during flocculation is the intensity and the time of mixing. (Librizzi and Lowery 1990).

Agglomeration of suspended particles is induced by suppression of the repulsive electrostatic forces. This procedure is known as destabilisation and may involve the following mechanisms (Sundstrom and Klei 1979):

I. Charge neutralisation by adsorption of counter ions
II. Reduction of surface charge by repression of the double-charge layer
III. Entrapment by a sweeping flock
IV. Bridging between particles by polymers.

The polymers involved in bridging contain many active sites where colloids can interact and become adsorbed. Destabilisation results mainly by slowing the particle motion due to the bridges, which are formed between the colloid particles. Re-stabilisation can result when excess polymers are present because then each colloid particle has its own polymer molecule and few bridges are formed. Charge neutralisation can be followed by charge reversal when overdoses of a coagulant are added to the wastewater (Sundstrom and Klei 1979).

When ions are added to wastewater they increase the ionic strength of the solution and repulsive forces are dissipated over shorter distances between suspended particles. Eventually a salt concentration is reached where the distances of counter ions surrounding the colloid particles is small enough so that two particles can interact and aggregate through the participation of Van der Waals forces. Since ionic strength depends upon the square of the ionic charge, it follows that the concentration of salts required to achieve destabilisation decrease as the cation changes from Na^{+} to Ca^{2+} to Al^{3+} (Sundstrom and Klei 1979).

Applications

An example of paper plant that utilises a full-scale waste colour control process is the Hodge pulp and paper mill. Their process is capable of 95% colour removal via coagulation and subsequent flotation separation of the coloured materials. Organic poly-electrolytes are preferentially used as coagulant and the sludge generated by this process

can be treated for partial coagulant recovery. Operating costs decreased appreciably when compared to the costs incurred with the operation of the previously used lime colour removal system (Ackel 1988).

Cationic polymers were more effective than non-ionic polymers as coagulants of lignin from pulp and paper industry wastewaters. The use of both polyethyleneimine (PEI) and hexamethylene diamine epichlorohydrin (HE) resulted in colour removal of about 80%, respectively, but only 30% total organic carbon (TOC) was eliminated from alkaline black liquor wastewater after 30 min of settling under gravity. However, chitosan, a natural coagulant, removed up to 90% of colour and 70% of TOC. Chitosan was more effective at a pH of 7.0 whereas the other coagulants performed better at pH 6.0. Greater amounts of alum, between 900 and 1 200 ppm and chitosan (1 200 and 1 800 ppm) were needed compared to the levels of HE (300 and 400 ppm) and PEI (400 and 500 ppm) to effect maximum removal efficiencies (Ganjidoust *et al.* 1997).

In another comparative study, addition of 8 ppm of a high polymer amine, Kemamine T 1902 D, at pH 3.0, gave a maximum colour removal value of 97%. In contrast, 1 000 ppm lime resulted in colour elimination approaching 90% (Gupta and Bhattacharya 1985).

OZONATION AS TREATMENT REGIMENT

Ozone selectively reacts with many colour-causing and halogenated organic compounds. The mechanism involves the conversion of high molecular mass compounds into lower mass organic acids and also changes in the molecular structure. Recent improvements in technology make ozonation of pulp mill effluent technically feasible and economically viable. This said, it should be remembered that ozonation is not widely used for the treatment of pulp mill effluents and further work is necessary to take this technology out of the laboratory and develop scaled up industrial systems. These efforts are hampered by the complexity of ozone chemistry and process design parameters are lacking, (Zhou and Smith 1997).

Zhou and Smith (1997) examined the process parameters for ozonation of kraft pulp mill effluents using fine bubble contactors. This basically consisted of a plexiglas column fitted with a fine diffuser, placed 5 mm above the bottom of the contactor through which ozone was bubbled. An ozone gas mixture was generated from extra dry pure oxygen by a corona discharge ozone generator. In laboratory tests, gas flow rates ranging from 1 000 to 2 000 ml/min were employed and the inlet ozone concentrations varied from 1.1 to 3.0% (w/w %). Pilot continuous ozonation tests were carried out at ozone inlet concentrations ranging from 3 to 5.3% (w/w %) and gas flow rates from 1 000 ml/min to 3 730 ml/min. In both pilot and laboratory batch tests colour removal of about 80% was achieved. AOX removal varied between 40 and 60% with lower removal efficiencies being recorded for the pilot tests. However, BOD concentrations after ozonation increased which indicated that biological treatment would be required to control this parameter.

Hostachy *et al*. (1997) examined advanced catalyst-ozone technology and achieved 60% COD removal with an efficiency ratio of 1.2 kg O_3/kg COD removed. Also ozone treatment converted a part of the COD to BOD.

Oeller *et al*. (1997) estimated the costs for ozonation treatment of an effluent with residual COD of 400 mg/l and a COD removal efficiency of 80%. Specific ozone consumption was set at 1.5 and 2 kg O_3/kg COD eliminated. The authors regarded their cost analyses to indicate that ozonation is an economic viable choice compared to other higher costing treatment processes such as adsorption or flocculation. Only biologically pre-treated effluent with a COD < 500mg/l and with a BOD_5/COD < 0.2 could be favourably influenced by ozonization with up to 1.8 g O_3/l wastewater. Ozonation of these biologically treated effluents brought about a rise in the BOD_5/COD ratio from an initial < 0.05 to a maximum of 0.37. This indicated a definite increase in biodegradability. Post-treatment of pulp and paper wastewater with ozone, was studied in a bubble reactor. The colour in bleach pulp effluent was reduced by 82% at a ozone consumption dose of 110 mg/l. Further increase in the ozone dose had no additional effect. The AOX was decreased by 75% at the same ozone dose and the COD was

reduced by 40% (Kallas and Munter 1994). A cost comparison between adsorption, using powdered activated carbon, and ozone treatment, indicated that to reach 80 to 90% purification by adsorption would incur costs l0 to 20 times higher than treatment by ozonation. For example at a cost of 0.2 US $/m^3 wastewater treated, an 80% AOX removal may be achieved by ozonation instead of a 15% removal by adsorption, for the same expenditure. However, it should be born in mind that activated carbon is an expensive adsorbent. Furthermore, the efficiency of ozonation depends on the type and the structure of the chemicals compounds treated, whereas adsorption using powdered activated carbon does not depend on these variables to the same extent (Kallas and Munter 1994).

ULTRA-FILTRATION

Ultra-filtration is a membrane separation process. Among the advantages of using ultra-filtration are that it is a relatively low energy consuming process, low in operating and maintenance costs (Anonymous 1996). However, membrane fouling appears to be a common and serious problem that leads to a rapid drop in permeate flux (Urbantas *et al.* 1986).

The process consists of feedwater that is pressurised by a pump and passes across a membrane in the cross flow mode. The feedwater is separated into a permeate which flows through the membrane while the remainder of the influent, termed the retentate stream, contains the material rejected at the membrane surface (Sierka and Bryant 1994). Apparently ultra-filtration is still not well accepted in the paper industry, however one of the most well known applications is for the recovery of ligno-sulphonates from sulphite spent liquor (Urbantas *et al.* 1986). Also, ultra-filtration of the alkaline stage effluent seems to give good results at a reasonable cost. Unfortunately the large volumes of the chlorination stage effluent prevent the use of ultra-filtration for the treatment of the entire bleach plant wastewater (Eriksson 1991). Application of ultra-filtration to treat the first E-stage effluent from a softwood kraft bleach pulp mill reduced the total TOCl by around 35 to 45% (Eriksson 1991).

Frostell *et al*. (1994) found that membrane filtration of the E-stage effluent gave a significant contribution to the overall treatment performance with 75% chlorine dioxide substitution being used in the first bleaching stage. However, with 100% chlorine dioxide only marginal improvement was noticed with ultra-filtration. The plant utilised a two-stage filtration unit with a concentration factor of 10 to 15. During experiments, polyether sulphone membranes with a cut-off of 25 000 Dalton were employed. A COD and AOX removal of between 40 and 50% were achieved.

IRRADIATION TREATMENTS

Photo-catalysis treatment of effluent, utilising irradiation of titanium dioxide (TiO_2) with radiation wavelengths below 390 nm, have been reported. Either the sun or ultraviolet (UV) from lamps may serve as sources of radiation. The radiation stimulates the valence band electrons in TiO_2 and the energised electrons jump to higher energy levels, leaving holes in the valence bands. These electrons react with dissolved organic or inorganic constituents in water or recombination reactions. The formation of hydroxyl radicals from water and hydroxyl ions through the inter-mediation of these holes have also been postulated. These radicals can completely oxidise organic compounds and their chlorinated derivatives (Sierka and Bryant 1994). Mobius and Cordes-Tolles (1997) however reported that the colour of wastewater in most cases were to dark to justify the use of UV-radiation as a treatment option.

Irradiation treatment employing high energy electron beam (EB) has been investigated and 40% as well as 70% AOX removal were obtained by 10 Kgy and 50 Kgy dosages, respectively. Because of its penetration power, treatment of effluents by EB can be used even if the wastewaters are turbid. The high energy consumption of EB treatment would however preclude total oxidation of organic material in effluents by this method (Berge *et al*. 1994).

COMBINATION OF PHYSICAL, CHEMICAL AND BIOLOGICAL TREATMENT STRATEGIES

In an effort to solve the complex problems surrounding effluent treatment posed by the pulp and paper industries, various treatment methods were tested in combination since no one treatment strategy can consistently solve all the problems. Of prime importance is the development of a practical, efficient and economic treatment process.

Vuoriranta and Remo (1994) used activated granular carbon not only as an adsorbent, to treat bleach kraft mill effluent, but also as a support material for biomass to facilitate biodegradation. Physical adsorption and biodegradation were studied in the granular activated carbon (GAC) system and a reduction in dissolved organic carbon (DOC) totaling 57% was attained over a period of 18 weeks of treatment. AOX removals of 70 to 90% were also achieved. Biodegradation and bioregeneration accounted for a stable removal of 20 to 30% in DOC levels. Saturation of the fluidised granular activated carbon column was not reached even though heavy loading with effluent was implemented. Apparently adsorption on GAC and biomass were followed by desorption and biodegradation. This seems to be the mechanism responsible for the rapid partial regeneration of the fluidized bed when treating bleach kraft mill secondary effluent. Therefore, bioregeneration seems to be responsible for maintaining the removal capacity during these studies, beyond the time that would normally be expected. Reduction in the removal rate of the GAC did occur with time, but this could be partially offset by the addition of nutrients to a nutrient deficient feed applied to the column (Vuoriranta and Remo 1994).

In one investigation ozonation was combined with biofiltration (Mobius and Cordes-Tolle 1997). The AOX reduction in the ozone reactor itself averaged 67%. In the subsequent biofilter, the AOX levels were diminished, on average, by a further 15%. Ozonation thus improved biological breakdown of persistent compounds, therefore increasing the BOD_5 and for this reason the effluent must be subjected to further biological treatment. This study indicated that the cost of treating wastewater by this method would incur costs of 1.5 US $/kg COD removed. The process has proven

satisfactory as a post-treatment strategy of biologically treated effluent where COD, AOX and colour levels still exceed the legal limits.

Sierka and Bryant (1994) investigated the changes in kraft bioeffluent quality as a function of increasing chlorine dioxide (ClO_2) substitution and the effect of ultra-filtration as well as heterogeneous photo-catalysis treatment on the condition of the effluent stream. Substitution of 10% ClO_2 for 50%, led to a more readily biodegradable waste.

For 10% ClO_2 substituted E-stage wastewater, best results, in terms of toxicity reduction, colour removal and COD elimination were recorded with a pretreatment strategy that included exposure to sunlight + TiO_2 + O_3, followed by ultra-filtration. After pre-treatment the effluent was subjected to aerobic treatment for 7 d. Nevertheless pre-treatment following the sequence: ultra-filtration then exposure to sunlight + TiO_2 + O_3, resulted in a bioeffluent with the lowest AOX level. In terms of TOC concentration levels, the lowest value was recorded after a pre-treatment sequence that included exposure to sunlight and TiO_2, followed by ultra-filtration. From these results it is clear that the sequence in which the different treatments were applied had an important impact on the various parameters studied. When improvements relative to conventional treated kraft bioeffluent (7 d aerobic treatment, without pre-treatment), quality is compared, for the different pre-treatment strategies, then the superiority of using 50% ClO_2 and ultra-filtration + UV (365 nm) + TiO_2 + O_3, was noticeable. Interestingly, the best physical and chemical treated product never corresponded to the best final bioelluent. This could possibly indicate that important modifications in pollutant biodegradability are brought about during membrane + photo-catalysis treatment.

4. CONCLUSIONS

In general, the high volumes and complexity of effluent produced by the pulp and paper industry present formidable problems for the treatment of these wastewaters. Biological treatment methods that utilise ligninolytic microorganisms, especially white-rot fungi, have potential, but the requirement for a co-substrate and the need for aeration would seriously hamper their utilisation from an economic perspective. Cultivation conditions have an important impact on the efficiency of white-rot fungi during biological treatment of bleach plant effluent. Greater biodegradation efficiencies are required in order to develop a practical biotreatment process. Physical treatment methods such as biosorption of chromophores from bleach effluent could have several advantages. These could include the availability of a relative inexpensive adsorbent material on site, the fast rate of the adsorption process and the option of incineration of the spent adsorbent after use. Because of the complexity of treating paper mill effluent, combinations of physical, chemical and biological treatment strategies might lead to a synergistic beneficial outcome that would facilitate the development of economic and efficient treatment procedures.

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CHAPTER II

PHYSICO-CHEMICAL ANALYSIS OF BLEACH PLANT EFFLUENT

1. SUMMARY

Bleach plant effluent obtained from the alkali extraction stage (Eo) of a dissolving sulphide pulp mill was chemically characterised by various methods. It was necessary to examine the chemical nature of the bleach plant effluent to devise effective treatment methods. Routine chemical analyses of effluent included: alkali-lignin, COD and pH determinations. Additional measurements involved physico-chemical techniques such as adsorption spectra analysis and determination of colour intensities of the wastewater. Effluent used during the biological treatments was studied in greater detail and analysis also included: AOX (absorbable organic chlorine), chlorophenol, gel permeation chromatography, nitrogen, phosphate and sugar determinations. Effluent collected at different periods from the bleach plant of the pulp mill usually differed significantly in chemical composition. Effluent employed during biological treatment studies contained trace amounts of nitrogen as well as carbohydrates however, no ortho phosphate was detected in the wastewater. Overall, the pH of the various effluents studied was in the alkaline range, colour intensities varied between 5 500 PCU to as high as 15 700 PCU and COD levels of 7 061 to 14 567 mg/l were measured. These methods provided crucial information that was essential for the planning of experiments conducted during this study. Furthermore, it was also used to measure the efficiency of the treatments methods applied.

2. INTRODUCTION

Bleach plant effluents, produced during chemical treatment of pulp by chlorine compounds, can contain approximately 300 different low molecular mass chlorinated byproducts of the bleaching process (Kallas and Munter, 1994). These effluents are also highly coloured mostly because of the presence of high molecular mass chlorolignins. According to literature the chromophore-bearing compounds are highly acidic and polymeric (Sundman and Kirk 1981). From the preceding section it is evident that bleach plant effluents contain complex mixtures of chemicals that can influence the biological treatment of these wastewaters. Studies by Christov and Steyn (1998) indicated that fungal treatment of Eo-effluents was sensitive to changes in effluent composition. Therefore, results may vary depending on initial parameters such as pH and colour employed during treatment of these effluents. The levels of macronutrients in Eo-effluent such as nitrogen and phosphate as well as the carbohydrate content could have a significant effect on the biological treatment of this wastewater**.**

3. MATERIALS AND METHODS

Adsorption spectra analysis and absorption measurements

Adsorption spectra of effluent were drawn after dilution and filtration using wavelengths varying from 200 nm to 800 nm with a Unicam He?ios B spectrophotometer (Cambridge, UK). Absorption measurements were also taken at fixed wavelengths, after filtration and dilution.

Alkali-lignin analysis

The pH of the effluent was adjusted to a value of 1 with concentrated HCl (Hernández *et al*. 1994) and left over night at 4ºC. Precipitates that were obtained after centrifugation were washed repeatedly and determined gravimetrically after drying to a constant weight at 50ºC in an oven.

AOX determinations

After filtration on a Büchner funnel the pH of AOX samples (300 ml) were adjusted to a value of 1.80 with concentrated HNO_3, transferred to brown bottles, frozed at a temperature of -20ºC and stored in a freezer until analysis were performed. AOX content was determined using a Euroglas AOX EC S 1000 analyser (Delft, The Netherlands) according to the manufacturer's instructions.

Carbohydrate determinations

A Dionex DX 500 high performance liquid chromatograph with pulse amperometric detection (Dionex Corporation, Sunnyvale, CA) was used for carbohydrate analysis. The Dionex was equipped with a CarboPac PA-10 column (Dionex Corporation) and the flow rate of eluent (1.8 mM NaOH) was set at 1 ml/min. After 25 min the column was regenerated for 10 min. The system was operated at 30ºC. After dilution, samples were filtered through 0.22 µm filters and injected, without further treatment. To measure oligo and polymer sugar content of effluent, samples (20 ml) were first treated with 1 ml concentrated sulphuric acid in a boiling water bath for 2 h. Samples were then cooled on ice, neutralised with NaOH and filtered through Whatman filter paper. Lastly, samples were also filtered through On Guard Ba and P columns before analyses using the Dionex HPLC were performed.

Chlorophenol analyses

Chlorophenols were extracted from bleach plant effluent by application of dichloromethane as organic solvent. Prior to extraction the pH of the samples were adjusted to 3.0 with concentrated sulphuric acid. Effluent was agitated by magnetic stirring in the presence of dichloromethane for 4 h in sealed bottles. The organic and water phases were separated in a separating funnel and the water extract was further extracted with a fresh batch of dichlorophenol for another 4 h. Thereafter the extracts were combined. Following extraction, the organic phase of each sample was dried over anhydrous Na_2SO_4 and then concentrated under a stream of nitrogen gas. Chlorophenol analysis was performed using a HP6890 (Palo Alto, CA) gas chromatograph (GC) with

flame ionization detector. The GC was equipped with a Chrompac (Middelburg, The Netherlands) 7542 column (10 m x 0.53 mm) utilising nitrogen as carrier gas (1 ml/min) and a linear temperature programme operating at a rate of 5 ºC/min up to 240ºC. Programmed temperature increase was implemented after an initial 1 min period at 90ºC. To identify chlorophenols in the effluent, extracts were spiked with standard compounds and compared to chromatograms of extracts obtained without spiking. An increase in peak area (of a peak with a retention time similar as the original peak in the sample that was obtained without spiking) after spiking indicates the presence of the spiked compound in the effluent.

COD measurements

Effluent samples contained in COD vials were digested in a Hach digester (Loveland, Colorado, USA) for 2 h, cooled down to room temperature and then the COD of the samples were determined at 620 nm with a Hach DR/2000 spectrophotometer. A COD standard comprising dried potassium acid phthalate, prepared by dissolving 425 mg potassium acid phthalate and making up to 1 litre with demineralised water in a volumetric flask, was used to check and adjust COD readings obtained.

Colour determinations

Samples for colour determinations were filtered through a 0.45 µm Millipore membrane filter after the pH was adjusted to 7.6 with sulphuric acid. The colour was detected with a Hach DR/2000 spectrophotometer at 455 nm using APHA (American Public Health Association) Platinum-Cobalt standard with a range of 0 to 500 colour units (PCU). One color unit equals 1 mg/l platinum as chloroplatinate ion. By adjusting the pH of effluent and analysing the colour levels of bleach plant samples as a function of pH the effects of pH on colour intensities was determined.

Nitrogen determinations

Total nitrogen in effluents was measured by a Kjeldal method according to standard procedures described in Tappi methods (T418). A Dionex 4500i ion chromatograph equipped with anion separator column (ion pac AS 9) and conductivity detector was used for nitrate determinations, according to methods set out in the manual of the instrument. As eluent a mixture of 0.125 g/l sodium carbonate and 0.125 g/l sodium bicarbonate was employed.

Phosphate analyses

Ortho-phosphate was determined using ion-exchange chromatography similarly as described for nitrate determinations in the preceding section.

Molecular mass analyses

Gel permeation chromatography (GPC) was performed using a Waters high performance liquid chromatograph equipped with a 250 ultrahydrogel column. Sodium nitrate (0.1M) served as eluent and was delivered to the column at a flow rate of 0.6 ml/min (Smith *et al*. 1994). Peaks were identified by refractive index measurements. Polymeric sugar compounds of various molecular mass, used as standards, were prepared according to instructions received from the suppliers (Showa Denko K.K., Japan). These standards were used to estimate the molecular mass of compounds in the Eo effluent.

4. RESULTS AND DISCUSSIONS

Physico-chemical characteristics of bleach plant effluents

In Table 2.1 a summary of the physico-chemical characteristics of bleach plant effluent obtained at different periods from the pulp mill are presented.

Table 2.1: Physico-chemical characteristics of bleach plant effluents

Parameters	Eo-effluents		
Colour (PCU)	5 500	9 550	15 700
pH	8.6	7.6	8.1
COD (mg/l)	7 061	9 750	14 567
Alkali lignin (mg/l)	253	288	420
Colour[a] (PCU)	3 825 (31)[c]	5 450 (43)	9 075(42)
Absorption 254 nm[b]	0.64	0.79	1.26
Absorption 280 nm	0.46	0.56	0.88
Absorption 455 nm	0.04	0.06	0.08
Absorption 254 nm[d]	0.54 (16)	0.56 (29)	0.91 (28)
Absorption 280 nm	0.37 (20)	0.36 (36)	0.66 (25)
Absorption 455 nm	0. 03 (25)	0.03 (50)	0.04 (50)

[a]Colour after alkali lignin extraction with HCl

[b]Absorption before alkali extraction

[c]Values in brackets indicate percentage reductions in values after alkali lignin extraction

[d]Absorption values following alkali lignin extraction

Chemically there were significant differences in the composition of effluent obtained at different periods from the pulp mill. These differences result from various wood types that are processed during dissolving pulp manufacture so that the effluent composition changes over time. COD levels recorded in the effluent were relatively high; this is an important parameter as far as loading of biological reactors systems is concerned.

As could be expected for an alkali extraction stage effluent, the pH of this wastewater was in the alkaline range. The effect of pH on effluent colour is shown in Fig. 2.1. Colour intensity of Eo-effluent is significantly affected by pH and therefore colour determinations have to be conducted at a specific pH values for results to be comparable.

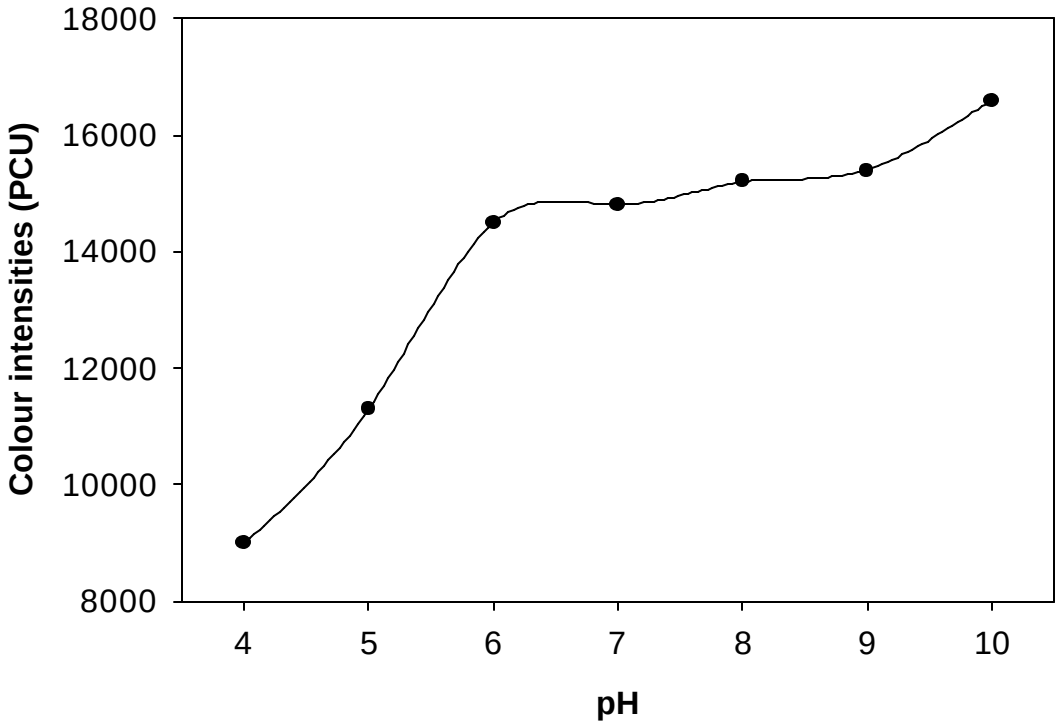

FIG. 2.1: Plot of effluent colour against effluent pH

The alkali lignin fraction presents compounds that are insoluble at acid pH values. Levels of alkali lignin extracted from the effluents differed. Colour reductions following alkali lignin extractions were also almost the same at 43% for the 9 550 PCU effluent and 42% for the 15 700 PCU effluent. However, the percentage decolourisation (31%) following alkali lignin removal from the 5 500 PCU effluent was somewhat lower. Therefore, in general, the alkali lignin fraction comprises a significant portion of the amount of coloured compounds present in Eo-effluent. Absorption values obtained at fixed wavelengths of 254 nm gives an indication of the aromatic content of the effluent, whereas the absorption readings obtained at 280 nm reflect phenolic composition (Ziobro 1990). Absorption levels were also taken at 455 nm, since this is the wavelength used for

the determination of colour in the effluent. Reductions in absorption levels recorded at these wavelengths, following alkali extraction, also differed.

Physico-chemical characteristics of effluent used during biological treatments

Physico-chemical characteristics of effluents studied in bioreactors are shown in Table 2.2.

Table 2.2: Physico-chemical attributes of the Eo-effluent that was used during biological treatment of the wastewater

Parameter	Value
AOX (mg/l)	13.8
Alkali lignin (mg/l)	303
Colour (PCU)	12 200
Carbohydrates (not hydrolysed) (mg/l)	27.6
Carbohydrates (hydrolysed)[a] (mg/l)	81.7
COD (mg/l)	11 717
Total nitrogen (mg/l)	16.0
Nitrates (mg/l)	10.4
Ortho-phosphate (mg/l)	ND[b]
2,4-dichlorophenol (mg/l)	3.1
pH	8.1

[a] Effluent treated with sulphuric acid (see materials and methods).

[b] Not detected

The AOX content was relatively low when compared to values reported for other bleach plant effluents (Paasivirta *et al*. 1992; Nagarathnama and Bajpai 1999). In contrast, both colour and COD levels were high as would be expected for this type of effluent. The sugar content of the effluent was low. However, following acid treatment carbohydrate concentration increased, indicating that oligo and/or polymeric sugars were present in the effluent. Phosphate (measured as ortho-phosphate) could not be detected in the Eo-effluent and nitrogen levels were low. This indicated that nitrogen, phosphate and

carbohydrate would most probably have to be supplemented during biological treatment of Eo-effluent for optimal bioremediation to be achieved. The only chlorophenol that could be identified in the effluent was 2,4-dichlorophenol.

Absorption spectrum of bleach plant effluent

The absorption spectrum of effluent is presented in Fig. 2.2. Absorption peaks were observed at the lower ultraviolet range as well as at about 280 nm. This absorption peak at 280 nm is typical for lignin as well as chlorolignin because of the presence of phenolic groups in these compounds (Wang *et al*. 1992).

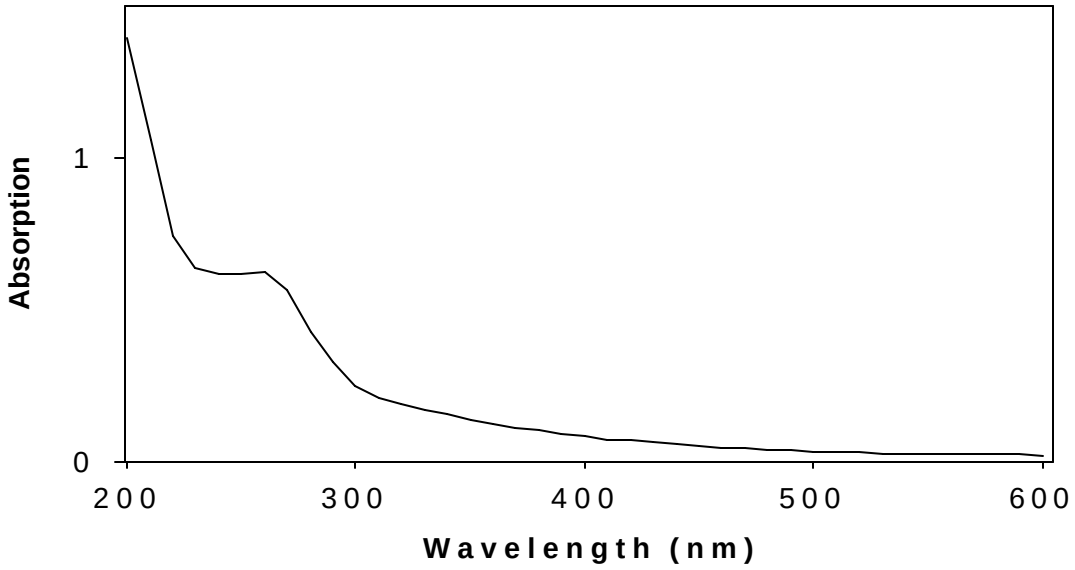

FIG. 2.2: Adsorption spectra of diluted effluent.

Molecular mass distribution of bleach plant effluent

The relationship between retention time and the log of the molecular mass of standard compounds is shown in Fig. 2.3. This data was used to estimate the molecular mass of compounds detected in raw Eo-effluent.

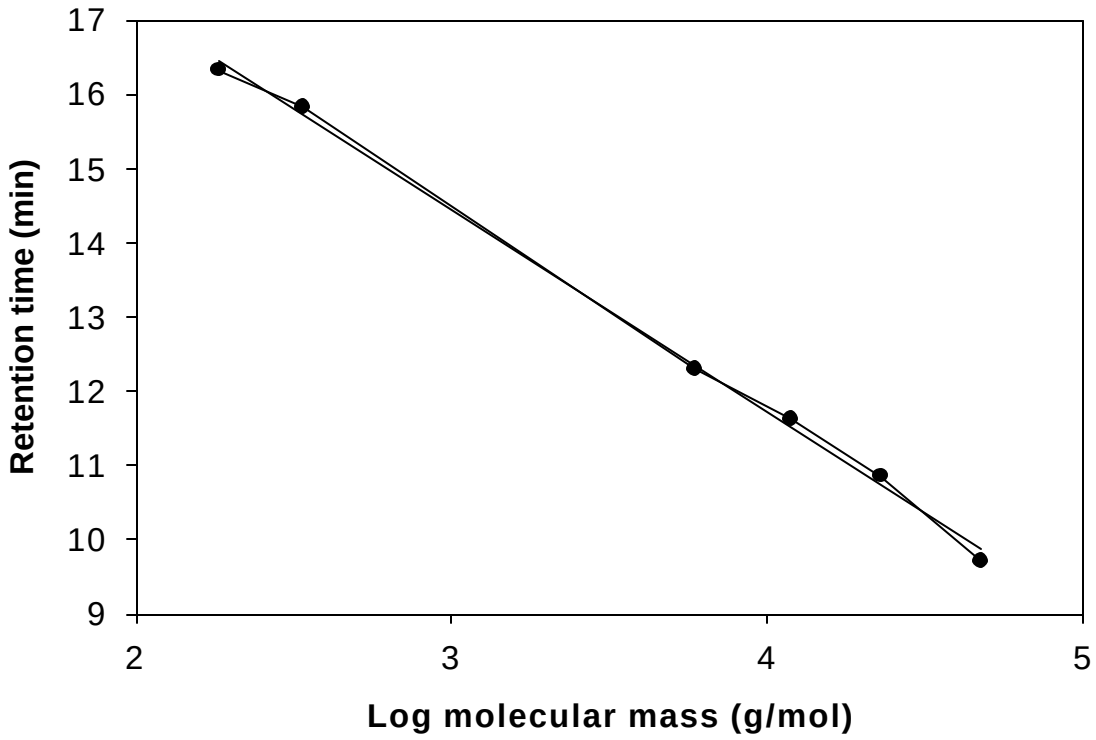

FIG. 2.3: Plot of retention time against log molecular mass using standard molecular mass compounds.

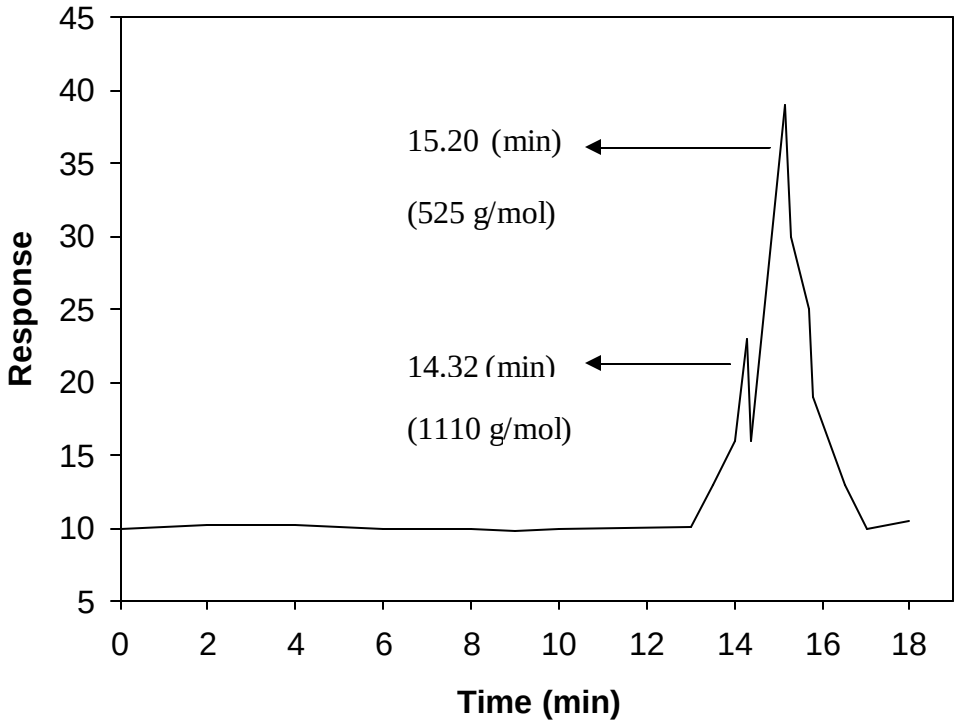

FIG. 2.4: Gel permeation chromatogram showing apparent molecular mass content of raw bleach plant effluent**.** Retention times and corresponding molecular mass, shown in brackets, have also been included.

Molecular mass content of raw effluent is shown in Fig. 2.4. The retention time for the integrated peaks corresponds to apparent molecular weights of about 1110 g/mol to about 525 g/mol, with the most prominent peak belonging to the 525 g/mol fraction.

5. CONCLUSIONS

Bleach plant effluents are complex mixtures containing a high colour and COD content and with compounds of various molecular mass being present. Nitrogen, phosphate and sugar concentrations found in these effluents in general are low or non-detectable. Therefore, these nutrients will have to be supplemented before effective biological treatment can be implemented. Effluent composition of different batches received at various times from the mill varied significantly and therefore treatments will have to be executed using a single batch for each study.

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CHAPTER III

PHYSICO CHEMICAL TREATMENT OF BLEACH PLANT EFFLUENT

1. SUMMARY

Adsorption-, chemical-, flocculation-, ion-exchange and ultraviolet irradiation treatments were evaluated for the remediation of Eo-effluent. The best decolourisation activities were obtained using adsorption as treatment method, with activated carbon removing almost all colour from effluent (> 99%). Chitosan (81%) and chitin (77%) could remove appreciable levels of colour from bleach plant effluent, followed by biomass from *Rhizomucor pusillus*, a mucoraelean fungus (71%). Chitosan and chitin could be major components of the cell wall of *R. pusillus* and these compounds might therefore be involved in the decolourisation ability of this fungus. Effluent pH was inversely related to effluent decolourisation when *R. pusillus*, chitosan or chitin was used as adsorbents. This might in part be due to the contribution made by acid catalysis during nucleophilic addition reactions, during which amino groups of chitin/chitosan react with carbonyl compounds present in Eo-effluent. Also amino groups present in chitin and chitosan can be protonated under acidic conditions and therefore acquire a positive charge that can interact with the chromophores found in Eo-effluent. However, pH exerted no significant effect on decolourisation when activated carbon was employed as adsorbent of effluent colour. Decolourisation employing commercial adsorbents seemed to be mainly due to chemisorption. Flocculation of coloured compounds from Eo-effluent by chitosan containing solutions resulted in a maximum decolourisation activity of 75%. Anion-exchange treatment was also extremely effective for the removal of colour (96%) from Eo-effluent. Ultraviolet irradiation could decolourise the Eo-effluent by about 42 to 43% after 160 min of treatment. Decolourisation using organic solvent extraction proved to be

the least effective treatment method with a maximum colour removal of only 21% being achieved when methanol was used as solvent.

2. INTRODUCTION

Bleach plant effluent from the pulp and paper industries that utilise chlorine compounds as bleaching agents contain toxic chlorinated organic materials and are also highly coloured (Bajpai and Bajpai 1994). Legislation is becoming stricter in an effort to control the impact of effluent on receiving waters. Therefore these effluents require remediation prior to discharge (Christov *et al*. 1999). Although physical-chemical treatments have been used successfully to decolourise bleach plant effluents, it is generally accepted that such strategies are too expensive for commercial application. Recently interest has been shown in fungal adsorption of coloured compounds from a bleach plant effluent (Jaspers and Penninckx 1996). In general terms, adsorption has been shown to be a good method to remove colour contamination (Liversidge *et al*. 1997). Although adsorbents such as activated carbon are effective in removing colour from effluents, they are too expensive from an economic perspective (Poots *et al.* 1976). Therefore a considerable effort has been made to discover alternative, inexpensive adsorbents (Liversidge *et al*. 1997). Fungi are extensively employed in a variety of large-scale fermentation processes and can therefore be regarded as a potential source of adsorbent material (Kapoor and Viraraghavan 1995).

It has also been reported that certain physico-chemical methods have improved cost wise and therefore could be promising for effluent treatment (Kallas and Munter 1994). Thus various physico-chemical treatment methods were evaluated for effluent decolourisation, with special emphasis on colour adsorption and the use of alternative/unconventional adsorbents for bioremediation purposes. The mechanisms involved in adsorption were furthermore investigated.

3. MATERIALS AND METHODS

Biomass production

Rhizomucor pusillus RM7 (referred to in the text as RM7) were maintained on potato dextrose agar at 4ºC. Fungal strains were obtained from the Sappi Biotechnology culture collection at the University of the Free State. For inoculum preparations, four 1x1 cm cubes were cut from fresh plates and transferred to sterile 500 ml Erlenmeyer flasks containing 200 ml growth medium of the following composition in g/l: glucose, 20; malt extract, 20; peptone, 20; yeast extract, 8. The pH of the medium was adjusted to 5 prior to sterilisation. After 4 days incubation on an orbital shaker at 30ºC, 40 ml culture broth was aseptically transferred to sterile 1l Erlenmeyer flasks containing 400 ml growth medium. After 2 days incubation at 30ºC on an orbital shaker, mycelium was transferred aseptically to Erlenmeyer flasks (500 ml containing 200 ml growth medium) and cultivated under identical conditions as described above. *Saccharomyces cerevisiae* was obtained commercially (Anchor Yeast, Industria, South Africa), in the form of dried yeast material. Yeast biomass was washed with distilled water and dried before processing and use as an adsorbent.

Adsorbent preparation

Adsorbents were ground into a powdered form. Thereafter the material was sieved using a 710 µm aperture sieve and dried at 50ºC to constant weight (were applicable). Biomass (used wet without drying) was washed with distilled water before the moisture content was determined with a HG 53 halogen moisture analyzer (Mettler Toledo, Greifensee, Switzerland).

Cell wall fractionation and chitosan extraction from *Rizomucor pusillus*

Dry biomass (4 g) was treated with 100 ml 1 N NaOH at 121ºC for 30 min. The alkali-resistant fractions were subsequently treated for chitosan extraction according to the method of Arcidiacono and Kaplan (1992). This method involves the homogenisation of alkali-extracted material in the presence of 2% acetic acid followed by reflux in 2% acetic acid for1 h. Thereafter the pH of the supernatant was adjusted to 8.5 with NaOH. The

resultant precipitate (chitosan fraction) was washed with deionised water. The alkali-resistant, chitosan and the residual fractions, obtained after chitosan extraction, were used in adsorption experiments.

Effluent

The effluent was obtained from the alkali extraction stage (Eo) of the bleach plant of Sappi Saiccor, Umkomass, South Africa. Effluent was stored in closed containers at 4ºC. The Eo-effluent had a colour intensity of 10 000 PCU, a COD of 10 200 mg/l and a pH of 8.2.

Adsorption experiments

Before use in adsorption experiments, fungal biomass was washed thoroughly with distilled water on Buchner funnels. Dried RM7 and *S. cerevisiae* biomass were used at a charge of 4g/100 ml Eo-effluent. The alkali-resistant, chitosan and the residual fractions, obtained after chitosan extraction was completed, were washed, dialysed against deionised water, dried at 50ºC to a constant weight and used in adsorption experiments. Adsorption experiments were conducted in shake flasks at 30ºC to treat 100 ml of Eo-effluent. The Effluent pH was adjusted to a value of 7.4 before adsorption experiments commenced. Charges used for the cell wall fractions were 0.332 g per 100 ml effluent, for the residual fraction, 0.145 g per 100 ml effluent, for the chitosan fraction and 0.615 g per 100 ml effluent, for the alkali-resistant fraction. These charges were based on the amount of cell wall material extracted from 4 g dry *R. pusillus* biomass. Wet RM7 biomass was also employed as adsorbent at a charge of 4 g dry equivalents per 100 ml effluent in shake flasks at 30ºC. Samples were filtered through Millipore filters before readings were taken.

Adsorption experiments using activated carbon, commercial chitin and chitosan as well as other adsorbents used in screening experiments were conducted in a similar way as described for the fungal biomass, except where indicated otherwise.

Adsorption experiments were also conducted with activated carbon, chitin, chitosan and dry RM7 as adsorbents at different ionic strengths. This was accomplished by the inclusion

of various Na_2SO_4 concentrations (0 to 1 M) in Erlenmeyer flasks during these investigations. Colour and pH values were monitored regularly.

Furthermore, the effect of pH on decolourisation during adsorption experiments was determined using activated carbon, chitin, chitosan and *R. pusillus* biomass as adsorbents. The charges used during these tests were 4 g/100 ml effluent. Effluent pH was tested over the range of 4 to 10 (except for chitosan since it dissolves at pH levels lower than 6, the range tested in this instance therefore were from 6 to 10). During tests the pH of effluent was monitored and adjusted when necessary, furthermore compensation were made for the dilution effect brought about by the addition of acid or base when colour intensities were determined.

Desorption experiments

The sorbent materials (activated carbon, chitosan, chitin and RM7-biomass) were tested in desorption experiments to establish whether they could be regenerated. Colour release during alkali treatments was followed over time to establish effective treatment periods. Furthermore, various concentrations of NaOH were tested to determine the alkali concentration necessary for colour release from adsorbents to proceed maximally. Based on results, 1N NaOH was employed for colour removal. Treatment with 1 N NaOH was carried out at 30ºC for 24 h on an orbital shaker. Colour desorbed was measured and compared against the amount of colour initially adsorbed onto the respective adsorbents.

Cell wall hydrolysis

Cell wall components (alkali-resistant, chitosan and residual fractions) obtained from *R. pusillus* biomass as well as commercial chitosan (Fluka) were hydrolysed in two steps. Initially samples were treated with 5 ml of 72% (v/v) sulphuric acid for 1 h at 75ºC. Thereafter 40 ml of distilled water was added and the samples were hydrolysed at 121ºC in an autoclave for 40 min. After hydrolysis, the samples were cooled on ice, neutralised using NaOH and sample volumes were adjusted to 100 ml with deionised water.

Ion-exchange treatment

Dowex-1, a strong anion-exchange resin (OH^--form) and Bio-Rad AG 50WX12, a strong cation-exchange resin (H^+-form) were used for the treatment of effluent conducted on rotary shakers at 30 ºC. Removal of colour from Dowex (OH^--form) were attempted using 1 N NaOH. The charges applied were respectively 20 g/100 ml Eo-effluent (Dowex) and 18.4 g/100 ml Eo-effluent (Bio-Rad). Experiments were also conducted using Dowex ion exchange resin converted to various ionic forms. Dowex resin (Cl^--form) was treated with 0.5 M NaOH, Na_2CO_3 and H_2SO_4, to convert the resin to the OH^-, CO_3^{-2} and SO_4^{-2} counter ion containing forms, respectively. Thereafter the resins were washed with deionised water. Experiments were conducted in shake flasks at 30 ºC at a charge of 5.6 g/100 and an initial effluent pH of 6.

Isotherm determinations

Isotherms experiments were conducted by incubating 0.2 g of adsorbent material in 20 ml effluent (pH 7.4) of initial colour in the range 725 to 33 200 PCU. Effluent was concentrated by evaporation at 50ºC to obtain colour intensity levels of 33 200 PCU. Experiments were conducted in sealed tubes on a rotary shaker at 30ºC and 100 rpm. The contact time employed was determined in advance by kinetic experiments conducted in a similar manner as described for the isotherm determinations. Decolourisation was followed up to 120 h to ensure that equilibrium was attained.

Flocculation

The effect of concentration and molecular mass (M_r) of chitosan dissolved in 1% (v/v) acetic acid solutions (flocculent) on decolouring activity during treatment of Eo-effluent was also investigated. A 1:1 mixture of chitosan: Eo was employed. Colour was determined after the flocks that formed upon mixing (by magnetic stirring), settled out under gravity after 20 min. Experiments were performed at room temperature.

Extraction of effluent with solvents

Eo-effluent was dried in evaporation trays at 105 ºC to a constant weight. Dried effluent (2.4 g) was extracted with chloroform, methanol, propan-2-ol, and water, respectively in a

soxhlet-extractor for 9 h. In each case 200 ml of solvent were used during extractions. Colour and light absorption of samples were determined before and after extraction.

Ultraviolet irradiation of effluent

Samples (600 ml) undiluted and 1:1 diluted with distilled water were irradiated with an ultraviolet light (Philips lamp of 30 W). The distance between the light source and the samples were 6 cm. Samples were stirred by a magnetic stirrer during irradiation. A control sample was kept in the dark. Aliquots (2 ml) were withdrawn during irradiation as well as from the control for colour determinations. COD of untreated and samples treated after 160 min were determined.

Analytical methods

Colour readings of the effluent samples were performed using a Hach spectrophotometer at 455 nm after appropriate dilutions and pH adjustments to pH 7.6. Colour of samples was expressed as Platinum Cobalt colour units (PCU). Absorption readings of samples were also taken at 254 nm, 280 nm and 455 nm using a Genesis spectrophotometer. A Dionex DX 500 HPLC with pulse amperometric detector (Dionex Corporation, Sunnyvale, CA) was used for carbohydrate analysis. The Dionex was equipped with a CarboPac PA-10 column (Dionex Corporation) and the flow rate of deionised water, used as eluent, was set at 1 ml/min. Separations were carried out for 25 min and thereafter the column was regenerated for 10 min. The HPLC system was operated at 30ºC.

4. RESULTS AND DISCUSSIONS

Ion-exchange treatment of effluent

EFFECT OF VARIOUS COUNTER IONS ON DECOLOURISATION TREATMENT USING DOWEX ANIONIC RESIN

Eo-effluent was treated with Dowex anion-exchange resins containing the counter ions OH^- CO_3^{-2}, and SO_4^{-2}, respectively to study the effect of various counter ions on decolourisation by this resin. Results are presented in Table 3.1. The presence of different counter ions, in general, did not have an appreciable effect on decolourisation by the ion-exchange resin, with the possible exception when SO_4^{-2} was used as counter ion. During treatment pH of the effluent increased and thereafter remained fairly stable around 9.25 to 9.40. In subsequent experiments during this study the OH^- containing form of Dowex were used.

Kratochvil and Volesky (1998) discussed the importance of selection of specific ionic forms of resins for optimal performance of ion-exchange systems. It was, for instance, observed that when *Rhizopus arrhizus* biomass was converted from H- to Na- or Ca/Mg-counter ion containing forms, respectively, the performance of the biosorption system more than tripled (Fourest and Roux, 1994). Ion-exchange might play a role during decolourisation of effluent by *R. pusillus* and this was one of the reasons why ion-exchange experiments were included in this study.

Table 3.1: Anion-exchange treatment of Eo effluent employing Dowex resin containing various counter ions.

Time (h)	pH	% Decolourisation
(OH^--form)		
0.1	9.37	14
2	9.40	56
24	9.40	75
168	9.31	94
(CO_3^{-2} -form)		
0.1	9.32	12
2	9.35	55
24	9.35	76
168	9.34	93
(SO_4^{-2}-form)		
0.1	9.25	8
2	9.26	59
24	9.25	75
168	9.33	86

ANION- AND CATION-EXCHANGE TREATMENT OF EFFLUENT

Dowex (strong anion-exchange resin) removed almost all colour (96.1%) from Eo-effluent, whereas the Bio-Rad resin (strong cation-exchange resin) decolourised the effluent by only 23 % (Table 3.2). Therefore although most of the chromophores found in Eo-effluent exhibit a negative charge, there seem to be a fraction of the coloured materials that can carry both negative and positive charges. Otherwise, and also more probable, some of the coloured compounds can associate non-specifically with the polystyrene components of the resins.

Table 3.2: Decolourisation of Eo-effluent using anion- and cation-exchange resins.

Time (h)	Colour adsorbed (%)	
	Dowex (OH^-)	Bio-Rad (H^+)
0	52	0
2	82	17
4	86	23
8	89	23
24	92	22
48	94	20
72	96	20
96	96	18

EFFECT OF ANION EXCHANGER CHARGE ON DECOLOURISATION

Effluent decolourisation by Dowex resin was examined using resin charges that varied from 0.33 g/100 ml up to13.38 g/100 ml effluent. Decolourisation of effluent began to level off at a charge of 5.3 g/100 ml with a decolourisation activity of 93 % being attained. The colour was removed by 96% at a charge of 13.38 g/100 ml (Fig. 3.1). Anion-exchange treatment was highly effective for the decolourisation of effluent and compared favourable to the decolourisation level achieved by using activated carbon as adsorbent. NaOH (1 N) could not remove colour taken up by Dowex. Interactions between the anion-exchanger and the chromophoric material from Eo-effluent were therefore strong. Dowex contain quaternary amino groups that exhibits strong basic characteristics and are believes to be one of the active sites for interaction with the effluent.

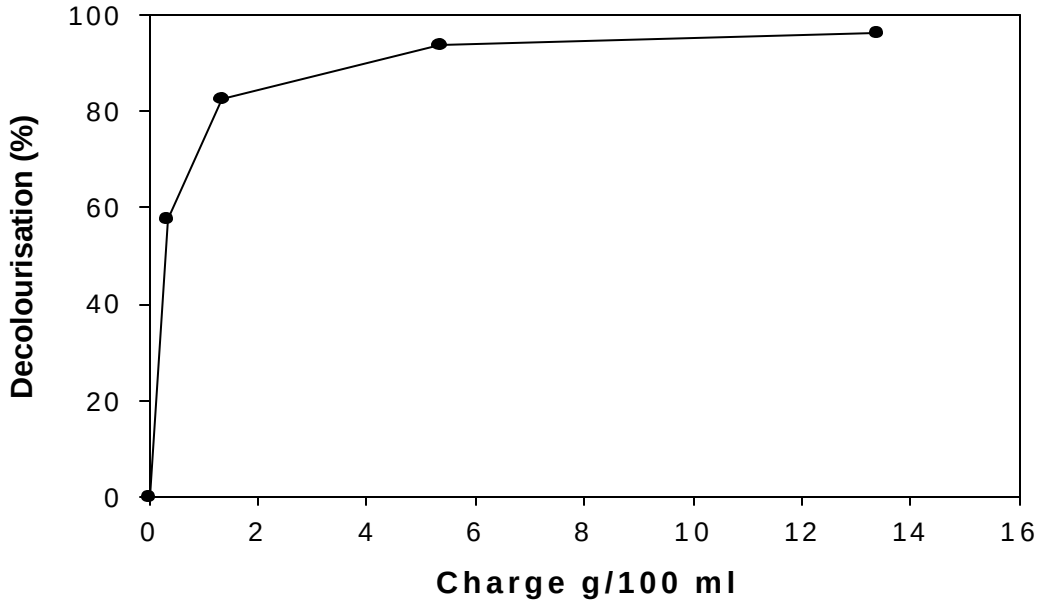

FIG. 3.1: Effect of Dowex anion-exchange resin (OH^--form) charge on decolourisation of Eo-effluent.

Colour adsorption

SCREENING OF ADSORBENTS

Various adsorbent were screened for colour removal from effluent, some of these results are shown in Table 3.3. Activated carbon proved to be the best adsorbent of colour in the effluent, followed by chitosan, a poly-aminosugar compound found in the cell wall of certain fungi. Activated carbon was included in this study as a reference adsorbent. Although it has been reported that barbecue charcoal could remove colour from textile plant effluent (Meyer *et al*. 1992), it proved unable to do so from bleach plant effluent. Cellulose, an analogue of chitosan, differing from chitosan in that an OH-group is found

at carbon two instead of a NH_2-group, decolouring activity when compared to chitosan. This seems to indicate that the amino groups present in chitosan was involved in the removal of colour from effluent. Wood shavings have been used for the removal of colour from dyes (Abo-Elela and El-Dib 1987) however, during treatment of Eo-effluent, wood material increased the colour (after 2h), probably because of the formation of chromophores by the action of alkali extraction of lignin present in the wood material.

Table 3.3: Adsorption of colour from Eo-effluent using various adsorbents

Time (h)	Decolourisation (%)				
	Adsorbents				
	Activated carbon	**Barbecue charcoal**	**Cellulose Powder**	**Chitosan**	**Wood material**
0	37	1	1	10	2
2	51	1	2	45	-8
4	61	0	3	54	-6
8	74	0	4	54	-22
24	94	0	7	62	-42

EFFECT OF COMMERCIAL ADSORBENT CHARGE ON DECOLOURISATION

Decolourisation was studied in relation to adsorbent charge. With chitosan and chitin a charge of 8 g/100ml seem to approach the optimum level necessary to achieve maximum decolourisation. However, decolourisation by activated carbon already begin to level off at a charge of 2 g/100 ml (Fig. 3.2) and almost all colour (> 99%) was removed at a charge of 8 g/l, indicating that it is a better adsorbent than chitosan or chitin. It was decided to conduct further adsorption experiments using a charge of 4 g/100 ml effluent, so as not to use excessive amounts of adsorbents during this study and for comparative purposes.

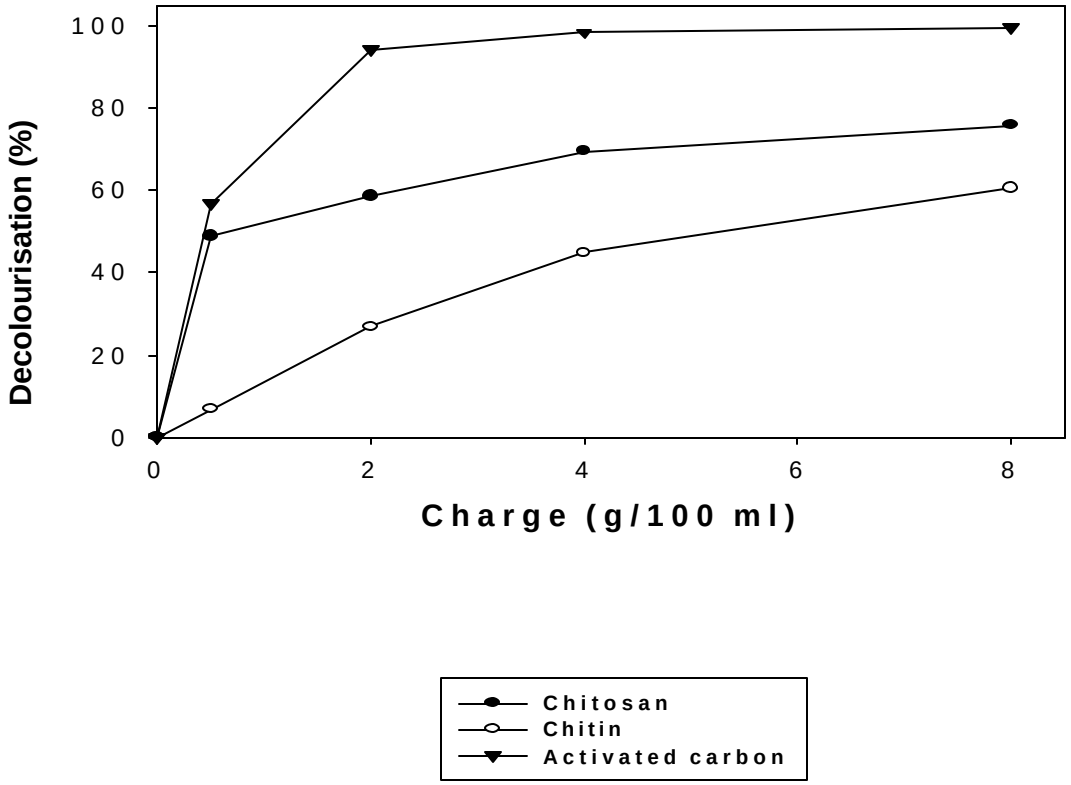

FIG. 3.2: Decolourisation expressed as a function of commercial adsorbent charges**.**

ADSORPTION OF COLOUR FROM EFFLUENT USING FUNGAL BIOMASS

Adsorption using intact biomass

During experiments using wet biomass, the maximum colour removal obtained was 41% after 24 h of treatment (Fig. 3.3). With dry biomass, the maximum decolourisation was

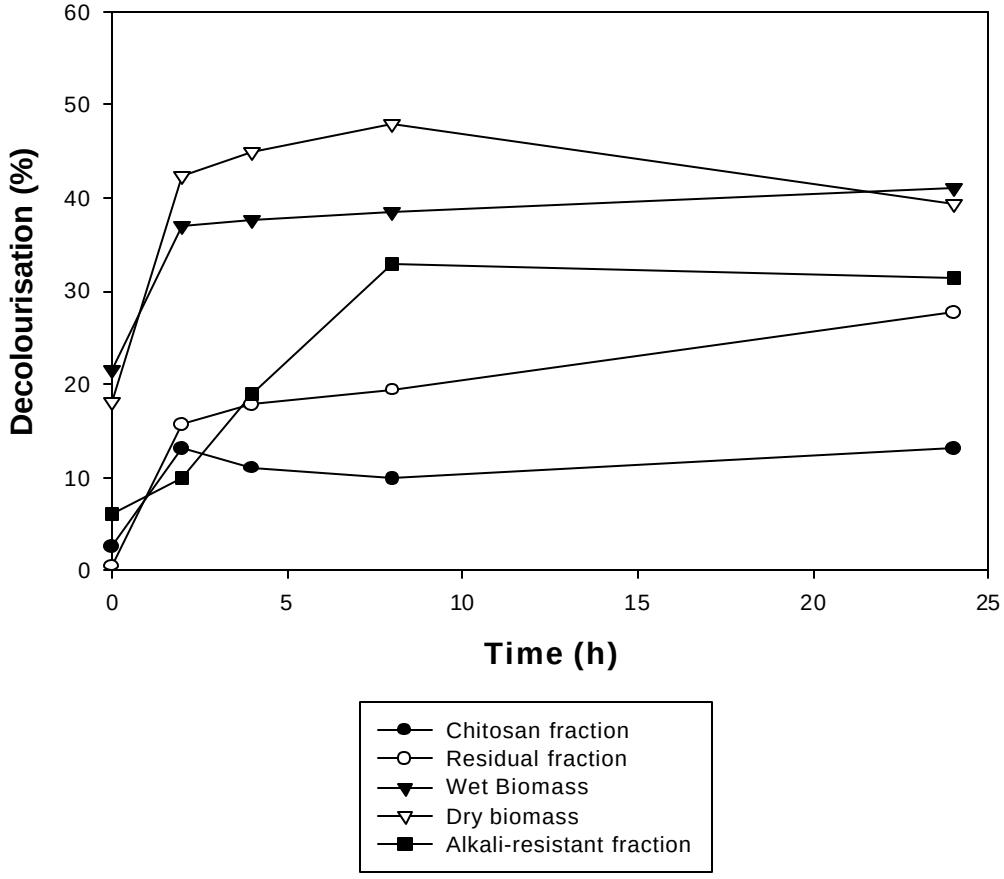

FIG. 3.3: Time profiles of decolourisation of bleach plant effluent by *Rhizomucor pusillus* biomass and cell wall fractions.

somewhat higher (48% after 8 h of treatment). Dried *S. cerevisiae* biomass adsorbed only 7% colour from Eo-effluent. This yeast is readily available commercially as 'bakers yeast' and was included as adsorbent for this reason. *S. cerevisiae'*s cell wall has been shown to contain only 3% chitin (Knorr 1984). The use of dry biomass has the advantage that it could be stored for longer periods of time at ambient temperatures until required. Additionally, dry biomass can be pretreated to improve attributes such as surface area and adsorption capacity. Processing however, would also contribute to the treatment costs. On the other hand, wet biomass can be used as obtained, nevertheless, it is less stable and has to be stored at lower temperatures.

Decolourisation levels as high as 51% were attained in earlier studies when wet *R. pusillus* RM7 biomass was used during the treatment of Eo-effluent (Christov *et al*. 1999). Jaspers and Penninckx (1996) found that non-growing mycelia (4 g/l) of *Phanerochaete chrysosporium* could adsorb more than 60% of the colour from a kraft bleach plant effluent after 24 h of treatment. It has been proven that colour removal from bleach plant effluent by *R. pusillus* proceeds mainly through adsorption of chromophores (Christov *et al*. 1999).

Generally, adsorption is a fast process that requires no or little energy inputs. In contrast, biodegradation and decolourisation using white-rot fungi are relatively slow processes that require nutrients and aeration (Bajpai and Bajpai 1994). Of practical interest is the ease with which adsorbed colour could be released from fungal biomass. Recent results indicated that fungal biomass could be reused as bioadsorbent after intermediate desorption of colour using alkali (Christov *et al.*1999).

***ADSORPTION OF COLOUR FROM EFFLUENT USING CELL WALL FRACTIONS OF* RHIZOMUCOR PUSILLUS**

Extraction procedures of cell wall fractions are shown in Fig. 3.4. The yield of the alkali-resistant fraction obtained from 4 g of biomass was 15% (w/w) whereas the yields of the residual and chitosan fractions were 8% and 4% (w/w), respectively (Table 3.4).

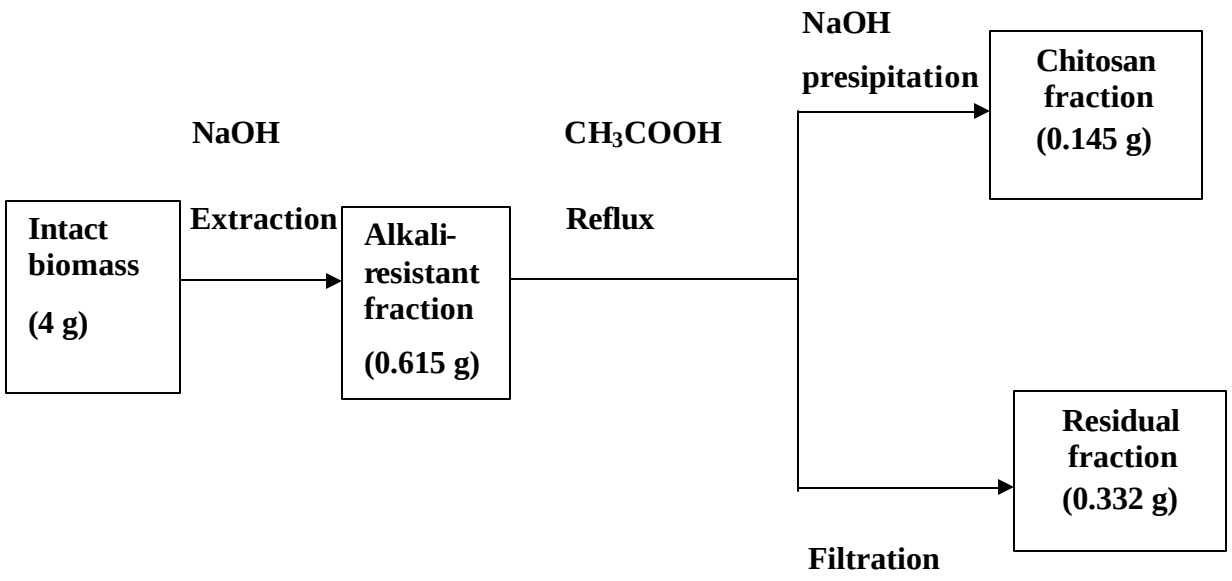

FIG. 3.4: Procedures followed during extraction of cell wall material from *Rhizomucor pusillus*.

The major portion of the decolouring activity of the intact biomass was retained in the alkali-resistant fraction (Fig. 3.3). This fraction exhibited the highest decolourisation characteristics when compared to that of the other fractions. One of the reasons for this would be the higher charge (0.616 g per 100 ml effluent) employed during adsorption experiments. Although both the residual fraction and the chitosan fraction combined represented 12% (w/w) of the total biomass (4 g) used in adsorption experiments, they could remove in total 40.8% of the colour from Eo-effluent following 24 h of treatment (13% of the colour removed by the chitosan fraction and 28% by the residual fraction) (Table 3.4). This indicated that these fractions could proportionally remove more colour from bleach plant effluent than would be predicted based on their yields obtained from 4 g of biomass (Table 3.4).

TABLE 3.4: Efficiency of colour removal from bleach plant effluent by biomass and cell wall fractions of *Rhizomucor pusillus*

Fraction	Yield (%)	Charge (g/l)	Decolourisation (%)
Chitosan	4	1.45	13
Residual	8	3.32	28
Combined[a]	12	4.77	41
Alkali-resistant	15	6.15	34
Intact biomass	100	40.00	39

[a]Chitosan and residual fractions combined.

***Analyses of cell wall fractions from* Rhizomucor pusillus**

Carbohydrate analysis revealed that the main product of acid hydrolysis of the alkali-resistant (Fig. 3.5), residual and chitosan fractions was glucosamine (data not shown). Furthermore, glucosamine was found in the acid hydrolysate of a commercial preparation

of chitosan (Fluka) as indicated in Fig. 3.6. Therefore, chitosan and/or chitin (both polymers yield glucosamine upon acid hydrolysis) seem to be principally involved in the mechanism of colour removal from Eo-effluent by *R. pusillus* biomass. Besides chitosan, some other compounds occur in the alkali resistant fraction, however these could not be identified.

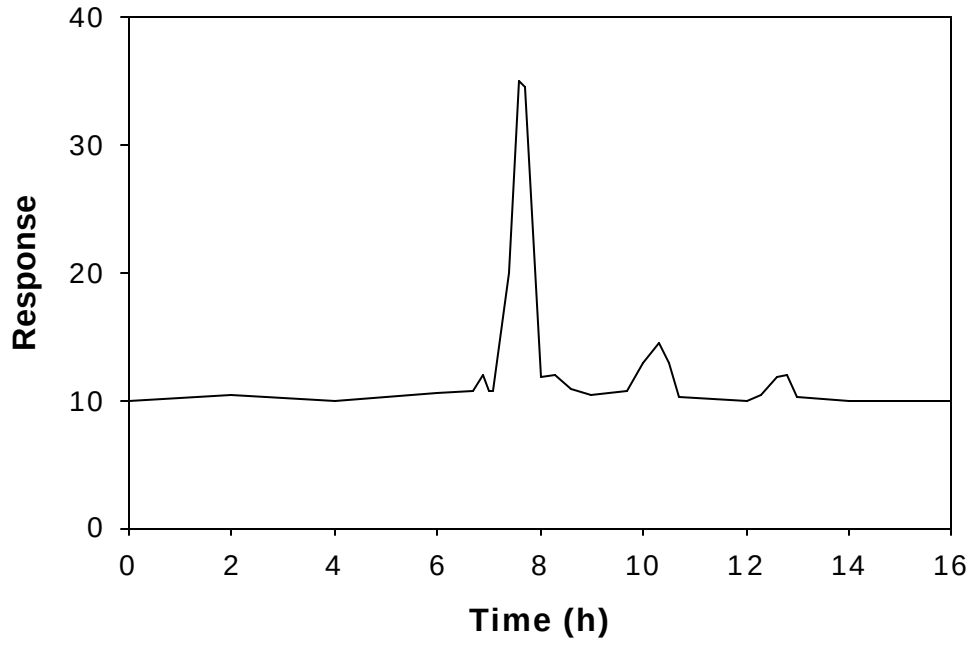

FIG. 3.5: Chromatogram of alkali resistant fraction following acid hydrolysis

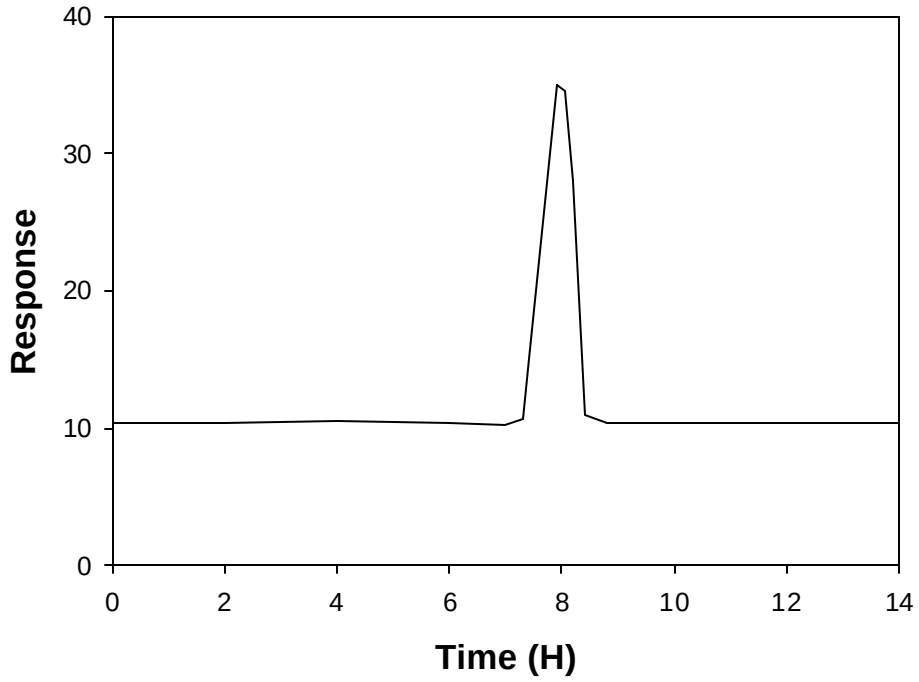

FIG. 3.6: HPLC chromatogram of Fluka chitosan sample after acid hydrolysis

Chitosan and chitin were found to be among the main constituents in the cell walls of mucoralean fungi (Bartnicki-Garcia, 1968) together with some polyanionic materials such as polyphosphates and polyuronides (Araki and Ito 1976). The latter could have, to some extent, a shielding effect on the amino groups of chitosan. Furthermore, under acidic

conditions (pH < 7.0), chitin and chitosan exhibit cationic characteristics (No and Meyer 1989, Anderson *et al*. 1997) and can therefore interact with negatively charged compounds such as the chromophores present in the Eo-effluent (Christov *et al*. 1999). However, at alkaline pH values (pH > 7.0), the amines behave as nucleophiles and the unshared electrons can react with various electrophiles (Kumar *et al.* 1999). Cellulose was inefficient in colour removal from effluent (Table 3.3) whereas chitin and, to a higher degree, chitosan exhibited decolourisation activity at pH 7.4 (Fig. 3.2). This indicated that the nitrogen groups of chitin/chitosan located in the cell wall of RM7 could be involved in effluent decolourisation. Hydrophobic interactions with chitin/chitosan are also possible due to the aromatic content of chromophoric materials in effluent. It has been demonstrated that anionic resins had the highest colour removal ability (Table 3.2). As shown earlier (Table 3.2), cationic resins could also remove colour from effluent therefore indicating that some of the effluent chromophores might interact hydrophobically with the polystyrene backbone of the resin. Also, a chemical interaction between chitin/chitosan and chloroorganic/oxidised lignin materials cannot be excluded. Quinone adsorption by chitosan was found to be rapid and strong (Payne *et al*. 1992). Quinone type colour was furthermore detected in E1 kraft effluent at a level of 60% (Durán *et al*. 1994). It has been suggested that effluent chromophores can be adsorbed through chemisorption by commercial adsorbents such as chitosan (Christov *et al*. 1999). By definition chemisorption implies a chemical reaction, usually involving covalent bonding, between adsorbent and adsorbant. Therefore, a complex mechanism of colour removal involving chemical and hydrophobic interactions between the effluent chromophores and cell wall material of *R. pusillus* might exist.

ADSORPTION CONDUCTED AT VARIOUS IONIC STRENGTH LEVELS

To investigate the mechanisms involved during adsorption, experiments were conducted using various Na_2SO_4 concentration levels. Results are shown in Tables 3.5-3.9. At an initial pH level of 6, the pH increased rapidly to values of between 7.26 and 7.35 after one hour, when chitin was employed. Under these conditions decolourisation by chitin was weak and essentially independent of the ionic strength used (Table 3.5). However, when experiments were repeated at initial pH levels of 4, decolourisation became dependent on the ionic strengths employed, with decolourisation being most favoured when no Na_2SO_4 was present (Table 3.6). Subsequently, all the other decolourisation experiments were conducted at an initial pH of 4, except where indicated otherwise. Thus at pH values < 7 the mechanism of colour removal by chitin involves ionic interactions, whereas at pH levels higher than 7, other modes of colour removal must be present. Since chitosan begins to dissolve at pH levels lower then 6, experiments were conducted at an initial pH of 6.0. Decolourisation were also enhanced dramatically when no Na_2SO_4 were present during the adsorption experiment (Table 3.7). The results once again indicate that ionic interactions are responsible for a fraction of the decolourisation activity exhibited by chitosan when experiments were conducted under acidic conditions. However, decolourisation experiments conducted with RM7 were independent of the ionic strength used (Table 3.8). This shows that in the case of RM7, decolourisation apparently does not involve ionic interactions. Whether RM7, chitin or chitosan where employed as adsorbents, decolourisation in the presence of 1 M Na_2SO_4 was mostly higher when compared to that achieved at 0.5 M Na_2SO_4. This could be indicative of hydrophobic interactions participating in decolourisation since hydrophobic types of interactions are usually favoured at higher ionic strength levels (Zhang and Sun 2001). Activated carbon colour adsorption was also independent of ionic strength (Table 3.9) and therefore colour removal was not instigated by ionic interactions.

Table 3.5: Decolourisation of Eo-effluent by chitin in the presence of various Na_2SO_4 concentrations (initial pH 6.0)

	Na_2SO_4 concentrations (M)					
	pH			**Decolourisation (%)**		
Time (h)	0	0.5	1.0	0	0.5	1
0.1	7.26	7.35	7.26	10	9	9
1	7.76	7.97	7.95	13	14	15
24	7.79	8.04	8.00	23	23	28
48	7.89	7.89	8.01	32	33	37

Table 3.6: Decolourisation of Eo-effluent by chitin in the presence of various Na_2SO_4 concentrations (initial pH 4.0)

	Na_2SO_4 concentrations (M)					
	pH			**Decolourisation (%)**		
Time (h)	0	0.5	1.0	0	0.5	1.0
0.1	4.44	4.45	4.40	10	10	10
1	5.03	5.08	5.09	41	35	42
24	5.15	5.29	5.30	67	51	56
48	5.20	5.36	5.34	67	58	60

Table 3.7: Decolourisation of Eo-effluent by chitosan in the presence of various Na_2SO_4 concentrations

	Na_2SO_4 concentrations (M)					
	pH			**Decolourisation (%)**		
Time (h)	0	0.5	1.0	0	0.5	1.0
0.1	6.10	6.09	6.11	23	25	23
1	6.78	6.90	7.02	79	52	57
24	6.86	7.06	7.14	81	56	57
48	7.19	7.21	7.21	80	65	66

Table 3.8: Decolourisation of Eo-effluent by RM7 in the presence of various Na_2SO_4 concentrations

	Na_2SO_4 concentrations (M)					
	pH			**Decolourisation (%)**		
Time (h)	0	0.5	1.0	0	0.5	1.0
0.1	4.14	4.21	4.19	12	13	13
1	4.23	4.22	4.19	43	41	45
24	4.26	4.29	4.24	54	51	59
48	4.30	4.38	4.22	53	51	56

Table 3.9: Decolourisation of Eo-effluent by activated carbon in the presence of various Na_2SO_4 concentrations

	Na_2SO_4 concentrations (M)					
	pH			**Decolourisation (%)**		
Time (h)	0	0.5	1.0	0	0.5	1.0
0.1	4.10	4.13	4.05	37	37	38
1	4.28	4.32	4.09	66	68	67
24	4.40	4.81	4.42	99	100	100
48	5.06	5.53	5.05	100	100	100

DESORPTION OF COLOUR FROM ADSORBENTS USING ALKALI TREATMENT

Adsorbed colour could be released to various levels from adsorbents following adsorption experiments. The decolouring ability of *R. pusillus* biomass was completely regenerated using 1 N NaOH (Fig. 3.7). On the other hand, a 51% restoration of the adsorption abilities of chitin was obtained. The amount of colour desorbed from chitosan and activated carbon decreased to 18 and 5% respectively. The differences in the levels of colour that could be removed from chitin and chitosan on the one hand and colour removal from RM7 on the other, by alkali treatment, shows that chitin/chitosan might not be exclusively involved in RM7's decolourisation abilities. Other cell wall compounds and the structural/spatial arrangement of chitin/chitosan in RM7 cell walls might also be important. Sentandreu *et al*. (1994) stressed the significance of spatial regulation on cell wall assembly and how critical important this is in determining the wall properties of cells. These properties therefore do not depend exclusively on their chemical composition but also on how different cell wall polymers interact.

Other workers also desorbed colour from biomass using alkali treatment. Following alkaline extraction (pH 12) 95% of colour adsorbed initially was released from *P. chrysosporium* biomass after treatment (Jaspers and Penninckx 1996). About half of the coloured adsorbing material from a kraft black liquor effluent could be recovered from *Polyporus versicolor* biomass after treatment with alkali (Marton *et al*. 1969).

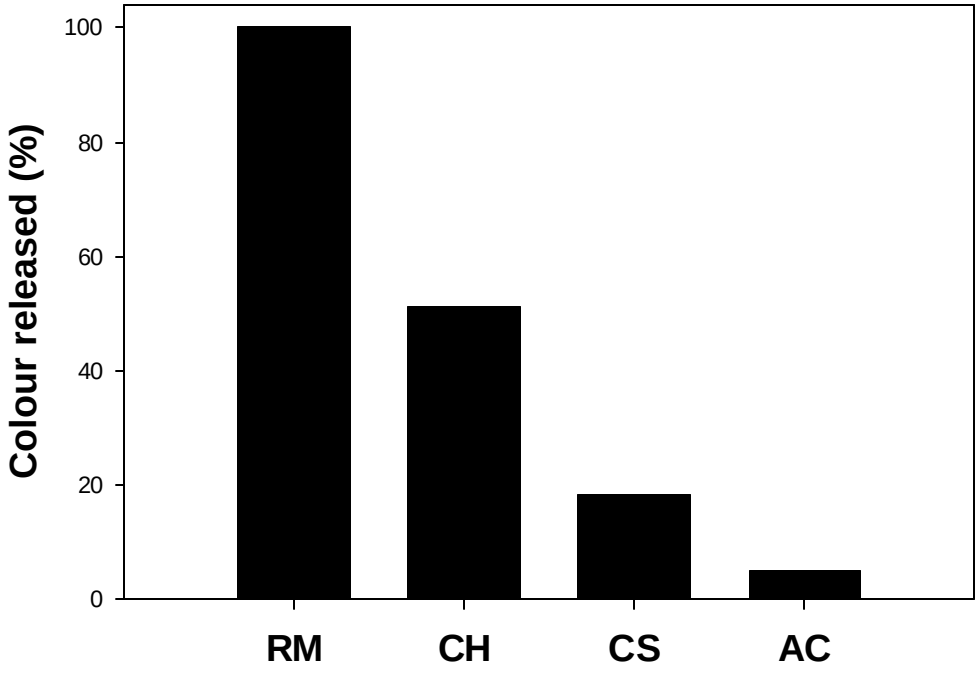

FIG. 3.7: Desorption of colour from various adsorbents using 1 N NaOH. RM, biomass; CH, chitin, CS, chitosan and AC, activated carbon.

COD REDUCTIONS FOLLOWING ADSORPTION EXPERIMENTS

Adsorption treatment employing activated carbon removed the highest level of COD from the effluent (40%), followed by Fluka chitosan (24%) and chitin (12%). The lowest

COD reduction was achieved using *R. pusillus* biomass (9%) (Fig. 3.8). Therefore, after adsorption, most of the COD was retained in the effluent. These results indicated that additional treatment would be required to effectively remediate the effluent after adsorbent treatments.

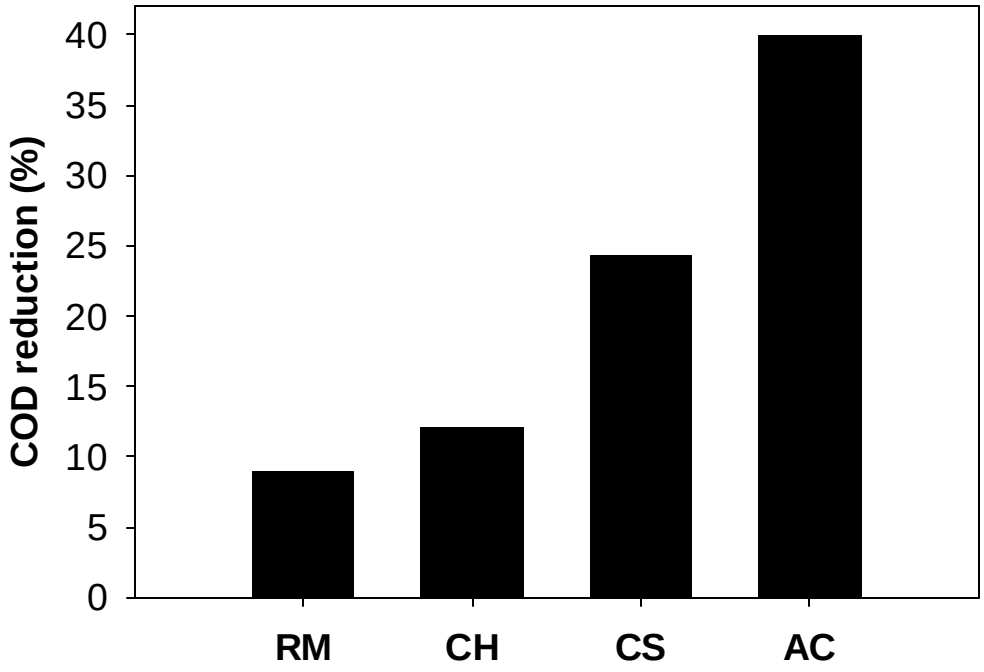

FIG. 3.8: Efficiency of COD reductions obtained after treatment of Eo-effluent with various adsorbents. RM, biomass; CH, chitin; CS, chitosan and AC, activated carbon.

ADSORPTION ISOTHERMS

Isotherm data for the various adsorbents are presented in Figs. 3.9 to 3.12. Freundlich isotherms were constructed, which gave good result as was evident from the regression

constants that were obtained for the fits of the Freundlich equation to the experimental data. The mathematical description of this isotherm has already been given in Chapter I. It is important to note that the value of the sorption equilibrium constant K contained in the Freundlich equation provides an indication of the adsorption capacity of an adsorbent (Kapoor and Viraraghavan, 1995). The K value of the Freundlich isotherm was the highest for activated carbon adsorbents, followed by that of chitosan, chitin and lastly RM7. Therefore the adsorption capacities of the adsorbents varied as follows activated carbon > chitosan > chitin > RM7.

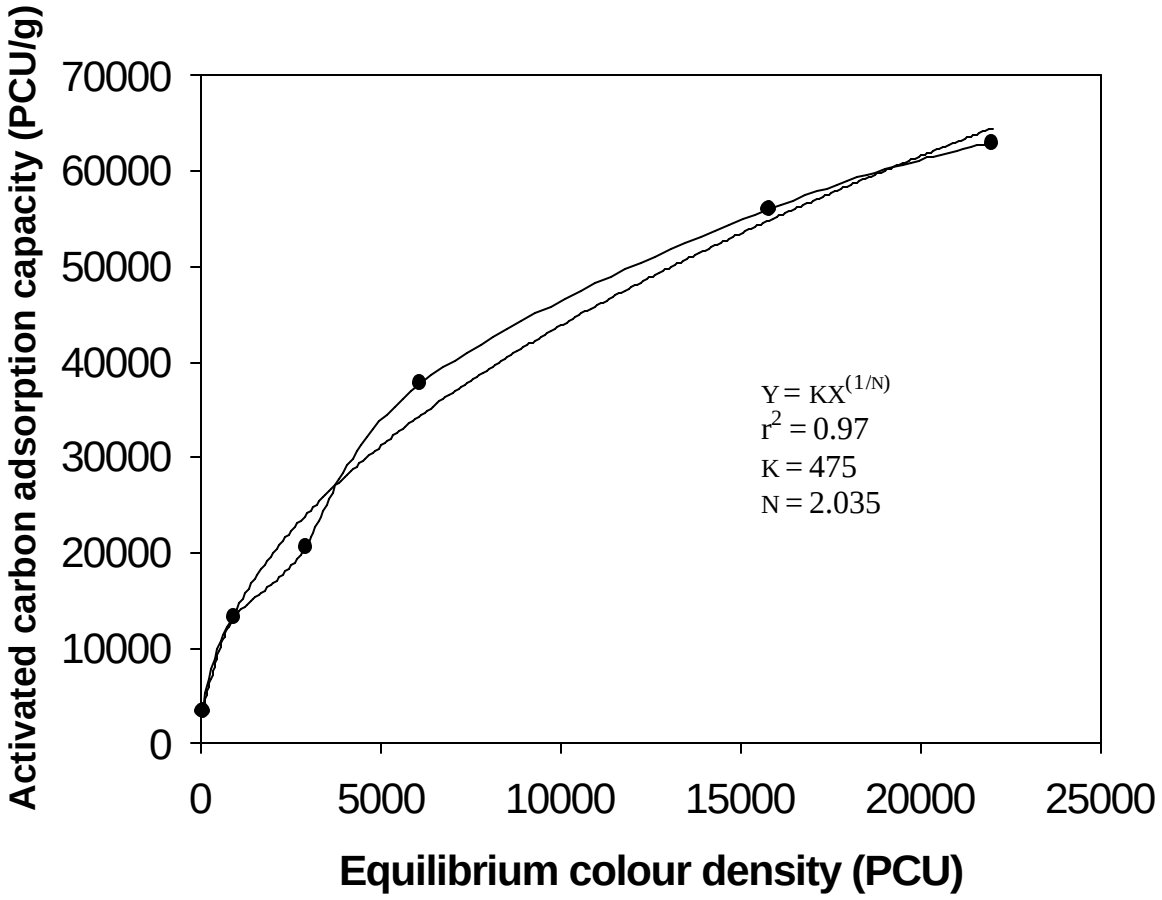

FIG. 3.9: Adsorption isotherm depicting decolourisation of Eo-effluent by activated carbon.

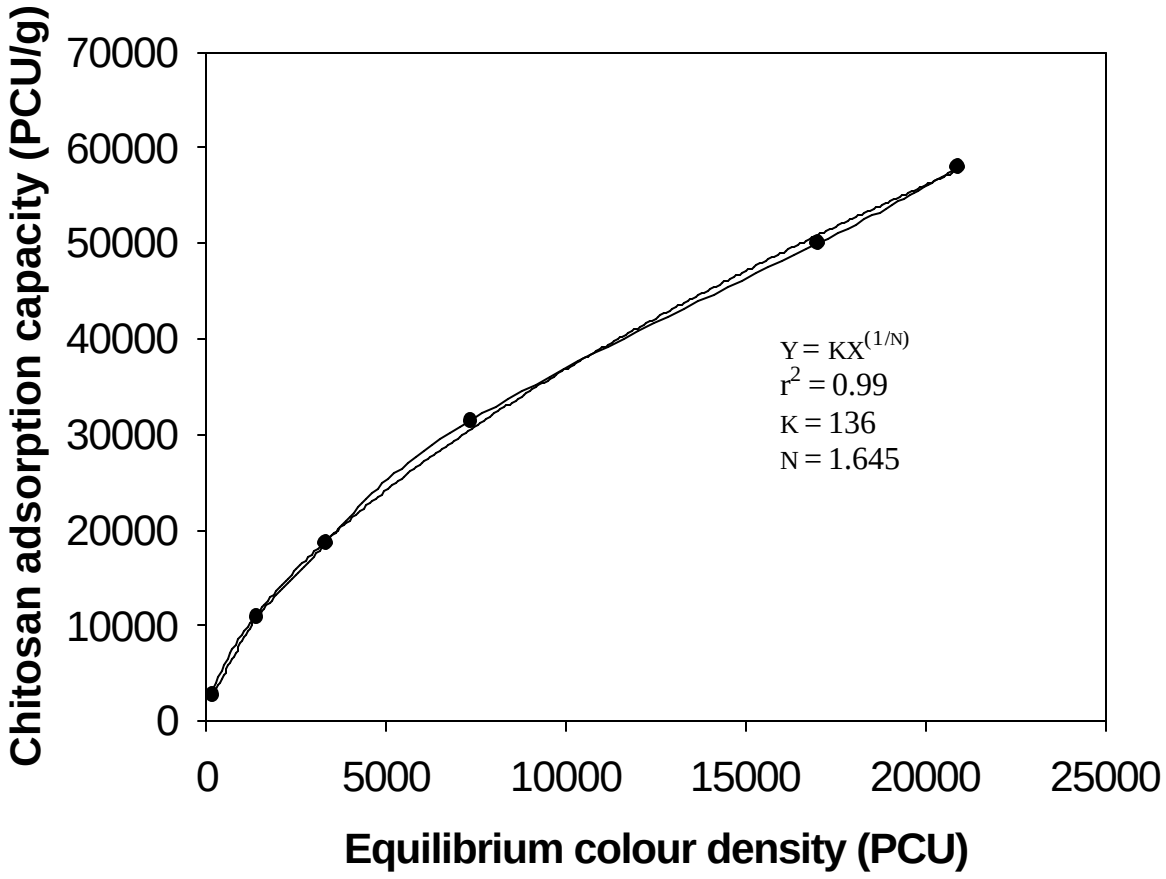

FIG. 3.10: Adsorption isotherm of colour removal from Eo-effluent by chitosan.

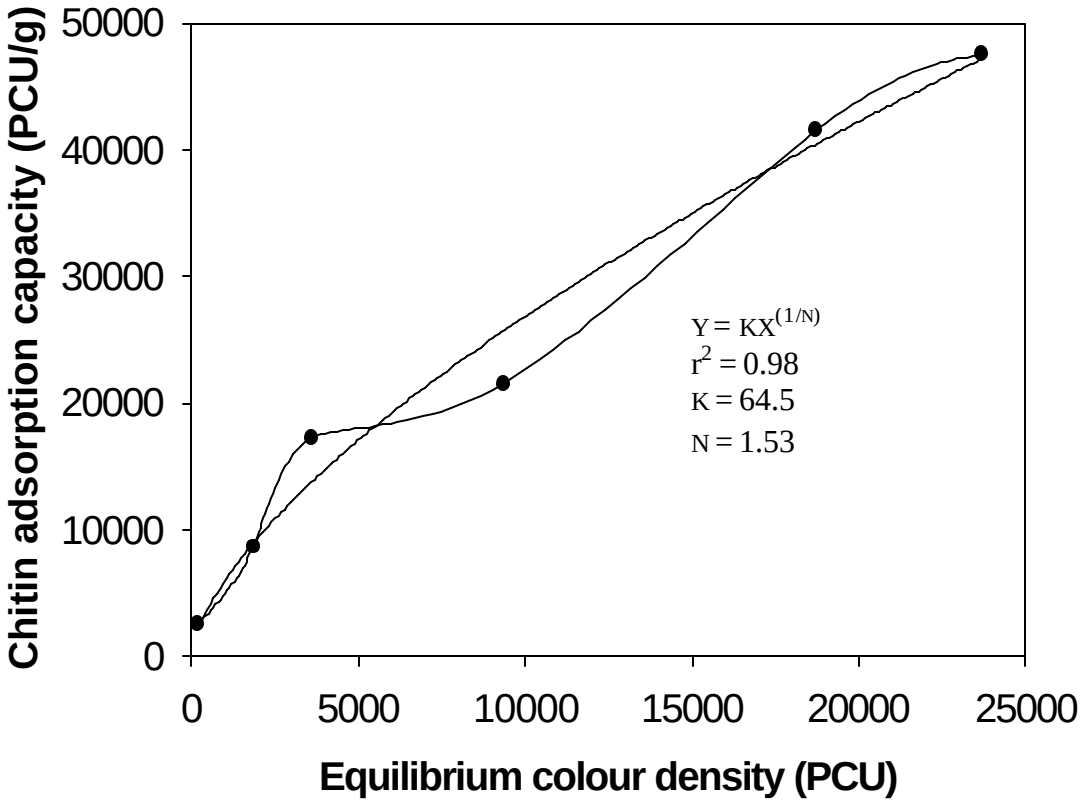

FIG. 3.11: Decolourisation isotherm using chitin as adsorbent

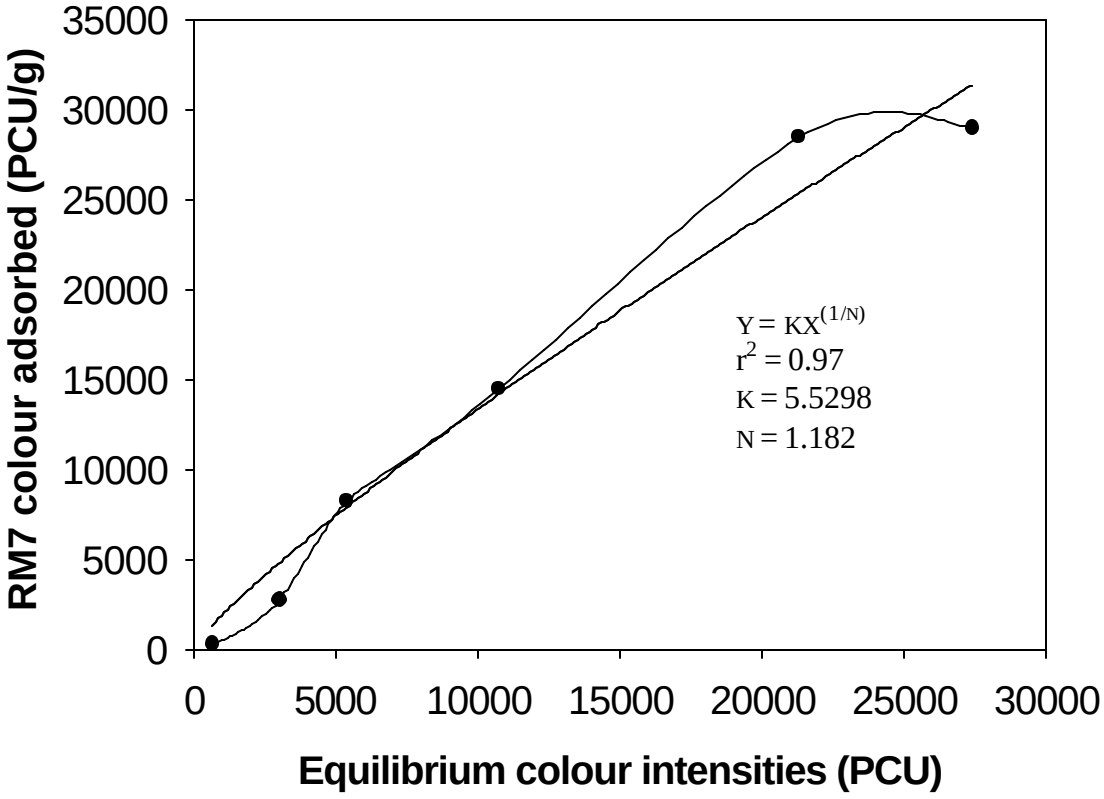

FIG. 3.12: Colour adsorption isotherm of *Rhizomucor pusillus* biomass

EFFECT OF EFFLUENT pH ON COLOUR ADSORPTION

It was observed that effluent pH exerted a major influence on the decolourisation that could be attained when chitin, chitosan and also *R. pusillus* biomass were employed as adsorbents of Eo colour. In contrast, decolourisation by activated carbon was not appreciably affected by pH (Fig. 3.13). This could be indicative of different mechanisms of adsorption being prevalent during colour removal by activated carbon as compared to the mechanisms operating when the other adsorbent were used. Linear regression analyses of effluent decolourisation as function of pH in the pH range 6 to 9 were performed. The data indicated that the slopes for chitosan (r^2 = 0.9996), chitin (r^2 = 0.9885), RM7-biomass (r^2 = 0.9319) and activated carbon (r^2 = 0.9553), were as follows: -18.48, -13.21, -7.78 and -0.61, respectively. These slopes quantify the extend to which adsorption was affected by pH when these adsorbents were used.

Chitosan's decolourisation activity was superior to that of chitin and *R. pusillus* biomass over the pH range of 6 to 9. However at pH 10 the decolourisation ability of the three adsorbents were essentially the same. As had already been discussed (p. 75, this chapter) decolourisation of effluent by chitosan and chitin should be inversely related to effluent pH, the same phenomenon was also observed with RM7. Therefore the mechanism of decolourisation of Eo-effluent by chitosan, chitin and RM7 seem to be similar. However, the cell wall composition of fungi is complex and contains various other compounds besides chitin/chitosan (Kapoor and Viraraghavan, 1995) that could be involved directly in colour removal or could modulate the decolourisation behaviour of the chitin/chitosan present in the cell wall. The possible chemical reactions participating in chemisorption of colour from Eo-effluent by adsorbents are shown in Fig. 3.14.

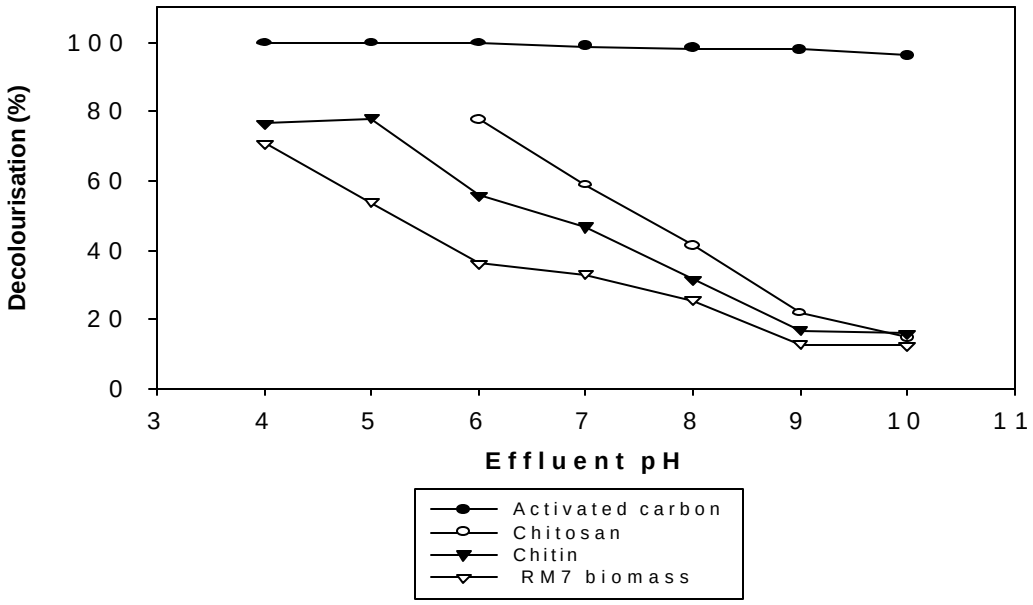

FIG. 3.13: Plot of effluent decolourisation against effluent pH

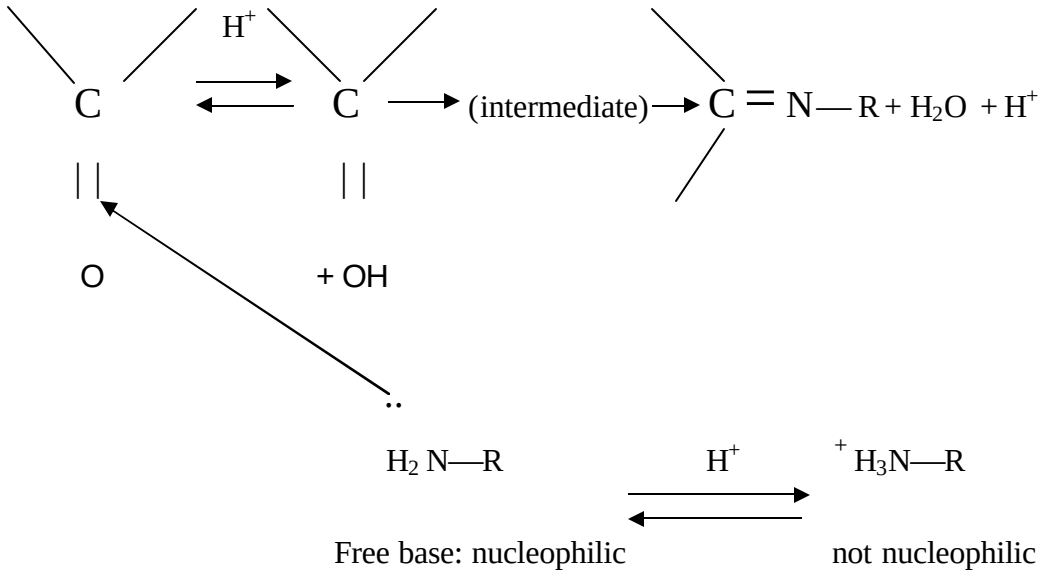

FIG. 3.14: Chemical pathways depicting possible reactions involved in sorption of chromophores from Eo-effluent by adsorbents containing functional amino groups. (Morrison and Boyd 1983)

Acid catalysis apparently increases the rate of nucleophilic addition reaction between carbonyl compounds of Eo-effluent and the amino containing groups of chitin/chitosan as well as those in the cell wall of RM7 by activation of the carbonyl (Fig. 3.14). Therefore, under specific/optimal acidic conditions chemical interactions will be enhanced. However, as pH levels increase towards neutrality and beyond, acid catalysis will be diminished. This will result in an overall reduction in colour removal abilities of the adsorbent with an increase in the pH levels as was also observed during this study. Furthermore, in the case of chitin and chitosan, as pH levels decrease, the free base form of the amino groups are converted to cationic salt containing groups which are not nucleophilic (Fig. 3.14). These salt-containing compounds can interact with the anionic groups found in Eo-effluent through coloumbic attractions. Since ionic interactions as decolourisation mode by RM7 has been shown not to occur, nucleophilic addition seem to be one of the main decolourisation mechanisms operating during fungal colour removal. In this instance and also probably to varying degrees, in the case of chitin and chitosan, it would appear that imine compounds produced by a reversible reaction (Muzzarelli *et al.* 1994 a) are formed during chemisorption that could be released by

alkali treatment. Furthermore, although chitin contains mostly nitrogen in the form of less reactive acetamide (Horowitz 1991) groups (secondary amino groups), it also contains some primary amino groups similar to that found in chitosan. The occurrence of these primary amino groups depends on the level of acetylation of chitin. According to Muzzarelli *et al.* (1994 a) chitosan can also react irreversibly and strongly with quinones to produce aminoquinones. It has been shown that quinones occur in bleach plant effluents (Durán *et al*. 1994) and can therefore react with chitosan. The different levels of primary amino groups present in chitosan and chitin can therefore possibly explain the different colour adsorption activities of these two compounds.

Colour removal from effluent using chitosan as flocculent

Chitosan can be dissolved in acids and applied in soluble form to treat effluents. Under acid conditions, the amino groups present in this poly-aminoglycan can be protonated (pK_a 6.2) (Muzzarelli *et al*. 1994 b), so that chitosan becomes polycationic and can therefore function as an anion exchanger. It was observed that chitosan, dissolved in acetic acid, could be utilised to flocculate coloured compounds from bleach plant effluent. When a 1:1 mixture of a high molecular weight chitosan-solution and Eo-effluent were mixed, it resulted in a maximum decolourisation of 75% being obtained. The pH of the mixtures varied from 4.08 to 4.36. Table 3.10 indicates that the medium and high molecular weight chitosan used during flocculation decolourised the Eo-effluent maximally at concentration levels of 1.2 and 1.6 g/l respectively. When adsorption experiments were conducted at pH levels of 6.0 and using a charge of 40 g/l medium molecular weight chitosan, comparable decolourisations activities (78%) were attained. This can partially be explained by the higher positive charge that chitosan would exhibit at a pH of about 4 as opposed to the charge density that it would show at a pH of 6. Flocculation thus requires a lower dose of chitosan when compared to the chitosan charge required during adsorption to achieve similar decolourisation activities. It was reported that molecular weight and charge density of chitosan played major roles in coagulation (Agerkivist *et al*. 1990).

The flocks that formed upon mixing of chitosan-solutions and Eo settled out under gravity after a treatment time of only 20 min. The high decolouring activity obtained over this short treatment time is beneficial for application on an industrial scale. Flocks can also be removed easily after settlement. Chitosan is a natural product obtained from chitin, and is therefore biodegradable and non-toxic, an attribute that could be beneficial for its utilisation in environmental applications (Pascual and Julià 2001). It has been indicated that next to cellulose, chitosan and chitin are the most abundant natural compounds (Knorr and Klein 1986). Chitosan has been tested previously as a coagulant in the remediation of an effluent (black liquor) from the pulp and paper industry and compared favourable with other synthetic coagulants employed (Ganjidoust *et al*. 1997). Disposal of the flocks formed after treatment could however present a problem.

TABLE 3.10: The effect of concentration and molecular mass (M_r) of Fluka chitosan on decolouring activity during treatment of Eo-effluent.

	Decolourisation (%)		
Chitosan Concentration (g/l)	**Low M_r[a]**	**Medium M_r[b]**	**High M_r[c]**
0.1	25	34	25
0.2	42	58	46
0.8	54	67	73
1.2	52	72	75
1.6	57	67	75
2.0	44	64	75

[a]Low M_r corresponds to 70 000 g/mol (data supplied by Fluka).

[b] Medium Mr corresponds to 750 000 g/mol

[c] High M_r correspond to 2 000 000 g/mol

Extraction of colour by organic solvent treatment of effluent

Colour was also extracted from Eo-effluent using various solvents (Table 3.11).

TABLE 3.11: Extraction of colour from Eo-effluent using solvents

Solvents	Absorbance and colour of solvent extracts			
	254 nm	**280 nm**	**455 nm**	**PCU[a]**
Water	39.350	30.750	3.250	10900
Methanol	18.300	13.900	0.750	2325
Propanol	0.841	0.666	0.015	61
Chloroform	0.206	0.149	0.000	0

[a]Platinum Cobalt colour unit

Distilled water and to a lesser extent methanol proved to be the best solvents of coloured and ultraviolet-absorbing materials. Whereas propan-2-ol extracted some colour, no chromophores were extracted with chloroform. Since polarity of these compounds decrease from water to chloroform this would indicate that coloured compounds also exhibits polar characteristics. Therefore, in general, organic solvents are not ideal solvents for effluent colour. Decolourisation of only 21% was obtained, using methanol. This compound exhibits polar characteristics even though it is considered to be an organic solvent. Because organic solvents are usually volatile, these compounds can be reclaimed by distillation after colour extraction and used again. This would be beneficial from an economic perspective.

Ultraviolet irradiation of effluent

Ultraviolet (UV) decolourisation of effluent has been studied, however penetration by the UV-radiation of the highly coloured effluent has been regarded as a problem that would render the process impractical (Möbius and Cordes-Tolle 1997). Nevertheless, biological treatment might necessitate the dilution of effluent so that UV-irradiation might become

feasibly as a pre-treatment step. Dilution of effluent resulted in a faster decolourisation being recorded initially. However, after 160 min of treatment the decolourisation activity attained was approximately the same for both the diluted and undiluted effluents (Fig. 3.15).

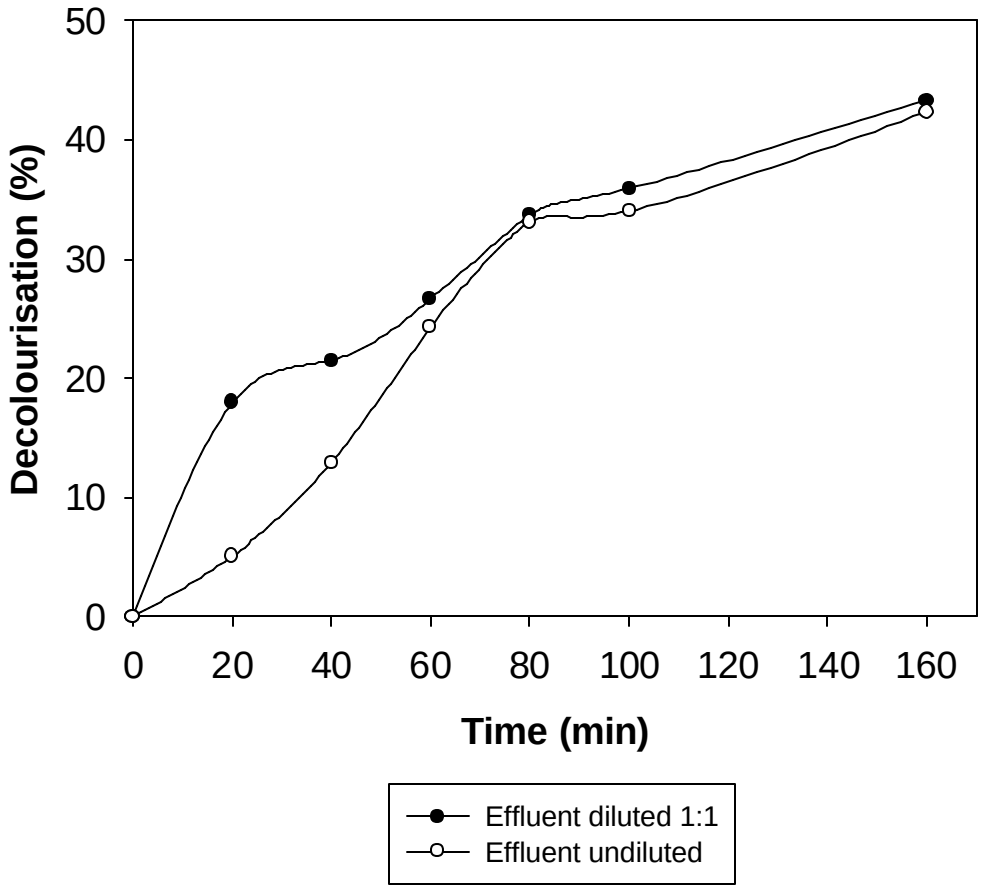

FIG. 3.15: Decolourisation of effluent by UV-irradiation as a function of time.

Comparisons of COD values taken before and after UV treatment of effluent (after 160 min) were almost identical (data not shown). This indicated that no appreciable degradation occurred during UV irradiation. Decolourisation of effluent by UV-

irradiation might proceed by the process of photo-oxidation and transformation of chromophoric species to colourless compounds or functional groups. Sunlight could be utilised as an inexpensive source of UV-radiation.

5. CONCLUSIONS

Commercial adsorbents such as activated carbon proved highly effective and could remove almost all the colour from bleach plant effluent. Anion-exchange treatment also rendered the effluent almost colourless. Biomass of *R. pusillus* compared fairly well with other physico-chemical adsorbents and could be regenerated by alkali treatment. In contract, only partial regeneration was possible using the commercial physico-chemical adsorbents, suggesting chemisorption of coloured compounds onto the sorbent material occurred during treatment. Effluent decolourisation was significantly higher at lower pH levels for all the adsorbents tested, except in the case of activated carbon. Isotherm data indicated that activated carbon was the best adsorbent of colour followed by chitosan, chitin and lastly *R. pusillus* biomass.

Adsorption experiments using *R. pusillus* to decolourise Eo-effluent have yielded results (41 to 48%) similar to some of the best decolouring activities reported in literature. Cell wall fractions extracted from the biomass of *R. pusillus* (alkali-resistant, residual and chitosan fractions) appeared to be principally responsible for the decolouring of Eo-effluent by this fungus. The major compound(s) of the cell wall fractions could be either chitosan and/or chitin, based on the release of glucosamine upon acid hydrolysis of these fractions. The residual and chitosan fractions, representing combined only approximately 12% (w/w) of the *R. pusillus* biomass, were able to remove more colour from the bleach plant effluent (about 41%) than the intact biomass (39%). A complex mechanism of colour removal involving chemical and hydrophobic interactions between the effluent chromophores and cell wall material of *R. pusillus* might exist. The decolourisation

activity of *S. cerevisiae* was much weaker than *R. pusillus*, probably because of differences in the cell wall composition between these two microorganisms.

Organic solvents tested during the study did not remove appreciable levels of colour from effluent. This could be due to the polar nature of chromophoric materials present in this wastewater.

Flocculation of coloured materials using chitosan dissolved in acetic acid, as flocculent resulted in a maximum decolourisation of 75%. A lower charge of chitosan was required as flocculent in order to obtain decolourisation activities comparable to that observed when chitosan was used as adsorbent of colour from bleach plant effluent.

Results indicated that certain physico-chemical treatments could effectively decolourise Eo-effluent but in most instances further treatment would be required. Biological treatment might therefore have to be considered.

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CHAPTER IV

BIOLOGICAL TREATMENT OF Eo-EFFLUENT

1. SUMMARY

The following biological methods for effluent remediation were investigated: 1) Activated sludge reactors, 2) Trickling filters and 3) Rotating biological contactor reactors (RBC). Special attention was given to the RBC treatment system. Various combinations (treatment using one system was followed by treatment in another system) of biological treatments methods were also employed.

Effluent treatment in an activated sludge reactor resulted in reduced toxicity, as determined by the bacterial growth inhibition test (BGIT), lower COD and chlorophenol levels. However, colour and high molecular mass compounds were not affected significantly by this method of treatment.

With trickling filters containing immobilised white-rot fungi, the highest decolourisation (61%) was obtained with *Coriolus versicolor*. This fungus required a co-substrate to efficiently decolourise the effluent.

Decolourisation was also studied in a rotating biological contactor (RBC) reactor using immobilised *Coriolus versicolor* and *Rhizomucor pusillus* strain RM7. Decolourisation by both fungi was directly proportional to initial colour intensities. It was found that the extent of decolourisation was not adversely affected by colour intensity, except at the lowest level tested. Decolourisation of 53 to 74% could be attained using a hydraulic retention time of 23 h. *Rhizomucor pusillus*, removed 55% of AOX compared to a 40 % AOX reduction by *C. versicolor*. Fungal treatment employing *R. pusillus* and *C. versicolor*, respectively, rendered the effluent essentially non-toxic. Addition of nutrients to decolourisation media stimulated colour removal by *C. versicolor*, but the decolourisation activity of *R. pusillus*

was not significantly affected by nutrient supplementation. Ligninolytic enzymes (manganese peroxidase and laccase) were only detected in effluent treated by *C. versicolor*. The effluent to some extent inhibited laccase activities. It seems that there are definite differences in the decolouring mechanisms between the white-rot fungus and the mucoralean fungus. This aspect was investigated in greater detail using gel permeation chromatography analyses in order to verify the mode responsible for the decolourisation activity in both types of fungi. *Rhizomucor pusillus* decolourised the effluent by adsorption and *C. versicolor* removed effluent colour by adsorption as well as by biodegradation. Decolourisation studies in the RBC were conducted in a recirculation system and also, in some cases, in continuous flow mode. Long-term experiments indicated that *C. versicolor* could decolourise the effluent for a period of 34 d whereas *R. pusillus* decolourised the effluent up to 54 d. Further improvements in effluent quality could be attained when treatment using one system was followed by treatment in another system, possibly because of effluent toxicity (BGIT) reduction in the pre-treatment step.

2. INTRODUCTION

Bleach plant effluents from the pulp and paper industry generated during bleaching with chlorine-containing chemicals contain toxic chloroorganics and coloured compounds that could cause serious environmental problems. Pre-treatment of the wastewater is therefore a necessity. A variety of chlorine-containing compounds are used as bleaching agents. This gives rise to the formation of coloured and chloroorganic materials in effluents (Nagarathnamma and Bajpai, 1999). Alkali extraction stage effluents from bleach plants can contribute up to 80% of the colour and 60% of the overall COD load in the total kraft paper mill effluent (Mehna *et al*. 1995). Physico-chemical treatment methods such as lime coagulation and ultra-filtration yield variable results and are also generally too expensive for application on an industrial scale (Royer *et al*. 1991). Conventional biological treatments such as activated sludge and aerated lagoons reduce COD load and toxicity of bleach plant effluents but cannot remove colour, which is due mainly to high molecular mass chloroorganic materials in the effluent (Royer *et al*. 1991). In contrast, biological methods employing white-rot fungi can reduce colour, toxicity and to some extend COD and BOD

(Royer *et al*. 1991). However, treatment with white-rot fungi usually requires prolonged retention times for sufficient decolourisation (Bajpai and Bajpai 1994).

In this study applications of various biological methods for effluent treatment were investigated**.** The emphasis was mainly on colour removal. However, AOX, COD, chlorophenol, toxicity reduction (BGIT) of wastewater and molecular mass distribution of the bleach plant effluent were also studied after treatment in activated sludge and RBC reactors.

3. MATERIALS AND METHODS

Maintenance and cultivation

Coriolus versicolor strain SCC-63, *Stereum illudens* SCC-31, *Pycnoporus sanguineus* SCC-92 and *Rhizomucor pusillus* RM7 were maintained on potato dextrose agar at 4ºC. Fungal strains were obtained from the Sappi Biotechnology culture collection at the University of the Free State. For preparations of inoculums, four 1x1 cm cubes were cut from fresh plates and transferred to sterile 500 ml Erlenmeyer flasks containing 200 ml growth medium of the following composition in g/l: glucose, 20; malt extract, 20; peptone, 20; yeast extract, 8. The pH of the medium was adjusted to 5 prior to sterilisation. After 4 d incubation on an orbital shaker at 30ºC, 40 ml culture broth was aseptically transferred to sterile 1l Erlenmeyer flasks containing 400 ml growth medium. The flasks were incubated for 4 d before homogenisation.

Effluent

The effluent used was obtained from the alkaline extraction stage (Eo) of the bleach plant of a sulphite dissolving pulp mill. Effluent was stored in closed containers at 4 ºC prior to treatment. The effluent had a colour of 12 200 PCU, COD of 11 717 mg/l and a pH of 8.1.

Design and operation of activated sludge reactors

Laboratory-scale activated sludge reactors (AS) were used for the treatment of Eo-effluent (Fig. 4.1). The aeration basin contained 760 ml effluent. The reactors were seeded with activated sludge from an activated sludge facility that treats bleach plant effluent produced at a paper mill (Sappi Enstra mill, Springs). Mixed liquor suspended solids (MLSS) was maintained at about 4 g/l. Air was supplied at a rate of 2.3 l/min, via an airflow meter fitted to an air compressor. Sludge age was generally maintained at about 19 days. Sludge was returned from the clarifier to the aeration basin at a controlled rate with the use of a timer fitted to a fish air pump. Initially one reactor was also run without the timer system and sludge was continuously returned from the clarifier to the aeration basin. Reactor feed (influent) pH was 7.3 and the COD concentration was set at 1000 mg/l, except where indicated otherwise. The reactors were operated at a temperature of 25ºC. Dissolved oxygen levels in the aeration basin was measured with an oxygen meter (YSI, model 55/12 FT, Yellow Springs, Ohio) according to instructions supplied with the instrument.

ESTIMATION OF RETURN SLUDGE FLOW

DSVI was used to estimate the return sludge flow rate Q_s as follows:

Q_s (ml/h) = DSVI/(100-DSVI) * Q

Q = reactor feed flow rate (ml/h)

EFFECT OF NITROGEN AND PHOSPHATE LEVELS ON ACTIVATED SLUDGE REACTOR PERFORMANCE

Urea was tested as a source of nitrogen, levels tested varied from no addition of urea to 160 mg/l $(NH_2)_2CO$ being supplemented to the effluent mixture feed of the AS. The reactor was adapted to changing conditions for three hydraulic retention times before results were recorded. Furthermore, the effect of KH_2PO_4 additions to effluent feed was tested in the range 0 to 200 mg/l.

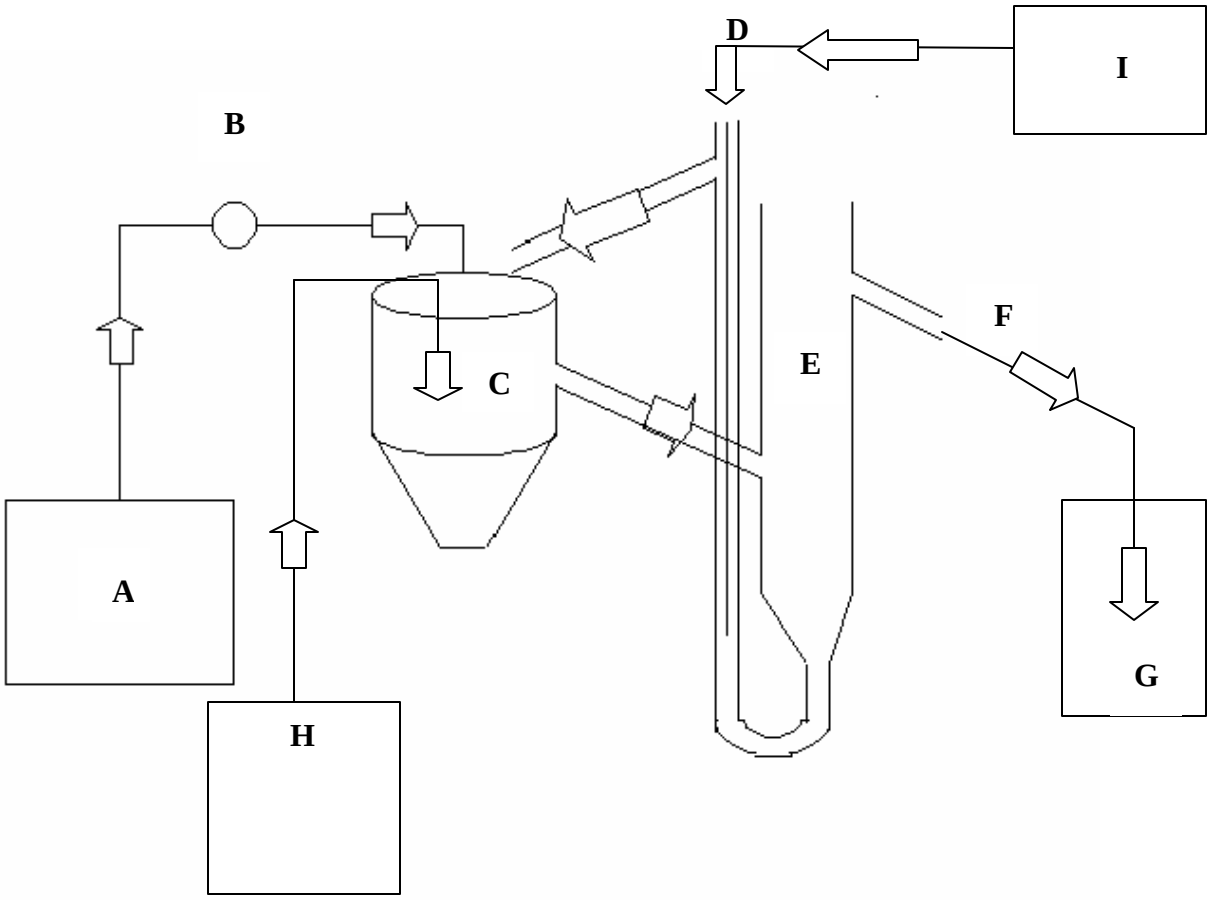


FIG. 4.1: Diagram of laboratory- scale activated sludge reactor.

A) Vessel with influent, B) Pump, C) Aeration basin, D) Air pumped into glass tube, E) clarifier, F) Effluent overflow, G) Vessel containing treated effluent, H) Air inlet and flow meter connected to air pump, I) Fish air pump connected to timer.

DETERMINATION OF HYDRAULIC RETENTION TIME ON ACTIVATED SLUDGE REACTOR PERFORMANCE

The effect of hydraulic retention time (HRT) on AS treatment was investigated using HRT values varying from 6 to 20 h. The system was adapted to changing conditions for three hydraulic retention times before measurements were taken.

THE EFFECT OF FEED TO MICROORGANISM RATIO (F/M) ON ACTIVATED SLUDGE REACTOR TREATMENT

The effect of various F/M values on AS performance were evaluated by varying the levels of bleach plant effluent included in the AS feed. During these investigations a constant ration of COD:N:P was maintained. F/ M values were calculated as follows:

F/M (1/d) = So/(?*X)

So = influent COD (g/l)

? = detention time (d)

X = MLSS (g/l)

SLUDGE-RESIDENCE TIME ESTIMATION

The following equation was used to estimate mean sludge-residence time (R):

R (d) = V_r/Q_w (Adrien 1998)

V_r = aeration basin volume (ml)

Q_w = waste sludge flow rate from the aeration basin (ml/d).

MLSS ANALYSIS

MLSS was determined by taking 25 ml (x2) from the aeration basin and filtering the samples through pre-weight glass filter disks, washing the disks with distilled water and then drying it at 105ºC to a constant weight. Thereafter the mass of the filters was determined, following cooling to room temperature in a desiccator.

DILUTED SLUDGE INDEX MEASUREMENTS

Diluted sludge volume index (DSVI) was determined by diluting 25 ml of the reactor sludge with 75 ml of the reactor outflow in a 100 ml measuring cylinder. The content of the measuring cylinder was stirred slowly with a thin glass rod for a minute. Thereafter the level (in ml) of diluted settled sludge (DSV) was recorded after 30 min had passed. The following equation was used to determine the DSVI (KHJ Riedel, personal communications):

DSVI (ml/g) = DSV * 10 (ml/l)/(MLSS (g/l)/4)

Treatment of effluent employing trickling filter reactors

MICRO-ORGANISMS EMPLOYED

The trickling filter reactors containing *Coriolus versicolor* (tube I), *Stereum illudens* (tube II) and *Pycnoporus sanguinens* (tube III), immobilised on polyurethane foam cubes.

FUNGAL IMMOBILISATION

Initially the fungi were homogenised and then inoculated under aseptic conditions in jars containing sterile 1% molasses solution and sterilised foam cubes (1x1 cm). Twice a day the contents of the jars were mixed to allow immobilisation to proceed. After 4 weeks visual inspection revealed that immobilisation had occurred and immobilised foam cubes were packed aseptically into the plastic tubes.

TRICKLING FILTER DESIGN AND OPERATION

Tubes of 1 m length and with a diameter of 4.5 cm containing immobilised fungi were equipped with reservoirs (Fig. 4.2) containing bleach plant effluent diluted 1:1 with 1% (m/v) molasses solution and the pH adjusted to 5.5. Peristaltic pumps were used to circulate the effluent-containing medium between the reservoirs and the tubes during the initial acclimatisation period. The flow speeds of the peristaltic pumps were set to maintain a contact time of 30 min between fluids pumped from the reservoirs and the

immobilised fungi. The volume of liquid contained in each tube was determined by subtracting the initial volume in the reservoir (at time zero) from the reservoir volume attained after no further change in reservoir volume was observed. One tube was packed only with foam and served as a control. The system was operated at 25ºC. $CuSO_4$-traps were installed to remove microorganisms from the air supplied via fish air pumps. After 5 d of treatment (when maximum decolourisation was attained), the decolourisation activity of tube I was recorded. A 8 d period (maximum decolourisation was attained) of cultivation under recirculating conditions passed before decolourisation studies using Tubes II and III in continuous flow mode were undertaken.

Procedures used during continuous flow treatment were as follows. Firstly the reservoir was removed and four, 1l batches, containing sterile 1:1 mixtures of Eo-effluent and distilled water, were consecutively pumped (4x1l) at a rate of 400 ml/h through each of the tubes. Samples were collected continuously and analysed for colour and light absorption levels at 280 nm. Thereafter the reservoir, loaded with fresh medium consisting of Eo-effluent diluted 1:1 with distilled water, was replaced and decolourisation studies continued under recirculating conditions until colour equilibrium was attained.

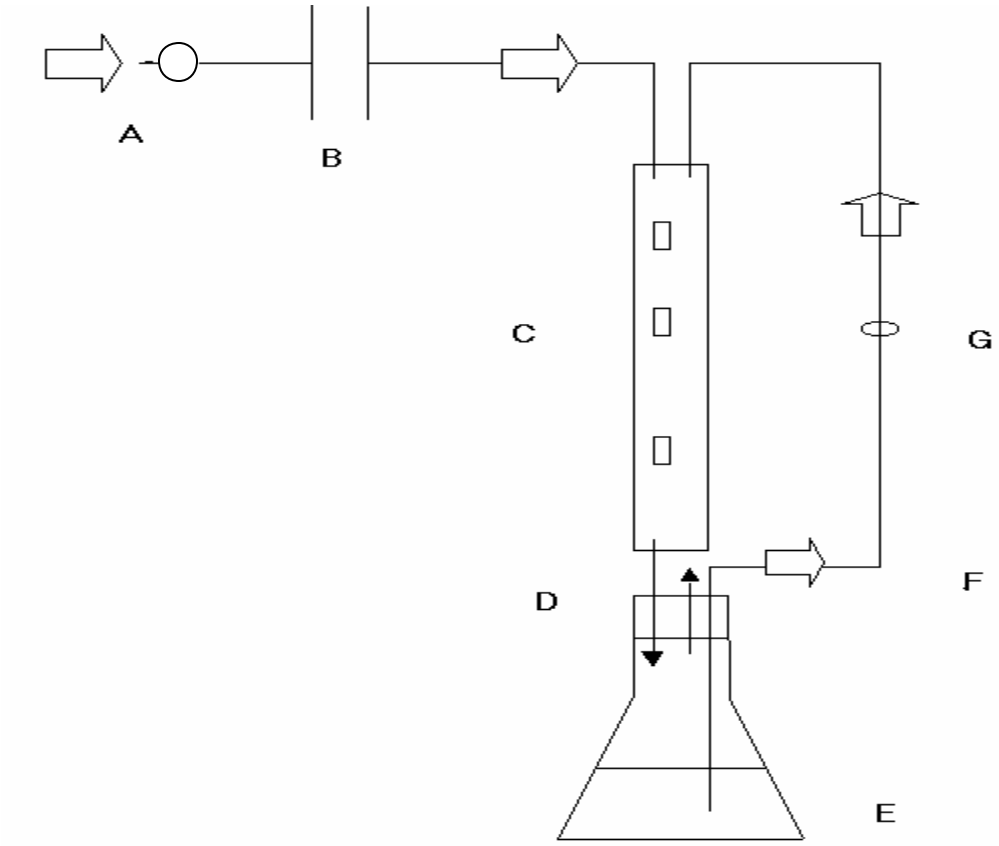


FIG. 4.2: Diagram depicting trickling filter reactor. A) Air pump, B) $CuSO_4$ trap, C) Reactor with foam cubes and fungi, D) Reservoir with inlet and air vent tubes, E) Effluent containing medium, F) Outflow from reservoir, G) Peristaltic pump.

DETERMINATION OF BIOMASS IMMOBILISED

At the end of the experiments the tricking filters were disassembled. The foam cubes were removed from each tube and washed with distilled water. Thereafter the cubes were dried at 50ºC, cooled to room temperature and the weight of immobilised biomass determined.

Treatment in a rotating biological contactor reactor

REACTOR DESIGN

The rotating biological contactor (RBC) reactors (Fig. 4.3) were constructed using 100 cm plastic tubes with a diameter of 7.5 cm. The tubes could be disassembled into 24 cm sections and each section contained 12 plastic disks with a spacing of about 1.5 cm between them. The plastic disks had an opening in the middle with a diameter of 2.5 cm. They were packed vertically into the tubes and held in place by o-ring containing plastic rings. Each disk was fitted with porous plastic material to serve as support for microbial growth. Tubes were mounted on rollers, powered by an electric motor and rotated at 4 rpm. Rollers were mounted in a frame that could be elevated to independently vary the volume hold capacity of the RBC system. Tubes were sealed at each end with caps containing inlet and outlet pipes. Inlet pipes were supplied with glass fittings. Reactors were connected to a peristaltic pump by silicon tubing attached to the glass fittings. Air was supplied to the reactors through side tubes, connected to the glass fittings by an air compressor via sterile air filters.

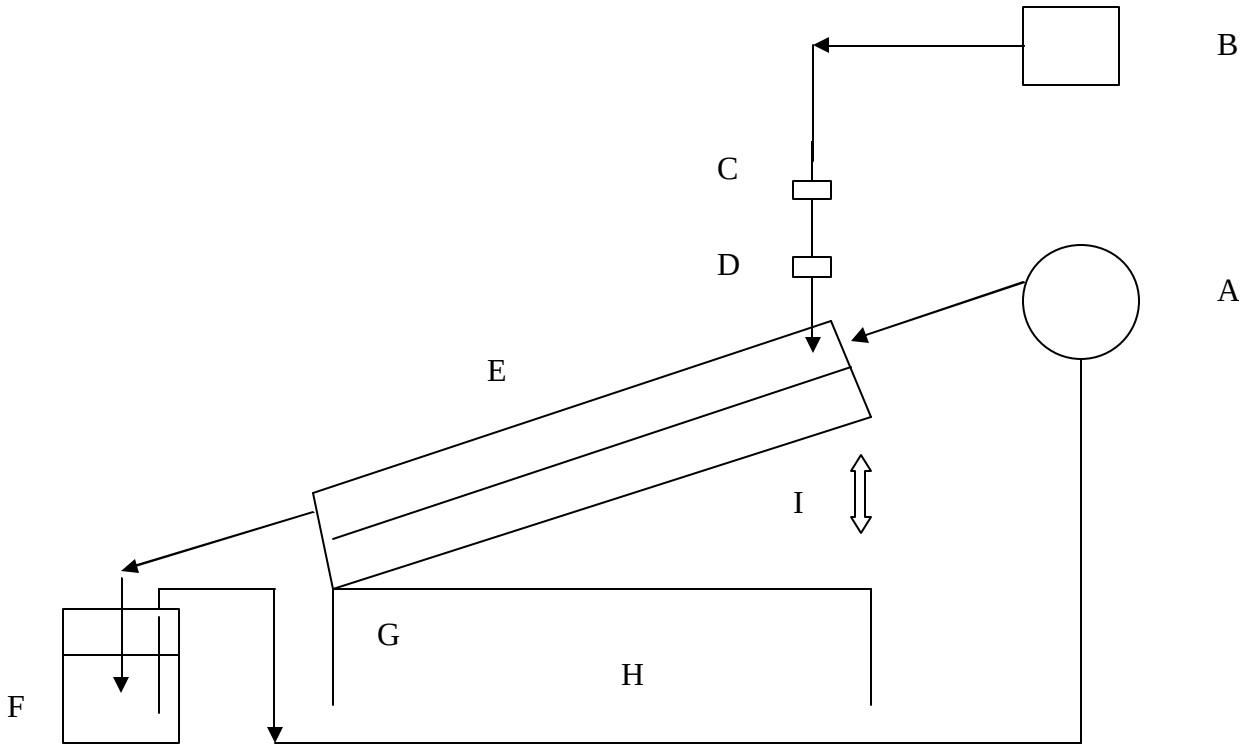


FIG. 4.3: Diagram depicting RBC. A) Peristaltic pump, B) Air compressor, C) Airflow meter, D) Air filter, E) Reactor with disks, mounted on rollers, F) Reservoir with Eo-effluent containing medium, G) Electric motor, H) Base of frame, I) Frame can move up or down for various angle settings.

IMMOBILISATION OF FUNGI IN RBC

Cell cultures (1.2 l) were homogenised for 60 s under aseptic conditions at a rate of 13 500 rpm with a homogeniser (Diax 900, Heidolph, Kelheim, Germany). Prior to immobilisation, the assembled RBC reactors were treated with 70% (v/v) ethanol for 1 h and then washed with sterile distilled water. The homogenised culture was pumped into the RBC reactors at a rate of 500 ml/h with a peristaltic pump. Thereafter growth medium (pH 3.5) was supplied to the reactors using a peristaltic pump. Following a week of

incubation at 25ºC, freshly prepared and homogenised culture was pumped into the reactors and this procedure was repeated a third time.

DETERMINATION OF HYDRAULIC CHARACTERISTICS OF RBC

The hydraulics of the RBC was characterised by feeding (30 ml/h) of a 50 ml NaCl solution (5 g/l) to the reactor before and after immobilisation. Samples (20 ml) were continuously taken during and after dosing and dispensed in vials that were numbered according to the order in which sampling proceeded. Conductivity of samples was measured with a Hanna conductivity meter (Hanna instruments, Padova, Italy) to follow NaCl elution fom the reactors. NaCl elution from the tubes was considered maximal when the conductivity of the samples reached a maximum level.

ANALYSIS OF NUTRIENT REQUIREMENTS OF RBC FOR OPTIMAL DECOLOURISATION

Nutrient requirements were studied with the RBC operated under continuous flow conditions at a HRT of 24 h. Levels of glucose, NH_4NO_3 and KH_2PO_4 were varied in the medium to determine the level necessary of each nutrient for best decolourisation to proceed. Glucose levels varying from 0 to 10 g/l were tested. NH_4NO_3 concentrations of 0 to 1.75 g/l were examined. Furthermore, the effect of KH_2PO_4 levels on decolourisation in the range of 0 to 0.5 g/l was also studied. Other nutrients that were included were based on the medium as described by Mhena *et al*. 1995, with the exception that $CaCO_3$ was used instead of KCl. The pH of the decolourisation medium was adjusted to 3.5.

REACTOR VOLUME DETERMINATION

After immobilisation of the fungi in the RBC, the volume of the reactors was determined. This was accomplished by dosing the inlet feed to the reactors with 50 ml of a sterile NaCl solution (5 g/l) similar as described in the section dealing with the determination of the hydraulics of the RBC above. Reactor volume was used to calculate hydraulic retention time. Hydraulic retention time was varied between 0.67 to 23 h to determine the

effect of this parameter on decolourisation. The hydraulic retention time used during investigations on the effect of initial colour intensities on decolourisation was 24 h.

DECOLOURISATION MEDIUM

Media with the following composition were used during decolouring experiments (g/l): glucose, 10; NH_4NO_3, 0.5; KH_2PO_4, 0.1; $MgSO_4$, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCl_2$, 0.5. The pH of the medium was adjusted to pH 3.5 with a Hanna pH-meter (Hanna instruments, Padova, Italy). Eo-effluent was diluted to various levels to determine the effect of initial effluent colour on decolourisation. Subsequently it was decided to supply effluent at 50% of full strength during further decolourisation experiments. Glucose, citrate buffer (0.01M) and Eo-effluent were heat-sterilised (121ºC, 20 min). The other constituents of the decolourisation medium were filter-sterilised using a 0.45 µm sterile Millipore filter (Bedford, MA, USA) and then aseptically added to the heat-sterilised portion to bring the final volume of the medium to 2 l in the RBC system.

OPERATION OF RBC

Following immobilisation, the fungi were adapted to the effluent-containing decolourisation medium by supplying the medium over a period of four hydraulic retention times before decolourisation experiments commenced. Sterile decolouring medium contained in a rubber stoppered 2 l Erlenmeyer flask was positioned at the outflow end of the reactor and served as reservoir during experiments conducted under recirculating conditions. The reservoir was connected to the reactor by a silicon tube. Medium was circulated between the reservoir and the reactor at a fixed rate by a peristaltic pump. The content of the reservoir was agitated by a magnet and magnetic stirrer (80 rpm). Samples were taken for analyses at the reactor outlet (at the junction between the reactor and the reservoir) as medium emerged from the reactor before mixing occurred in the reservoir. Samples were also taken from the reservoir. After 72 h of treatment the contents of the reservoir reflected the concentration in the reactor. Continuous flow experiments were conducted during which the reactor was not equipped with a reservoir. Medium that emerged from the reactor was not recirculated, but

collected in a closed container positioned at the outflow end. Samples were taken for analyses at the reactor outlet. After steady state was achieved, samples were also collected (total volume) on a daily basis (every 24 h) from the closed container, frozen and kept at -20ºC until required for further analyses. Air was supplied to the reactor by an air compressor through an in-line sterile Hepa-vent Whatman air filter (Ann Arbor, MI, USA) at a flow rate of 2.3 l/min. Reactors were operated at 25ºC.

ESTIMATION OF COLOUR ADSORBED TO BIOMASS

Colour adsorbed to biomass after completion of decolourisation experiments was determined by treatment of fungal cell mass with 1 N NaOH for 24 h. The cell mass (38.3 g, *C. versicolor*, and 47.2 g, *R. pusillus*) was obtained from the RBC by scraping it from the reactor disks at the end of the experiment. The mixture was agitated by a magnet via a magnetic stirrer. Prior to alkali treatment, the fungal biomass was washed with distilled water on Büchner funnels. The progress of decolourisation was monitored over time (72 h) in a preliminary experiment to determine the maximum colour that could be desorbed. Colour desorbed from *R. pusillus* and *C. versicolor* biomass was completed within 24 h and no further release of colour was observed beyond that treatment time. Experiments were also conducted in shake-flasks, using similar charges as was used during RBC treatment. Furthermore, short contact times between biomass and decolourisation medium were employed (5 min) so that the only mechanism of decolourisation would theoretically be due to adsorption. Thereafter, biomass was washed on Büchner filters and subsequently treated with NaOH as described above. The colour desorbed from the biomass under these conditions was then quantified.

KINETIC MODEL

An empirical model proposed by Desrochers *et al*. (1983) was used to describe the decolourisation kinetics of the RBC for one hydraulic retention time (23 h). The various parameters were determined by non-linear regression. This model was used since steady state is approached quickly because of adsorption. It was proposed that a n-th order chemical reaction rate are followed and that under plug-flow conditions the following equation holds:

$dC/dZ = -1/ U_0 /(k*C^n)$ ….(1), where:

U_0 = axial fluid velocity (cm/h)

-k = volumetric reaction rate constant (CPU/ h)

C = effluent colour intensity (PCU).

n = n-th chemical reaction rate, other parameters have been described in Fig. 4.4.

The parameters k and n were obtained by fitting equation 1 to a plot of dC/dZ vs C.

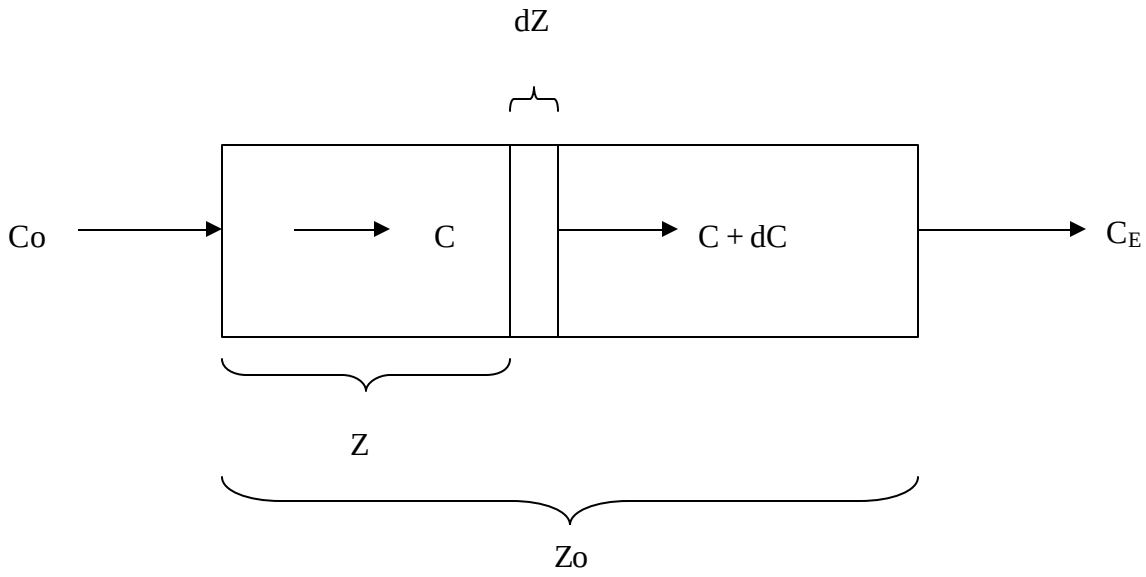


FIG. 4.4: Diagram depicting elementary section used in the model for calculation purposes. Legends: C_o , influent colour intensity; C, colour concentration at length Z; C + dC, colour intensity for the length Z + dZ; Zo total length of column; C_E, effluent colour intensity.

The differential model starts by obtaining a mass balance over a cylindrical section element with cross section of thickness dZ (Fig. 4.4).

ENZYME ACTIVITY DETERMINATIONS

Manganese peroxidase activity was assessed according to the method of Paszczynski *et al*. (1988). Manganese peroxidase was assayed using 0.1 M sodium tartrate (pH 5.0), 0.1 mM 2,6-dimethoxyphenol, 0.1 mM $MnSO_4$ and 0.1 mM H_2O_2 contained in a cuvet of 1 cm light path. Absorption changes monitored at 468 nm was followed over time at a controlled temperature of 30ºC. Enzyme activities obtained in the absence of H_2O_2 were subtracted from values obtained in the presence of this oxidant to account for non-peroxidase activity present during assays. Enzyme activities were repeated using various dilution of the cultivation broth to verify linearity of enzyme activity against dilution of enzyme broth used in the assays. Lignin peroxidase activity was determined according to procedures of Addleman and Archibald (1993) and laccase activities were also assessed by using 2,6-dimethoxyphenol as substrate (Font *et al*. 1997) The same protocol employed during MnP determination was followed except that $MnSO_4$ and H_2O_2 were not included in the reaction mixture. One unit (U) of enzyme activity converted 1 µmol of substrate per min.

Enzyme activities were also followed during shake flask experiments conducted at 30ºC on orbital shakers using the same decolourisation medium as described for RBC studies. Effluent was diluted to various strengths and included into the medium. This was done to determine the influence of effluent strength on enzyme activities during cultivation. The shake flasks were inoculated with active growing *C. versicolor* culture, pre-cultured on growth medium with pH adjusted to 3.5. The flasks contained 0.103 to 0.120 g/l of fungal biomass directly after inoculation. Enzyme activity was followed over a 20 d period.

Cell-associated ligninolytic enzyme activities produced in the RBC by *C. versicolor* were also determined as follows. Firstly, biomass was harvested and washed with distilled water after treatment of decolourisation medium in RBC for 23 h when highest decolourisation was attained. This was followed by extraction of washed fungal biomass with 0.5 M NaCl solution at 4ºC (Lackner *et al*. 1991). During extraction a magnetic stirrer operated at about 50 rpm and magnet was used to agitate the biomass. Ligninolytic

enzyme activities were assayed in the wash water and salt extraction solution, respectively.

LONG TERM DECOLOURISATION EXPERIMENTS

Experiments were conducted to evaluate the decolourisation potential of the RBC over longer time periods. During these investigations fresh media were supplied via the reservoir, after every 72 h of cultivation and decolourisation monitored, until a significant loss in the decolourisation activities, were noted.

Analytical methods

The RBC reactors were washed with distilled water before biomass was scraped from the disks upon completion of the experiments. Biomass was dried to a constant weight at 105ºC and then determined gravimetrically. Colour determinations and COD analysis were performed as described before (P. 47, Chapter II). Conductivity of samples was measured with a Hanna conductivity meter and expressed in µS/cm. Adsorbable organic halogen (AOX) analyses were performed using an Euroglas AOX EC S 1000 analyser (Delft, Netherlands) according to the manufacturer's instructions. Bacterial growth inhibition tests were performed to determine toxicity of the effluent (Slabbert 1986; Slabbert, 1988). *Pseudomonas putida* strain Berlin 33/2 (DSM, Teilsammlung, Bayreuth, Germany) was used as test organism. Tests were performed in 25 ml minimal medium (1.05 g K_2HPO_4, 0.45 g KH_2PO_4, 0.047 g sodium citrate, 0.1 g $(NH_4)_2SO_4$, 0.01 g $MgSO_4.7H_2O$ and 0.25 g glucose per litre of deionised water (pH 7.15) in 50 ml medicine bottles. A culture of *P. putida*, grown, as static cultures in an incubator, overnight at 27°C in minimal medium, was diluted with fresh minimal medium (to an absorption level of approximately 0.8), 30 min prior to inoculation of filter-sterilised (Millex, Millipore, Molsheim, France) test samples. The cell suspension was added aseptically, at a ratio of 1:4 to a 12.5-times concentrate of the minimal medium and used as 2.5 ml volumes for inoculation of 22.5 ml test samples. Test samples were incubated at 27ºC for 6 h after which growth was measured in terms of absorption. Absorption determinations were carried out spectrophotometrically at 600 nm. Solutions containing samples and medium only (without inoculum) were used to blank the spectrophotometer. Sterile deionised

water was used for control tests. Results were expressed as percentage inhibition or stimulation (%) over control. Growth inhibition of $\geq$10% was considered as a toxic effect. Analytical values represented the mean of at least two determinations.

4. RESULTS AND DISCUSSIONS

Activated sludge reactor

DETERMINATION OF RETURN SLUDGE FLOW

The diluted sludge volume (55.5 ml was the sludge volume recorded after 30 min (Fig. 4.5), that was used in the calculation below) was determined on a regular basis (twice a week) and the values used to calculate the return sludge flow as follows:

Q_s = 55.5/(100 - 55.5) * 76 ml/h = 94.8 ml/h

(The value 76 ml/h is used since in this example the hydraulic retention time was 10 h, based on the aeration basin working volume of 760 ml).

The flow rate was set by using a timer connected to a fish air pump and by varying the air flow rate of the fish air pump. The fish air pump forced air down a glass tube that then resulted in the transfer of settled sludge from the clarifier back into the aeration basin. An activated sludge reactor was also operated without a timer but problems with sludge bulking followed, that could not be solved satisfactorily. Upon fitting of the timer system no sludge bulking occurred, with the only exception being when problems with air supply to the aeration basin was experienced. This occurred infrequently and was not a major problem.

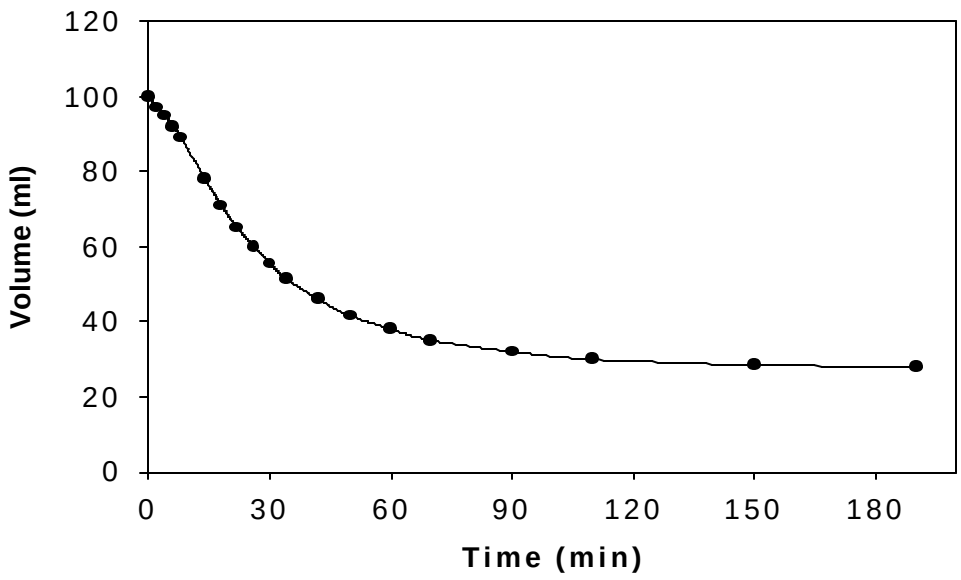


FIG. 4.5: Diluted sludge volume followed over time during settling of activated sludge under gravity in a measuring cylinder

NUTRIENT REQUIREMENTS OF ACTIVATED SLUDGE REACTOR

The effect of urea and KH_2PO_4 addition to effluent feed on COD removal by the AS reactor was assessed at a hydraulic retention time of 10 h and with a food to microorganism ratio of 0.6. Maximum COD removal was obtained when 100 mg/l urea was added to the effluent, which corresponded to 48 mg/l nitrogen (Fig. 4.6). The phosphate requirement of the AS in terms of COD removal is illustrated in Fig 4.7. Optimum COD removal was attained at a KH_2PO_4 level of 20 mg/l added to the effluent feed, which correspond to a phosphate concentration of 4.5 mg/l. The optimum COD:N:P ratio was therefore found to be 100:4.8:0.45. Saunamäki (1994) obtained best results with BOD:N ratios of 100:(3.0 to 4.5) and BOD:P ratios of 100:(0.3 to 0.5).

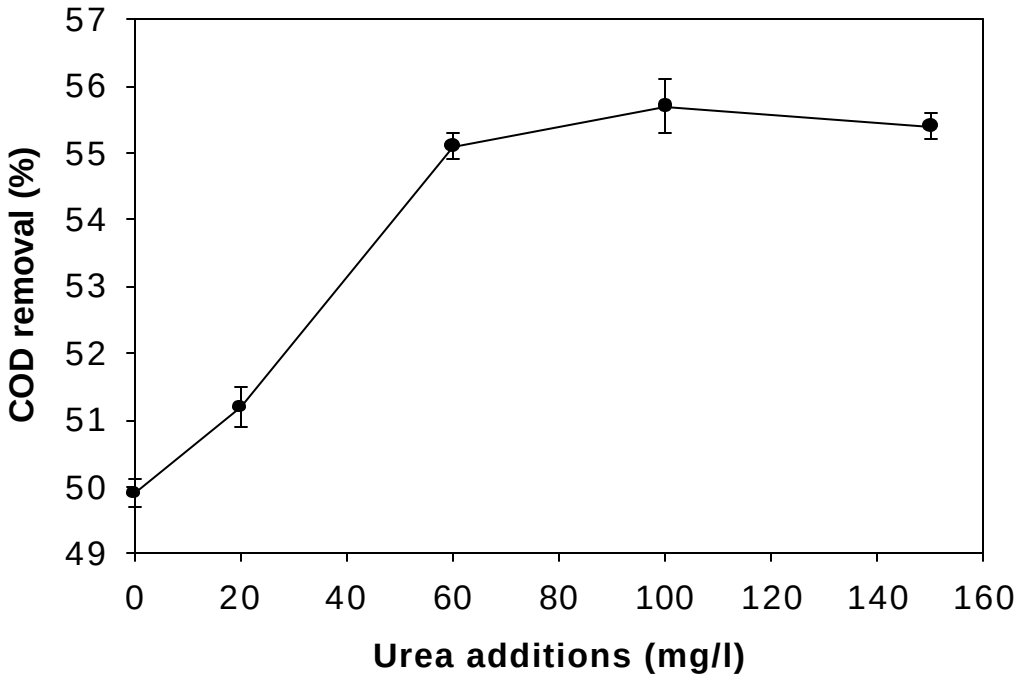


FIG. 4.6: Effect of urea additions to AS feed on COD reduction

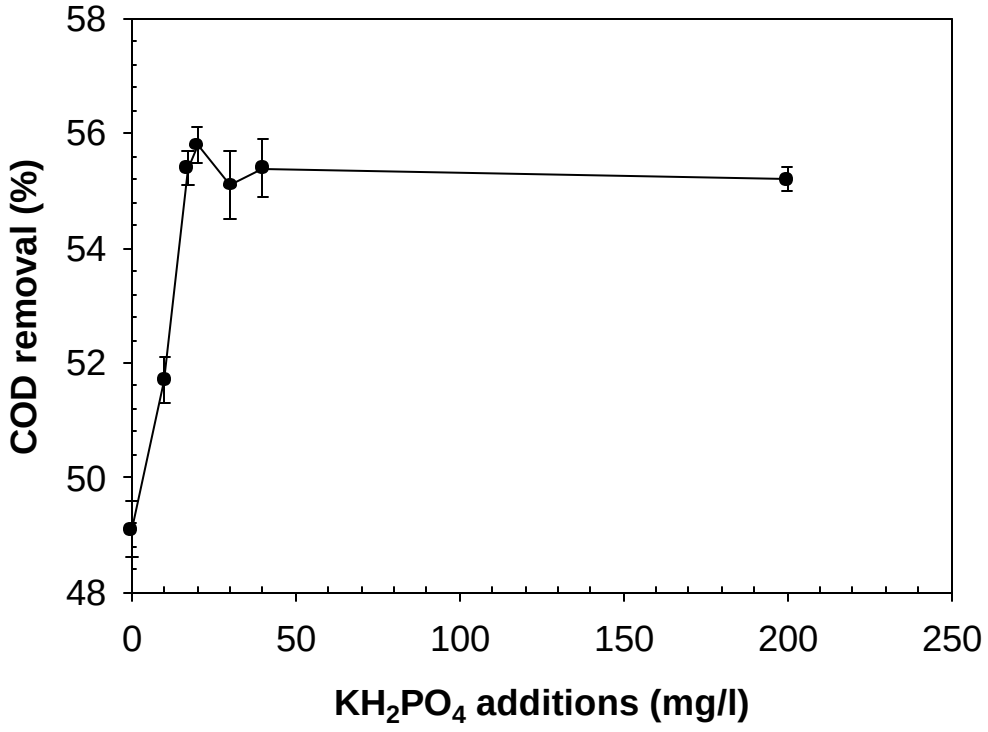


FIG. 4.7: COD removal as a function of KH_2PO_4 additions to AS feed.

EFFECT OF HYDRAULIC RETENTION TIME ON PERFORMANCE OF ACTIVATED SLUDGE REACTOR

The effect of hydraulic retention time on COD removal is presented in Fig. 4.8. At a hydraulic retention time (HRT) of 15 h COD removal efficiency began to level off. It was decided to conduct further experiments at a HRT of 12 h. A HRT of 15 h was deemed too long for operation of the AS-system on a practical level when treating bleach plant effluent and also because of technical problems experienced with the diaphragm pump that was employed initially.

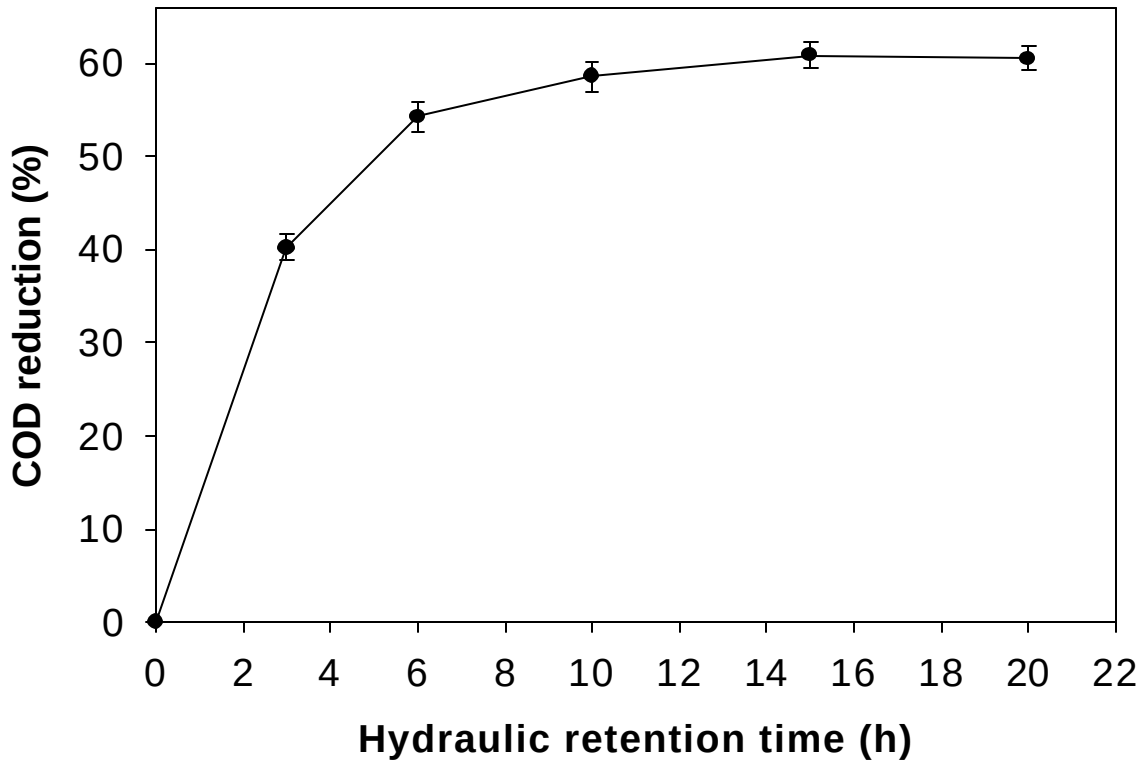


FIG. 4.8: COD removal plotted against hydraulic retention time of AS reactor

INFLUENCE OF MICROORGANISM TO FEED RATIO ON ACTIVATED SLUDGE REACTOR PERFORMANCE.

The effect of microorganism to food ratio was studied by using various effluent strengths supplied to the AS feed at a constant hydraulic retention time of 12 h. A maximum COD reduction of 61.3% was possible at low F/M ratios, but COD reduction decreased significantly as effluent loading increased, finally reaching COD removal levels of 25.5% at a F/M ratio of 4.07 (Fig. 4.9).

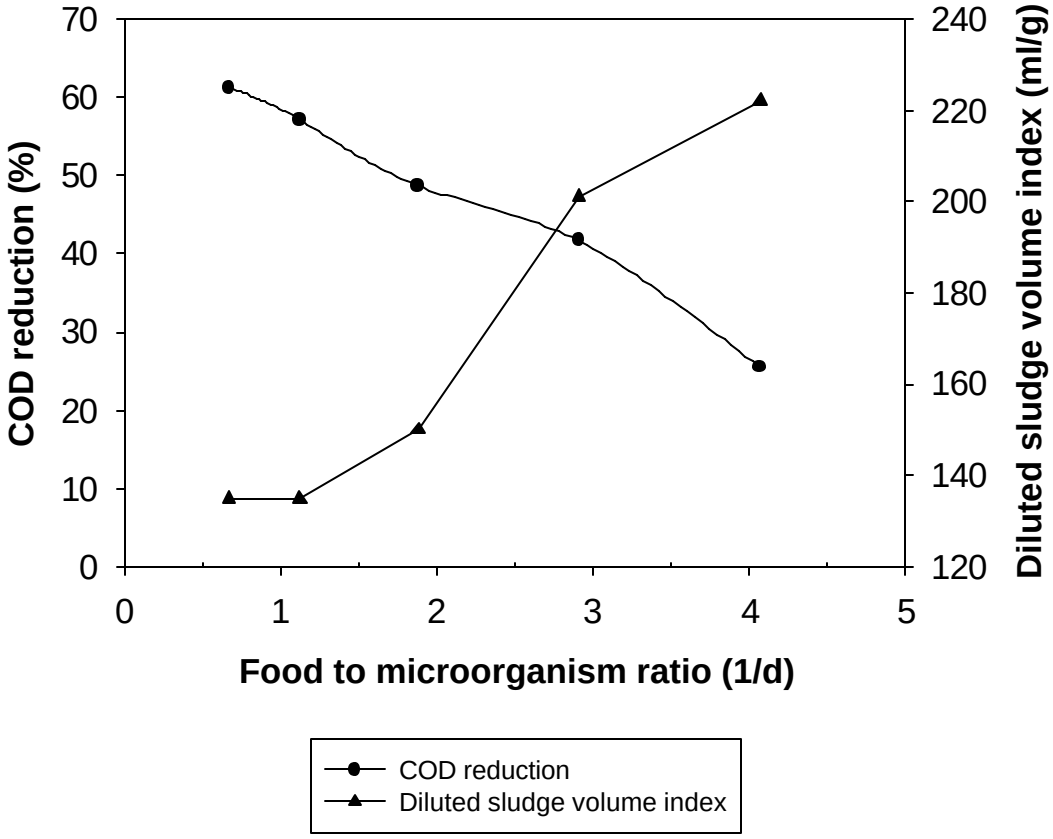


FIG. 4.9: COD reduction (circles) and diluted sludge volume index (triangles) plotted against the food/microorganism ratio.

As the effluent loading of the feed to the AS increased, the diluted sludge volume index also increased. This indicated the AS was operated under conditions that could result in sludge bulking. Therefore, the system has to be operated at lower loading conditions in order to maximise COD removal and to prevent sludge bulking that could lead to sludge loss.

COD REMOVAL IN ACTIVATED SLUDGE REACTOR OVER TIME

The AS was operated at a COD:N:P ratio of 100:4.8:0.45 and with a HRT of 12 h for several months without major problems. During operations a COD removal level of between 55 to 61% was possible. According to Haberl *et al*. (1991) reduction in COD percentages of between 30 to 40 % has been reported in literature. In this period the MLSS was maintained at about 4 g/l and the average sludge retention time in the reactor was about 19 days. Fig. 4.10 shows COD reduction and the dissolved oxygen levels measured during operation of the AS.

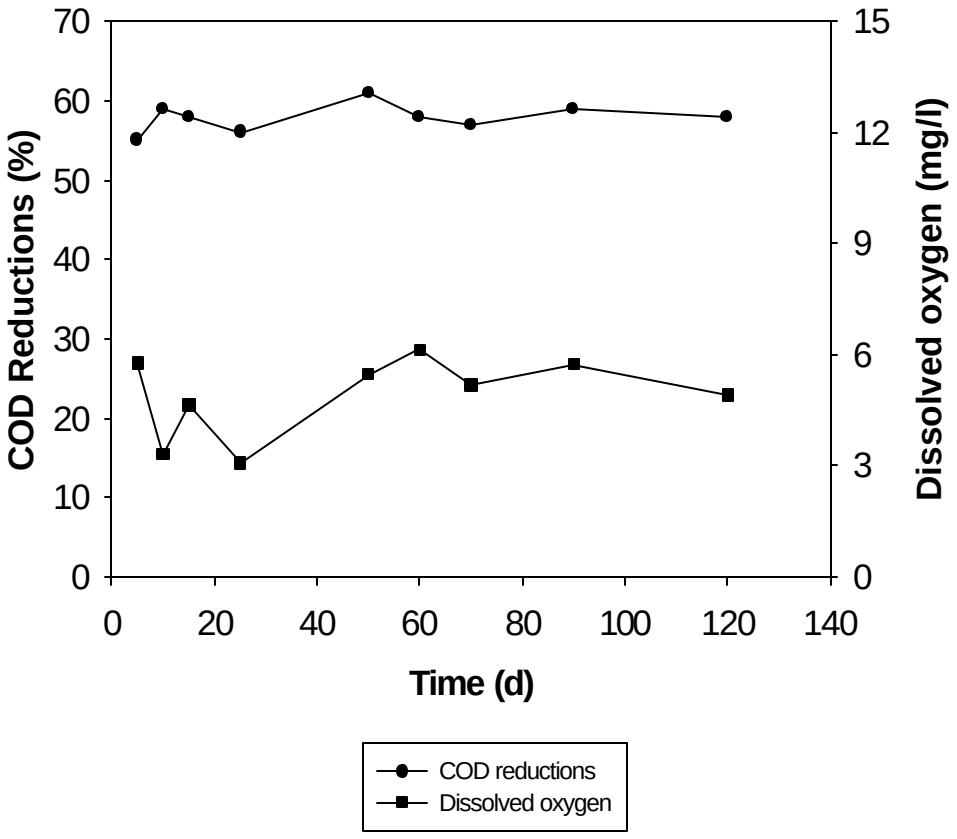


FIG. 4.10: COD removal (circles) and dissolved oxygen (solid squares) plotted against time during operation of AS.

IMPROVEMENT IN EFFLUENT QUALITY FOLLOWING ACTIVATED SLUDGE REACTOR TREATMENT

There was significant improvement in effluent quality in terms of COD and chlorophenol removal during treatment in the AS. The only effluent characteristic that was not appreciably affected was colour (Table 4.1).

Table 4.1: Effluent quality improvements after treatment in AS

Parameter	Reductions (%)
AOX	20
COD	58
Colour	10
2,4-dichlorophenol	65
Toxicity	9

Control: AOX, 1.2 mg/l; COD, 1 000 mg/l; Colour, 1 050 PCU; Toxicity, -11.7%; 2,4-DCP, 0.26 mg/l; pH 7.3.

Treatment in the AS rendered the effluent essentially non-toxic. AOX removals of between 20 to 30 % has been reported in literature (Haberl *et al*. 1991).

GEL PERMEATION CHROMATORGRAHPY

The molecular mass distribution before and after treatment in the AS was studied by gel permeation chromatography. Chromatograms of effluent before and after treatment are presented in Fig. 4.11 and 4.12, respectively. High molecular mass Compounds (> 1 000 g/mol) were not affected by treatment in the AS. These compounds are associated with the colour found in the effluent and explain why AS treatment cannot remove colour from effluent to an appreciable extent. High molecular mass compounds cannot enter the cell membrane of bacteria (Nagarathnamma and Bajpai, 1999).

Therefore, these compounds cannot be biodegraded by bacteria since these microorganisms lack an extracellular enzyme system capable of degrading chlorolignin.

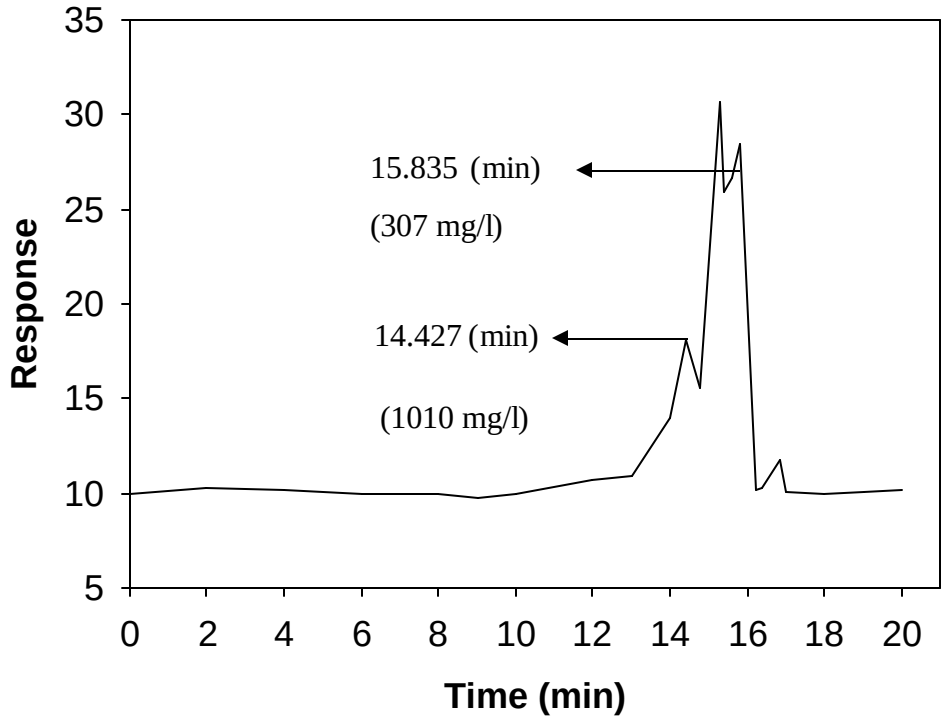


FIG. 4.11: Gel permeation chromatogram showing apparent molecular mass contents of effluent before treatment in the AS.

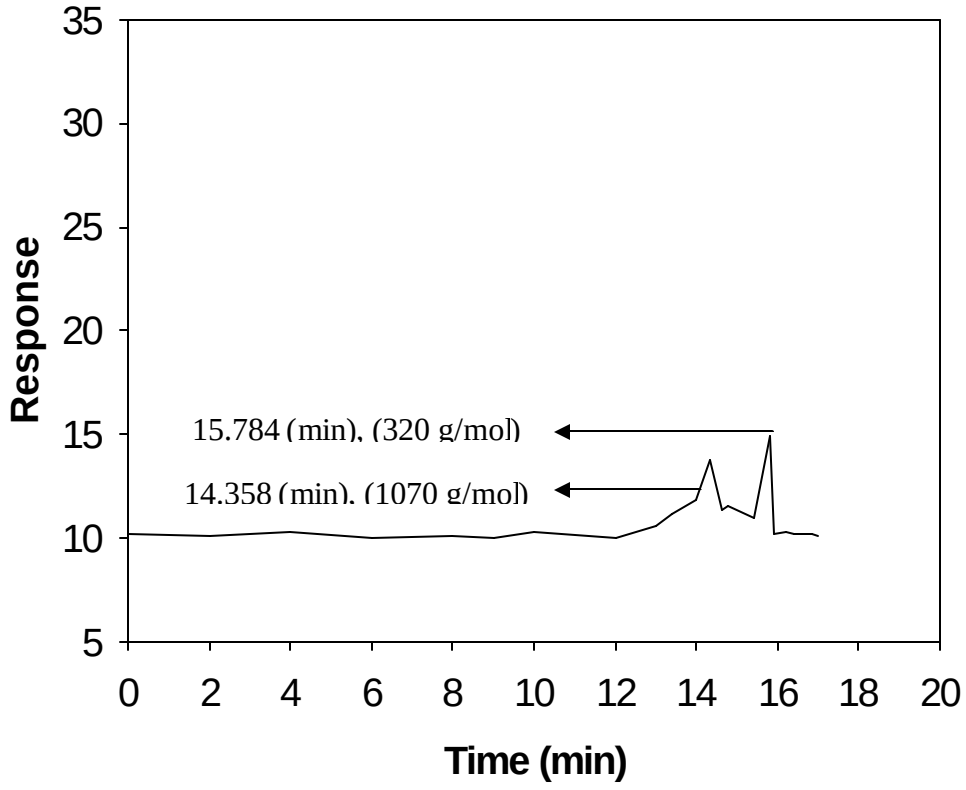


FIG. 4.12: Gel permeation chromatogram depicting apparent molecular mass content of effluent after AS treatment.

Trickling filter treatment

START UP OF TRICKLING FILTER EXPERIMENTS

Table 4.2 shows decolourisation and absorbency readings obtained after treatment of effluent containing medium with three white-rot fungi. *C. versicolor* produced the highest decolourisation after 5 d of treatment, followed by *S. illudens* and *P. sanguinens*. *C. versicolor* also lowered the absorbency readings to the greatest extent.

Table 4.2: Absorbency readings and decolourisation of Eo-effluent after treatment through trickling filter reactors with immobilised white-rot fungi.

Treatment	Absorption		Fungi
Time (d)	280nm	254nm	
5	1.04	1.58	*C. versicolor*
8	1.18	1.60	*S. illudens*
8	1.20	1.65	*P. sanguinens*
8	1.35	1.83	Control[a]

[a]The medium used in the control experiment consisted of Eo-effluent diluted 1:1 with 1.0 % molasses solution

CONTINUOUS FLOW CULTIVATIONS

After 5 d of treatment (when highest decolourisation was attained) decolourisation by immobilised *C. versicolor* was studied under continuous flow conditions. The effluent was decolourised by an average of 15%. The pH of the treated effluent increased slightly from an initial value of 4.77 to about 5.23 to 5.24 during the experiment. Since the initial pH of the feed was set at 5.5, the pH of the medium decreased slightly during treatment under continuous flow conditions (Fig. 4.13). Other workers also observed decreases in pH during treatment of bleach plant effluent or dyes by white-rot fungi (Royer *et al* 1991; Borchert and Libra 2001). Continuous flow experiments were also conducted with *S. illudens* and *P. sanguinens* after completion of 8 d of treatment under recirculating flow conditions. *Stereum illudens* decolourised the medium by an average of 11 % and *P. sanguinens* removed on average only 2% of the colour from the influent (Fig. 4.13). During treatment using *S. illudens* the effluent pH decreased from an average of 6.20, initially, to about 5.29 during the rest of the experiment. The initial pH measured early in the experiment conducted using *P. sanguinens* was on average 7.20 and decreased slightly to about 6.71 during the rest of the experiment (Fig. 4.13).

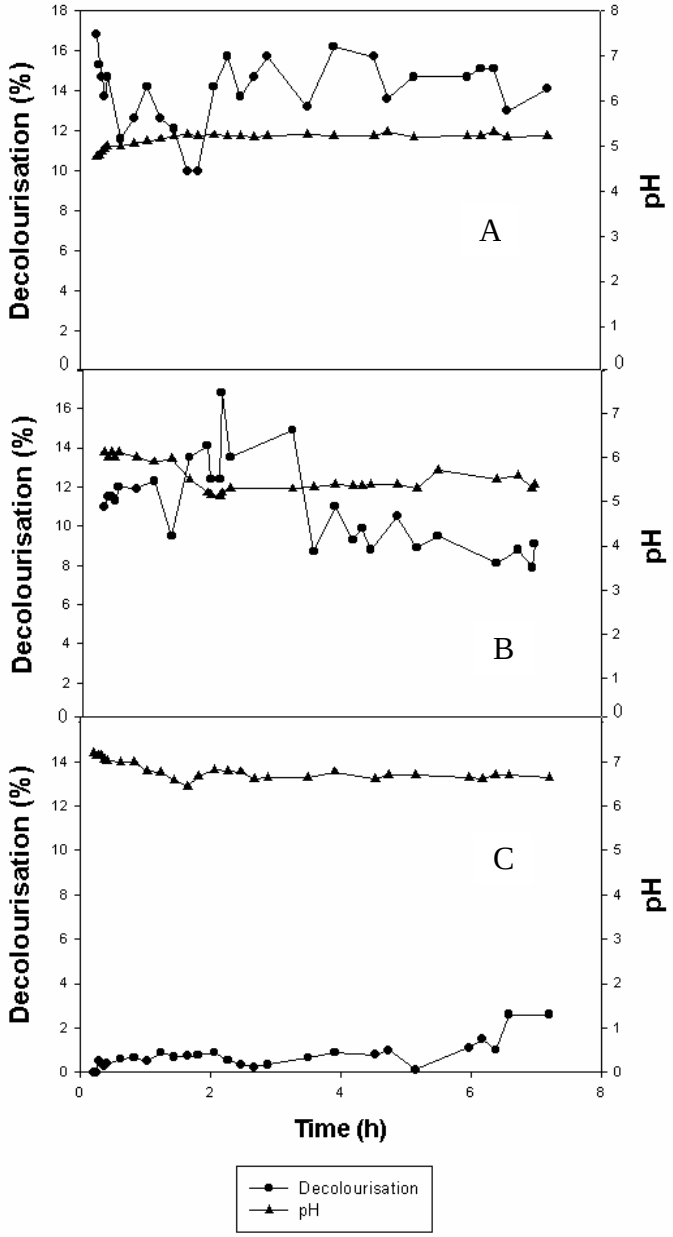


FIG. 4.13: Decolourisation and pH time profiles of effluent treated in trickling filters containing immobilised white-rot fungi obtained under continuous flow conditions. Panel A: *Coriolus versicolor*, Panel B: *Stereum illudens* and Panel C: *Pycnoporus sanguinens*

CULTIVATIONS WITHOUT ADDITIONAL CARBON COURCE

During an experiment conducted over a longer time period, the contents of the reservoir of the reactor containing *C. versicolor* was replaced with Eo-effluent diluted 1:1 with distilled water. Decolourisation of only 33 % (Table 4.3) was attained and the pH increased to 8.41 after 14 days of treatment under recirculating conditions. This indicated that *C. versicolor* required an additional carbon source to optimally decolourise bleach plant effluent. Molasses was included initially to serve as an inexpensive source of nutrients.

Table 4.3: Absorbency readings and decolourisation of Eo-effluent after treatment in a trickling filter reactor containing immobilised *C. versicolor* (Recirculating flow, medium: Eo-effluent diluted 1:1 with distilled water).

	Absorption			
Fungus	**280 nm**	**254 nm**	**Decolouring (%)**	**pH**
C. versicolor	0.75	1.71	33	8.41
Control	0.85	1.83	0	5.50

Absorption of the medium measured at 280 nm decreased following treatment in the trickling filter, indicating that lignin could have been degraded following treatment in the system under continuous flow conditions (Table 4.3). Treatment in trickling systems, operated under recirculating conditions, demonstrated that comparable decolourisation activities could be obtained when results were correlated to that obtained previously in shake flask cultures (results not shown). Because of technical problems optimisation studies were not conducted in the trickling filter system. Of the three white-rot fungi tested *C. versicolor* proved to be the best decolouriser of Eo-effluent and further studies using this fungus were therefore undertaken in the RBC.

BIOMASS IMMOBILISED IN TRICKLING FILTERS

After experiments were completed, the fungal mass immobilised in each tube was measured gravimetrically. The tube with *C. versicolor* contained 5.1 g biomass, 5.8 g of *S. illudens* and 3.2 g of *P. sanguinens* were immobilised, respectively.

Treatment of Eo effluent using RBC

HYDRAULIC CHARACTERISTICS OF RBC

The hydraulic characteristics of the RBC system are illustrated in Fig. 4.14.

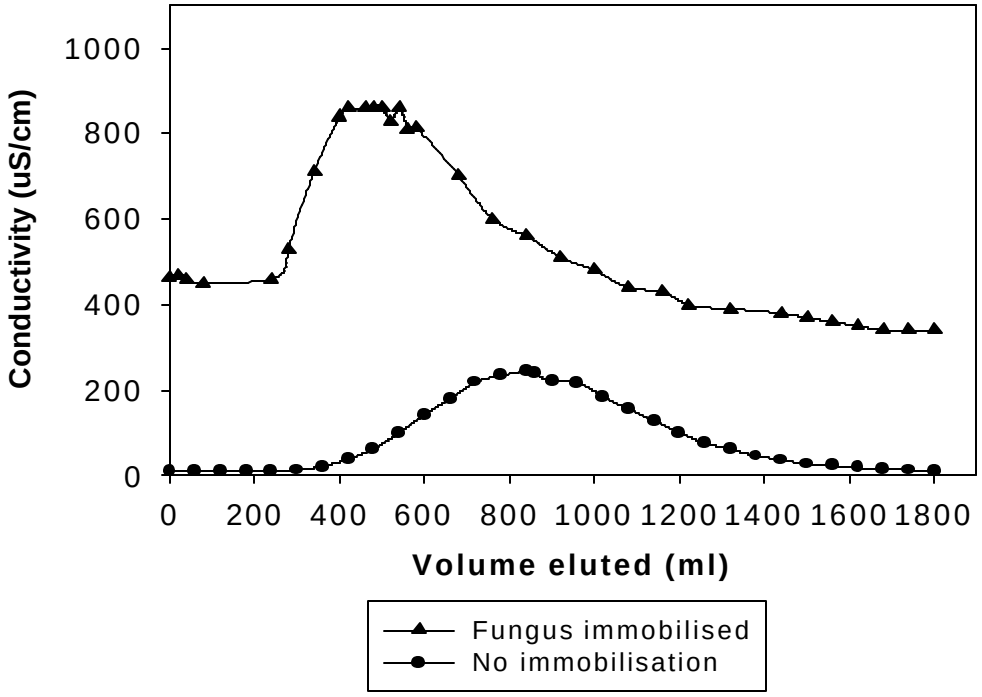


FIG. 4.14: Plots of eluent conductivity against volume eluted from RBC. (Plot with circles indicates RBC hydraulics without immobilised fungi whereas plot with triangles shows RBC hydraulics when *Coriolus versicolor* was immobilised in the system).

The salt elution profiles from the RBC, as measured by conductivity, indicate that the RBC system approximated plug flow hydraulic characteristics. With RBC containing immobilised fungi, higher conductivity levels were measured, this could indicate that proteins, salts or other conducting compounds are released from biomass during conductivity determinations. The elution profile obtained with no fungi immobilised in the RBC was also more symmetrical than the peak observed when biomass was immobilised in the RBC (Fig. 4.14). This might be due to irregularities brought about by the immobilised fungal cells in the RBC and also to some adsorption of NaCl on to the biomass. The release of adsorbed NaCl (or other conducting compounds) from biomass could lead to tailing effect in the elution profile of NaCl from the RBC.

BIOMASS IMMOBILISED IN RBC REACTOR

After studies on the nutrient requirements of the fungi were completed, the biomass immobilised in the RBC with *C. versicolor* was 36.1 g and 42.3 g for *R. pusillus.* The amount of biomass recorded after decolourisation experiments conducted at a retention time of 23h and colour levels of 6100 PCU was 38.9 g per RBC reactor immobilised with *C. versicolor* and 42.2 g in the case of *R. pusillus*. The biomass levels detected in the RBC reactor after examination of the effect of colour intensity on decolourisation were 44.0 g for *C. versicolor* and 68.5 g in the case of *R. pusillus*.

NUTRIENT REQUIREMENTS OF FUNGI DURING RBC OPERATIONS

Effect of glucose on decolourisation

Additional glucose was required by *C. versicolor* to decolourise effluent efficiently. In contrast *R. pusillus* required no additional glucose for optimal decolourisation (Fig. 4.15). It has been shown that *R. pusillus* decolourise effluent mainly by adsorption (Christov *et al*. 1999). Furthermore, white-rot fungi need an additional carbon source to degrade lignin. From the graph it can be seen that *C. versicolor* required 10g/l glucose to be added for the highest colour removal from effluent to occur. Following the experiment during which no glucose was added, glucose supplementation was resumed. Decolourisation levels increased and reach levels of about 60% when 10 g/l glucose was included in the decolourisation medium. This indicated that the fungus was still active and not detrimentally affected by glucose deprivation. Decolourisation levels used in Fig. 4.15

were the highest values obtained during each experiment. Hydraulic retention time of the RBC was set at 24 h during these investigations.

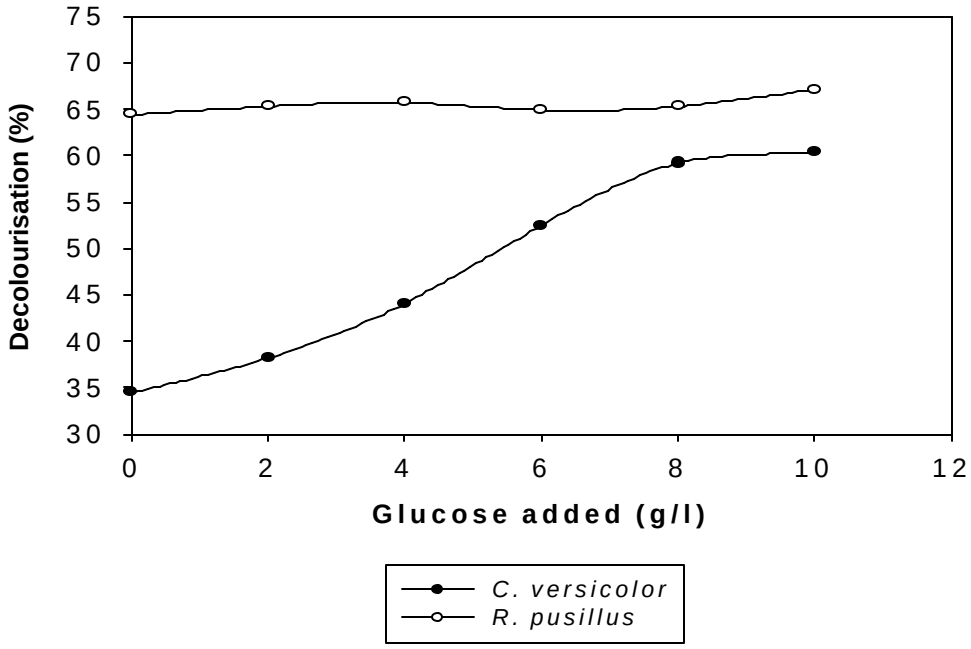


FIG. 4.15: Plot of decolourisation against glucose supplementation to decolourisation medium. (KH_2PO_4 0.5 g/l and NH_4NO_3 1.75 g/l).

Nitrogen requirements for optimal decolourisation in RBC

Nitrogen addition to the decolourisation medium was necessary for decolourisation to be improved. Best decolourisation was obtained at a NH_4NO_3 level of 0.5 g/l for *C. versicolor*. *Rhizomucor pusillus* decolourisation was not affected significantly by NH_4NO_3 additions (Fig. 4.16).

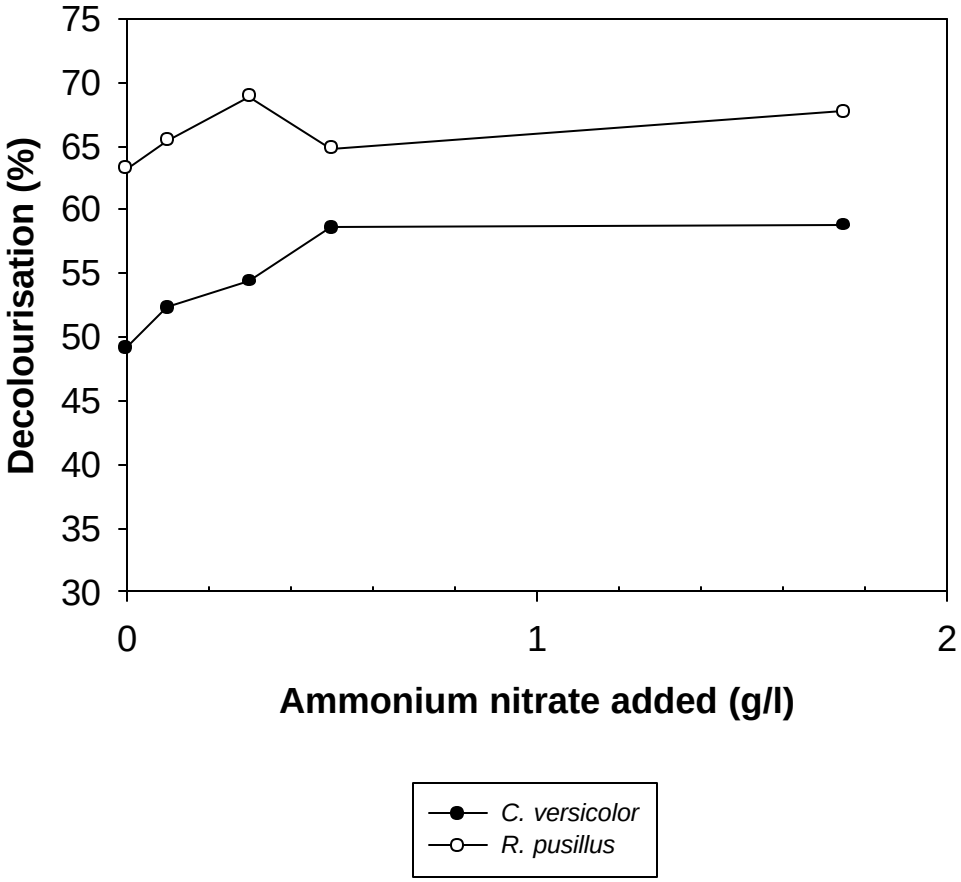


FIG. 4.16: Effect of NH_4NO_3 supplementation on decolourisation by *Coriolus. versicolor* (solids) and *Rhizomucor pusillus* immobilised in RBC. (KH_2PO_4 0.5 g/l and glucose 10 g/l).

Phosphate requirements of RBC

Decolourisation was studied during the supplementation of various KH_2PO_4 levels to the decolourisation medium (Fig. 4.17). *Coriolus versicolor* required 0.1 g/l KH_2PO_4 to decolourise the medium the best. However, KH_2PO_4 levels did not significantly affected decolourisation by *R. pusillus* during treatment.

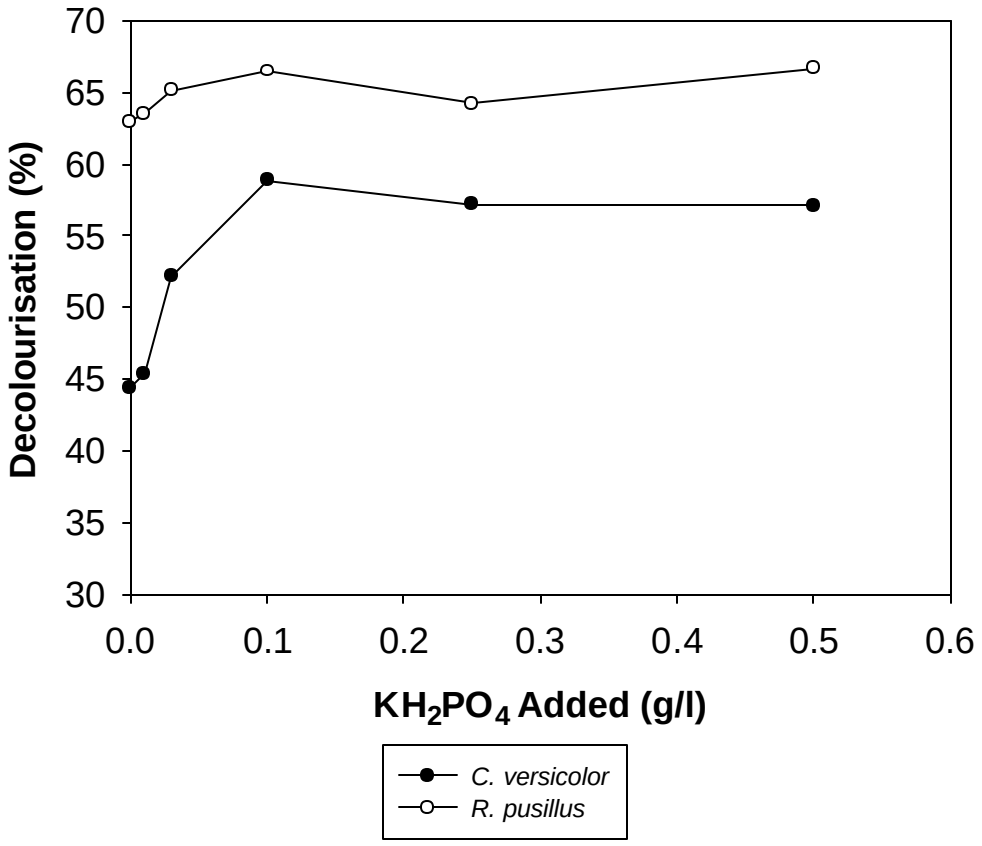


FIG. 4.17: Plot of decolourisation as a function of phosphate supplementation to RBC decolourisation medium. (NH_4NO_3 1.75 g/l and glucose 10 g/l).

EFFECT OF HYDRAULIC RETENTION TIME ON DECOLOURISATION

Hydraulic retention times varying from 0.67 h to 23 h were tested during continuous flow operation of the RBC. With *R. pusillus*, colour removal levelled off at a residence time of 23 h whereas *C. versicolor* required a hydraulic retention time of 18 h to reach the best decolourisation levels (Fig. 4.18). For comparative purposes, it was decided to conduct further investigations at a hydraulic retention time of 23 h for both fungi.

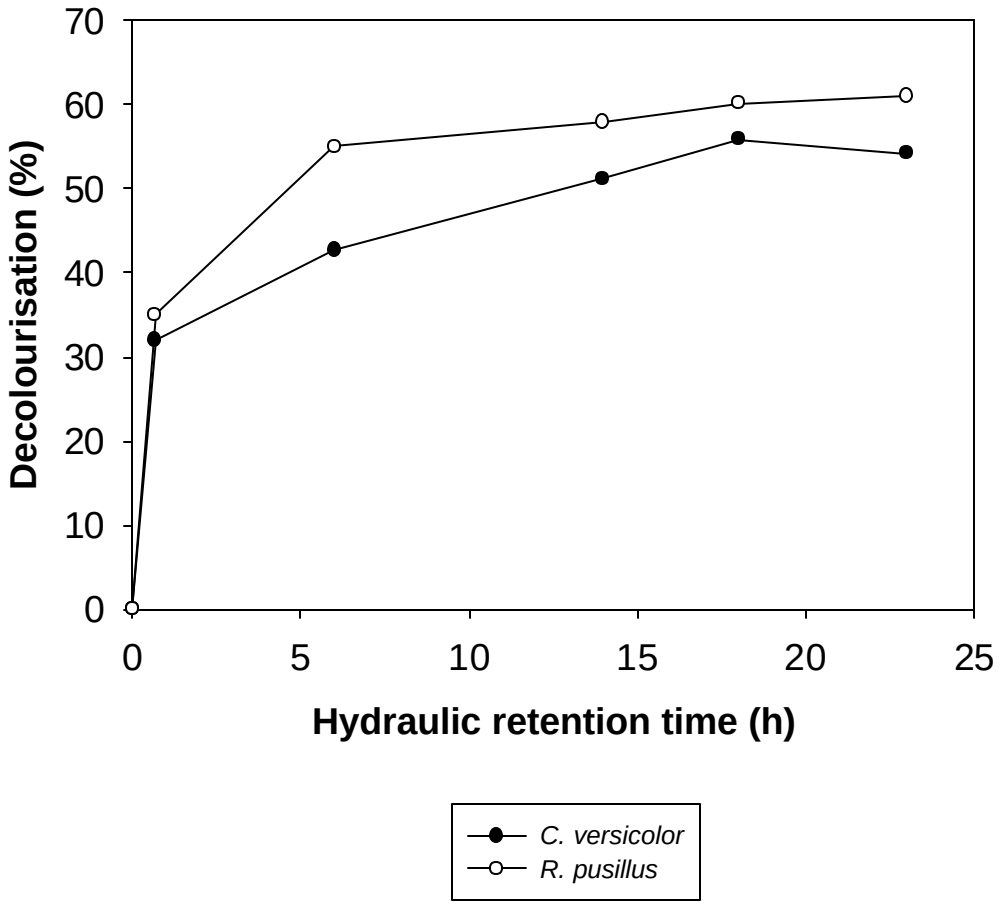


FIG. 4.18: Impact of hydraulic retention time on the extent of decolourisation of bleach plant effluent by *Rhizomucor pusillus* and *Coriolus versicolor* in RBC reactor.

The relative high decolourisation levels (32 to 55% decolourisation; Fig. 4.18) achieved at low hydraulic retention times (0.67 to 6 h) may be due to a rapid initial adsorption of colour by the biomass (Jaspers and Penninckx 1996). Yin *et al.* (1989) also found a rapid decolourisation during the first hour of contact using *P. chrysosporium* in a RBC. With a trickling filter system, the fastest degradation of bleach effluent occurred during the first 3 to 6 h, when about 50% of colour and AOX were removed by immobilised *P. chrysosporium* (Bajpai and Bajpai 1997). Royer *et al.* (1985) observed that the

process of decolourisation of bleach plant effluent using *C. versicolor* consisted of two phases: adsorption (within 24 h) followed by oxidation (after 48 h). Based on literature and the findings of this study, it seems that adsorption could be one of the first steps of decolourisation. Alginate-immobilised *C. versicolor* showed a colour removal of about 60% at a residence time of 30 h thereby the plot of decolourisation vs. residence time exhibited a hyperbolic profile (Desrochers *et al.* 1983.). Similar profiles were obtained during decolourisation runs in this investigation (Fig. 4.18). Pallerla and Chambers (1997) found that about 71% of feed colour was removed by *T. versicolor* at a residence time of 16 h. Although no significant difference between decolourisation levels at residence times of 16 (71%) and 24 h (72%) could be found, a significant decrease was noted when the residence time was lowered to 10 h (61%).

INFLUENCE OF COLOUR INTENSITY

Colour intensities were examined in the range of 1 550 to 10 650 PCU (Fig. 4.19). Apparently more colour was removed as colour intensities increased. However, decolourisation levels remained virtually constant at colour intensities higher than 3 250 PCU (Fig. 4.19). Similar results were reported by other workers (Mehna *et al.* 1995; Royer *et al*. 1991; Pallerla and Chambers 1997). The cause for this phenomenon is uncertain at present and needs further investigation. One reason could be that adsorption, the first step of colour removal, is significantly lower at reduced colour levels. Thus the interaction between chromophoric material and mycelium could be diminished and the biodegradation process not effectively initialised. According to Royer *et al*. 1991, contaminant microbial growth was stimulated at lower effluent strength and this could inhibit decolourisation by immobilised *C. versicolor*. A loss of decolourisation was also shown at high colour intensities, probably due to increased toxicity of effluent compounds (Royer *et al*. 1991). Furthermore, it was shown that decolourisation was lower at a colour inlet value of 1 000 colour units (CU) (41%), as compared to colour removal at 1 285 CU (69%) and 1 538 CU (66%) during decolourisation by *T. versicolor*. Apparently, low removal rates could be due to chromophoric compounds or chlorolignin becoming seemingly less accessible as substrates following effluent dilution (Pallerla and Chambers 1997).

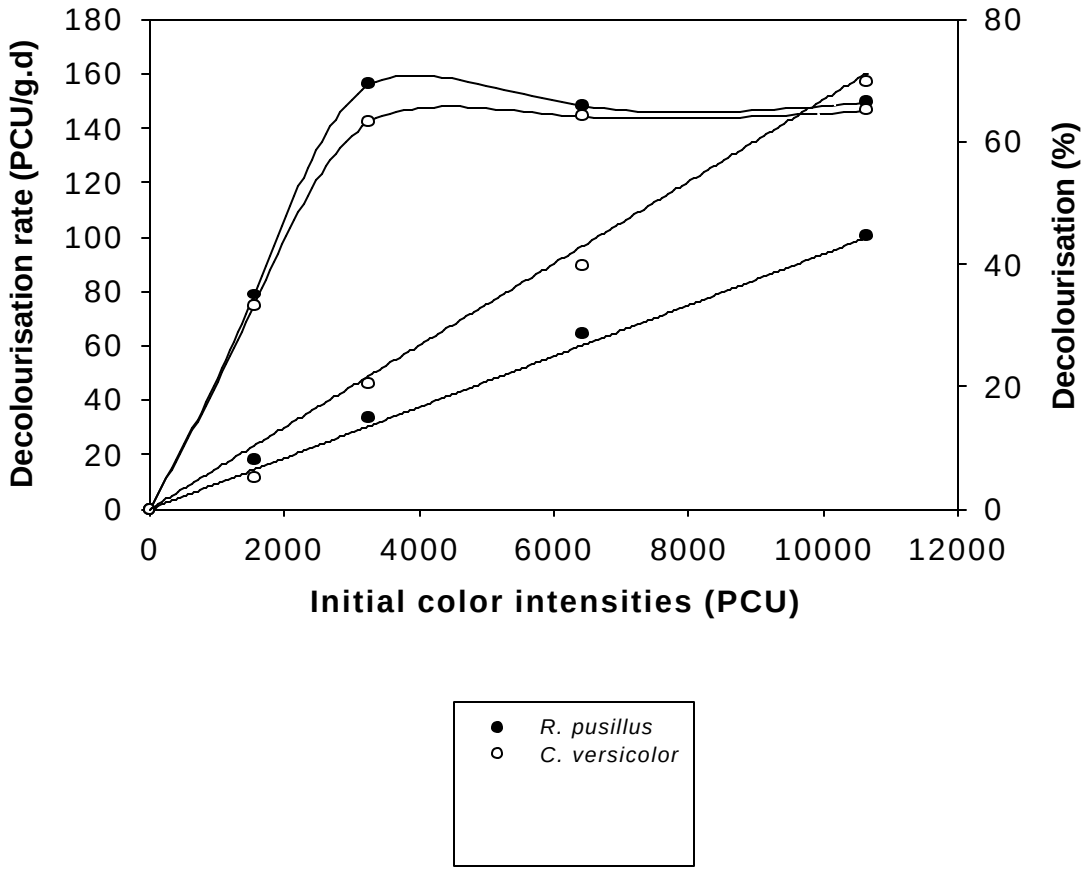


FIG. 4.19: Impact of initial colour intensities on specific decolourisation rate (straight lines) and extent of decolourisation (curves) of bleach plant effluent by *Rhizomucor pusillus* and *Coriolus versicolor* in RBC reactor (hydraulic retention time of 24 h).

Mhena *et al*. 1995, found using *T. versicolor* that no significant decolourisation effect was brought about by dilution of effluent, with colour intensities varying between 4 125 and 18 500 PCU. In this case, colour intensities tested were probably too high to affect decolourisation negatively. Initial colour intensities further influenced the decolourisation rate significantly. Initial colour levels were directly proportional to decolourisation rate expressed as change in colour/g.d in both fungi. Although this phenomenon has been described using white-rot fungi (Royer *et al*. 1991; Pallerla and Chambers 1997), as far as known, this is the first report where it has been demonstrated to occur using a mucorelean fungus for effluent treatment.

DECOLOURISATION USING PREDETERMINED PARAMETERS

Decolourisation experiments were conducted using diluted effluent with colour of 6 100 PCU (colour of raw undiluted and untreated effluent was 12 200 PCU), at a hydraulic retention time of 23 h. Under these conditions, *R. pusillus* decolourised the effluent to a level of 74% whereas *C. versicolor* removed 61% of the chromophores from the decolourisation media (Fig. 4.20). In the case of *C. versicolor*, there was a decrease in the decolourisation activity from 61 % at 23 h to about 53% at 72 h (data not shown). On the other hand, *R. pussilus* also showed a slight decrease in the decolouring activity from 74% (23 h) to 71% (72 h). Decolourisation with *R. pusillus* initially proceeded fast reaching 43% after only 0.67 h, whereas colour removal by *C. versicolor* was 34% for the same time period. Reducing sugar concentrations measured after one hydraulic retention time were 1.2 g/l for *R. pusillus* and 0.9 g/l in the case of *C. versicolor* (Fig. 4.20). Therefore, sugar depletion did not effect the decolourisation process negatively.

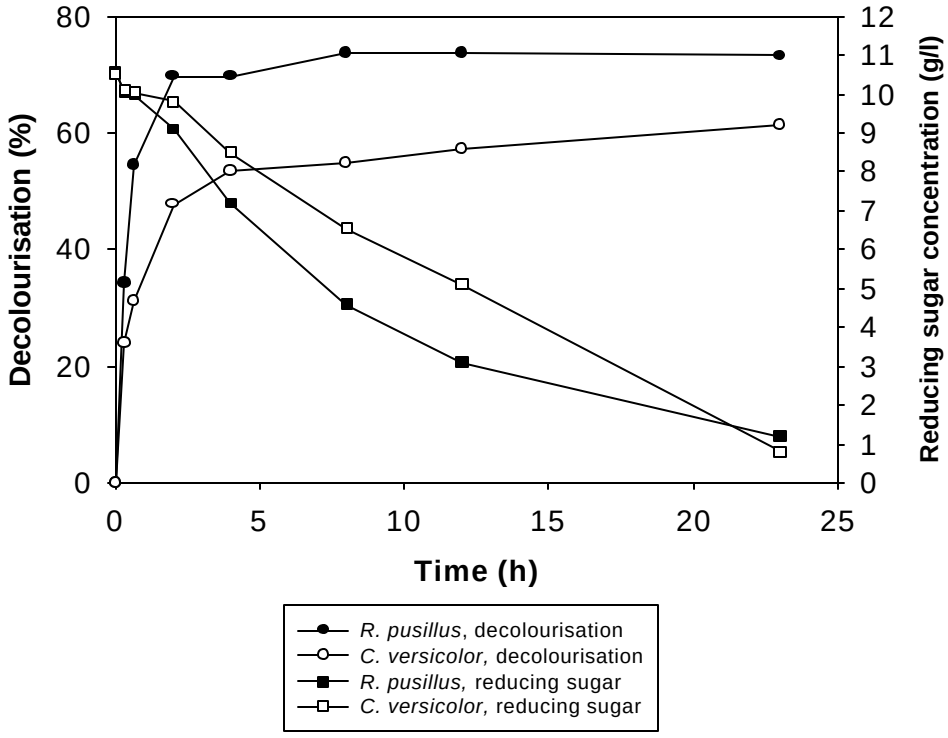


FIG. 4.20: Decolourisation and reducing sugar consumption profiles obtained after one passage of effluent containing media through the RBC using *Rhizomucor pusillus* and *Coriolus versicolor,* respectively (hydraulic retention time of 23 h; effluent colour intensity of 6 100 PCU).

PROPOSED KINETIC MODEL

Figure 4.21 indicate the results that were obtained when the model was applied to described the kinetic of decolourisation by *R. pusillus*. The n and k values were 2.6 and 1.5595×10^{-6} respectively. The n value of 2.6 does not indicate the chemical mechanisms involved since the model was developed empirically. The model describing the decolourisation kinetics obtained with *C. versicolor* is indicated in Fig. 4. 22. The n and k parameters were 2.7 and 1.3865×10^{-6}, respectively.

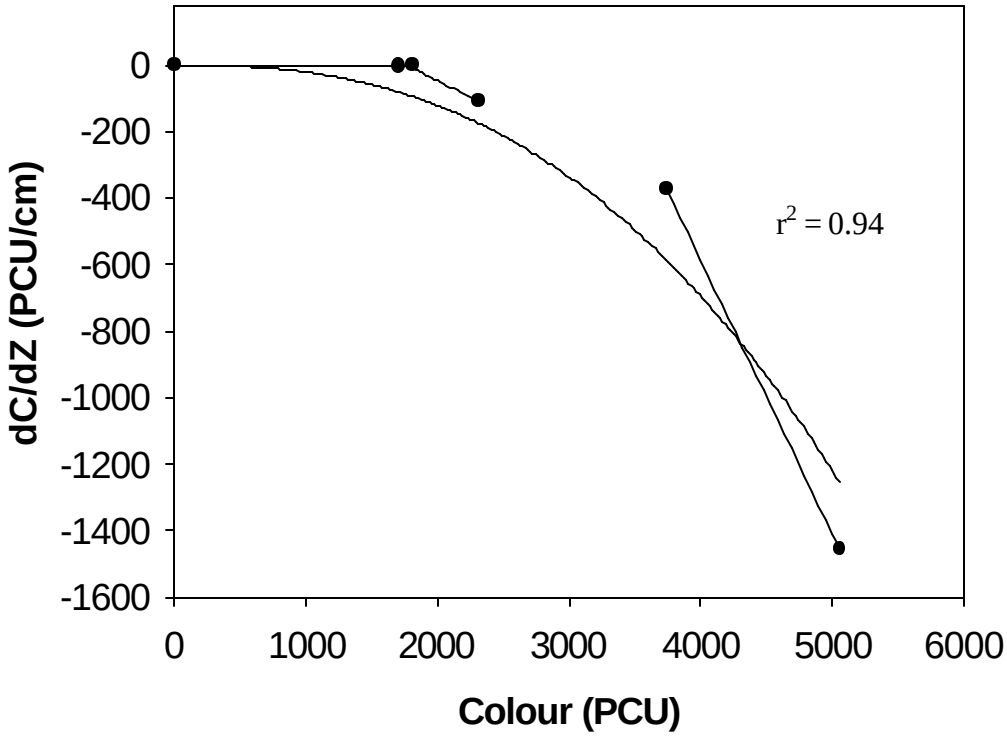


FIG. 4.21: Modelling of decolourisation kinetics of *Rhizomucor pusillus*

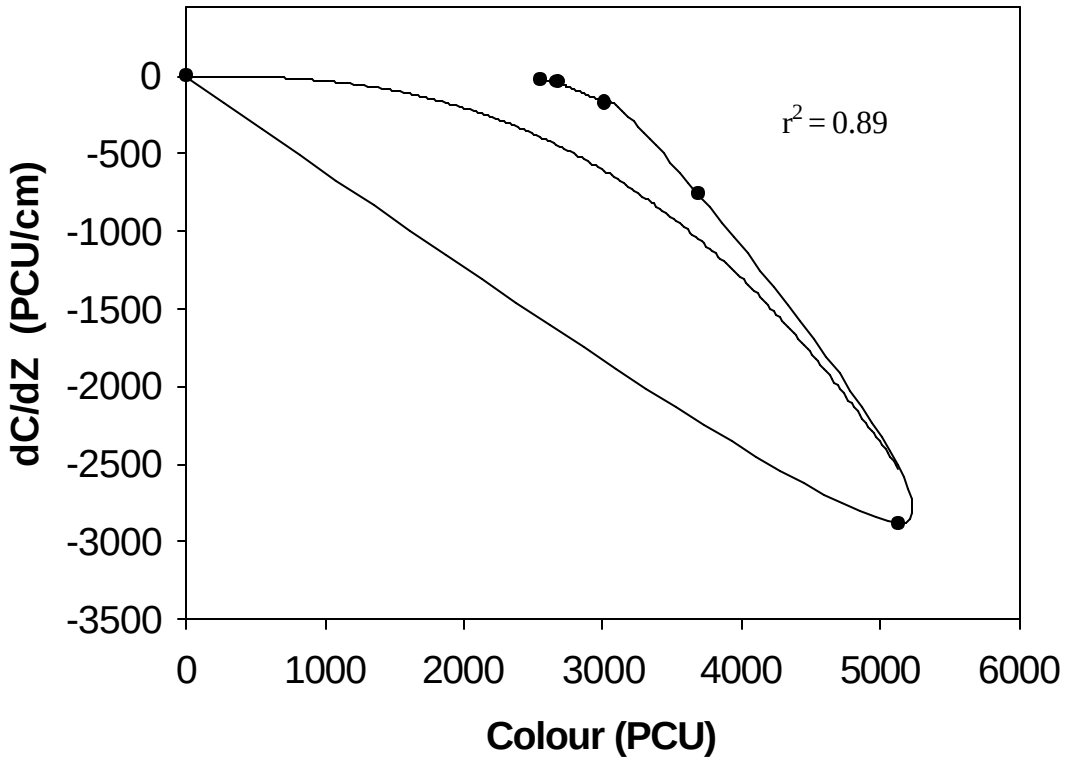


FIG. 4.22: Model describing the decolourisation kinetics of *Coriolus vericolor*

EFFECTS OF FUNGAL TREATMENT ON EFFLUENT QUALITY

In Table 4.4 the percentage removal of COD, colour, AOX and toxicity from effluent following fungal treatment is summarised. Overall, *R. pusillus* was more efficient in effluent bioremediation as compared to *C. versicolor*.

Table 4.4: Bioremediation of bleach plant effluent using *Rhizomucor pusillus* and *Coriolus versicolor* in RBC reactor

Fungi	Reduction over control (%)[a,b]					
	AOX	**COD**	**Colour**	**2,4-DCP[c]**	**Toxicity**	**pH**
Rhizomucor pusillus	55	71	71	16	26	3.8
Coriolus versicolor	40	59	53	40	-5	3.5

[a] Control: AOX, 6.9 mg/l; COD, 19 052 mg/l; Colour, 6 100 PCU; Toxicity, -43%; 2,4-DCP, 1.5 mg/l; pH 3.5.

[b] Samples were withdrawn from the RBC reservoir after 72 h of treatment and AOX, COD, colour and toxicity removal expressed as percentage reduction over control.

[c] 2,4-dichlorophenol

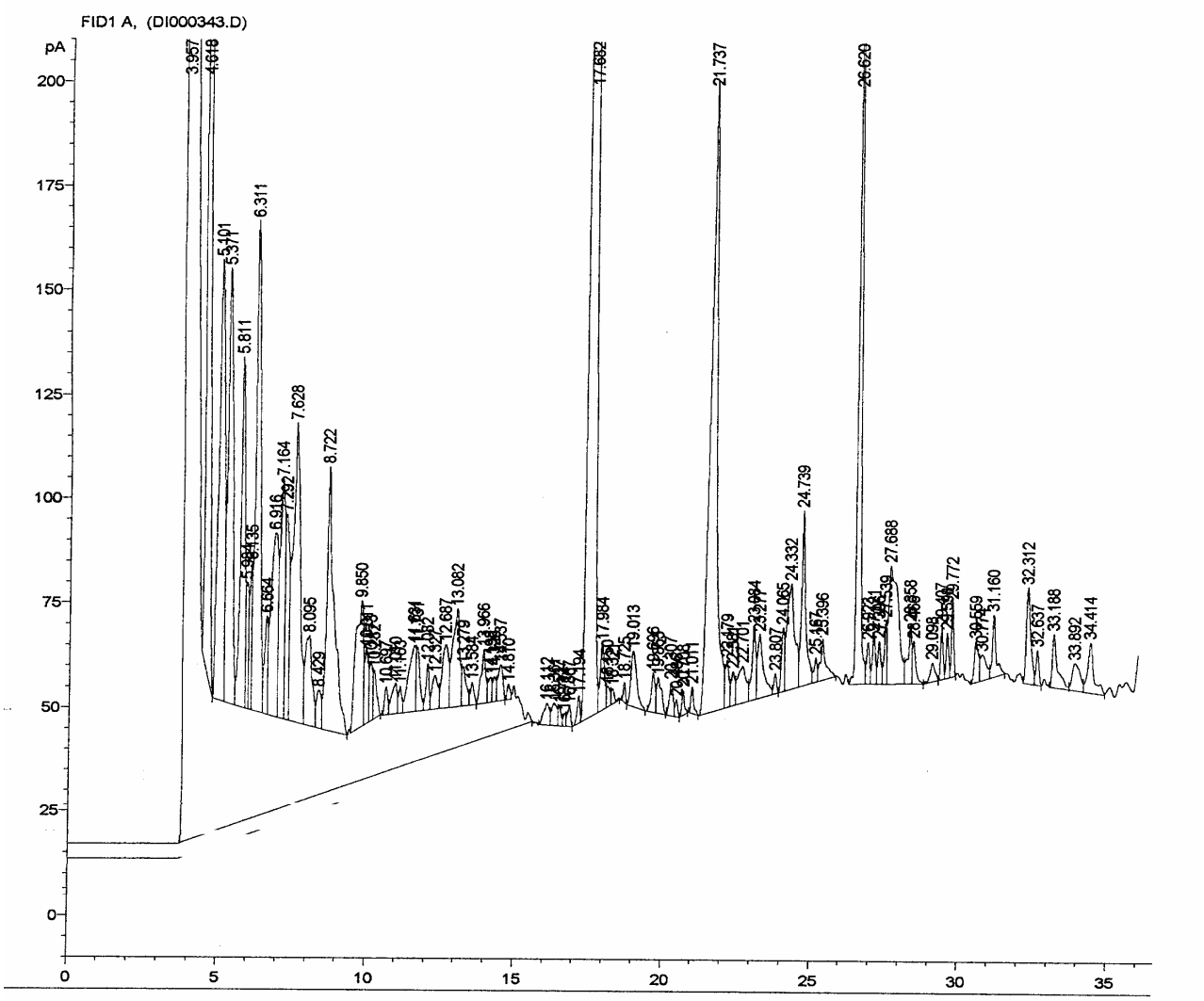
In general, decolourisation levels obtained here compared favourably with results reported by Pallerla and Chambers (1997), where 72% colour reduction was attained using *T. versicolor* at a residence time of 24 h and 1 842 CU. Decolourisation depends, however, on various factors such as the effluent, fungi, physico-chemical conditions employed, etc., thus making direct comparisons difficult (Garg and Modi, 1999). Mehna *et al.* (1995) indicated that their *T. versicolor* strain could decolourise E_1-effluent (18 500 PCU), to a maximum extend of 92 %, with a COD reduction of 69% over a 7 d period at pH of 4.5. The inoculum concentration employed was 5.0 g/l. Even

better results were obtained using a *Rhizopus oryzae* strain (Nagarathnamma and Bajpai, 1999). Interestingly, this fungus which is also a member of the order mucorales, decolourised bleach plant effluent by 92 to 95%, thereby removing 50% COD in 24 h at temperatures of 25 to 45 ^{o}C and at pH of 3 to 5 (Nagarathnamma and Bajpai, 1999). It was demonstrated that *C. versicolor* decolourised bleach plant effluent by 84% with a resultant removal of 39% AOX, during a 9 d incubation period (Bajpai and Bajpai, 1997).

A reduction in toxicity of 80% against *Daphnia magna* were obtained following one passage through a trickling filter containing immobilised *P. chrysosporium* (Bajpai and Bajpai, 1997). Nagarathnamma and Bajpai (1999) indicated that treatment of bleach plant effluent with *R. oryzae* rendered the effluent essentially non-toxic. The decrease in effluent toxicity demonstrated in this work (Table 4.4) correlated well with the AOX removal: *R. pusillus* removed 55% AOX and also showed the greatest improvement in toxicity reduction (–43% before treatment to +26% after treatment). Similarly, *C. versicolor* caused a 40% reduction in AOX accompanied by a 38% reduction in toxicity (–43% before treatment to a –5% after treatment). It was demonstrated that chloroorganic removal from effluent was accompanied by toxicity reduction (Nagarathnamma and Bajpai, 1999).

CHLOROPHENOL DETERMINATION OF TREATED DECOLOURISATION MEDIUM

Chromatograms of material extracted with dichloromethane before (Fig. 4.23) and after treatment with *C. versicolor* (Fig. 4.24) and *R. pusillus* (Fig. 4.25) are presented in Figs. 4.23 to 4.25. Figures 4.24 and 4.25 shows new peaks and peaks that became prominent following treatment by *C. versicolor* and *R. pusillus*. The presence of these peaks could indicate that the fungi produced new compounds during treatment. There was also a decrease in some of the area of peaks, showing that compounds were degraded during treatment. 2,4-dichlorophenol was one of the compounds identified that was degraded, during fungal treatment in the RBC.



Time (min)

FIG. 4.23: Chromatogram of compounds extracted from decolourisation medium before fungal treatment in RBC (analysis by liquid gas chroma tography)

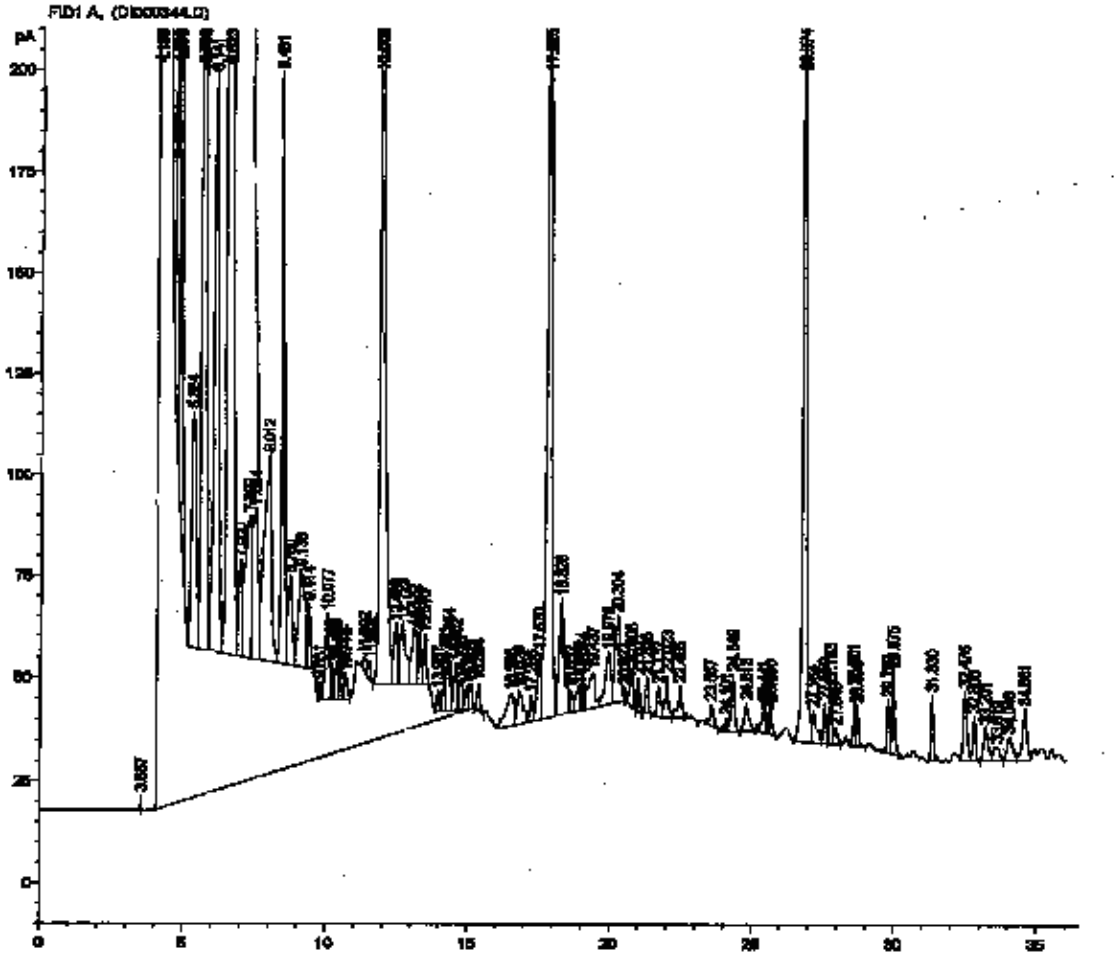
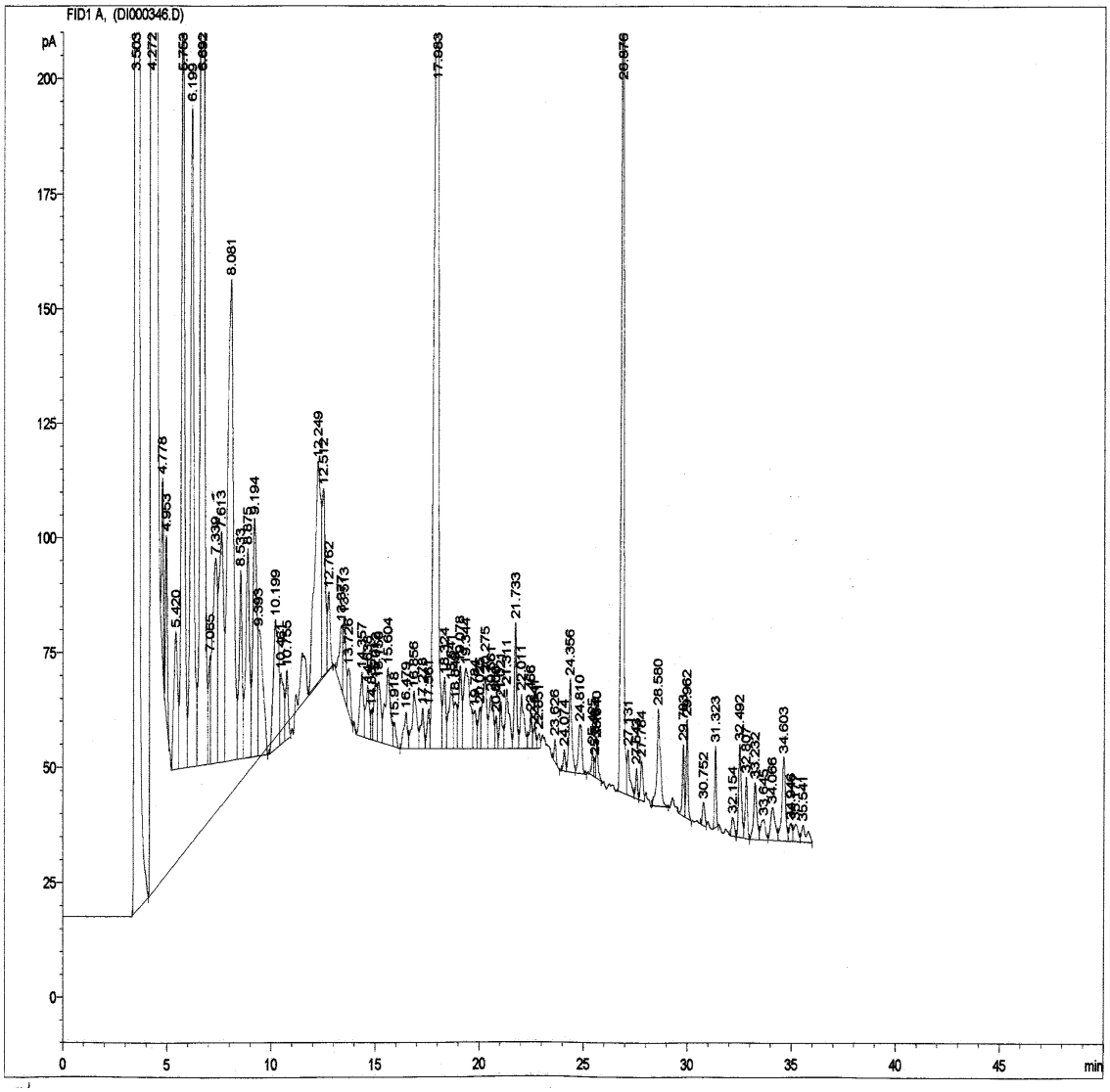


FIG. 4.24: Chromatogram of compounds extracted from decolourisation medium following treatment by *Coriolus versicolor* immobilised in RBC. (analysis by liquid gas chromatography)



Time (min)

FIG. 4.25: Chromatograph depicting compounds extracted from decolourisation medium following treatment by *Rhizomucor pusillus* immobilised in RBC.

ESTIMATION OF COLOUR ADSORBED

Colour adsorbed on biomass was estimated after decolourisation by alkaline desorption of chromophoric material from cell mass with 1N NaOH. Table 4.5 compares the abilities of both fungi to decolourise effluent as result of colour biodegradation and/or bioadsorption. The colour removed from the effluent during treatment with *R. pusillus* could be completely recovered from the fungal biomass by alkali desorption. However, only 45% of the colour removed by *C. versicolor* was released upon treatment of the biomass with NaOH.

Table 4.5: Mechanisms involved in colour removal from bleach plant effluent by *Rhizomucor pusillus* and *Coriolus versicolor* in RBC reactor

Fungi	Total effluent decolourisation (%)	Adsorption (%)
Rhizomucor pusillus	63	100
Coriolus versicolor	58	45

[a] The percentage of colour adsorption by fungi was calculated assuming complete release of colour from fungal biomass following treatment with 1 N NaOH (see Materials and Methods for details).

Adsorption of chromophoric compounds from bleach plant effluent onto fungal biomass has been determined using alkaline treatment of cell mass following decolourisation experiments (Christov *et al*. 1999; Jaspers and Penninckx 1996; Marton *et al*. 1969). It was demonstrated that 95% of colour adsorbed was released from *P*. *chrysosporium* after

alkaline treatment (Jaspers and Penninckx, 1996). Results obtained here indicated that all colour adsorbed by *R. pusillus* during effluent decolourisation was released from the fungal biomass following treatment with 1 N NaOH (Table 4.5). Therefore, the decolourisation mode of *R. pusillus* appeared to be due to physical adsorption.

Treatment of fungal biomass with decolourisation medium in shake flasks using short contact times (5 min) revealed that not all the colour (28%) could be recovered from *C. versicolor* by alkali extraction. Therefore the biomass of *C. versicolor* retained colour in contrast to the biomass of *R. pusillus* from which coloured compounds could be extracted stoichiometrically by alkali treatment. Increasing the concentration of alkali (10 N) used for colour extraction did result in the removal of this fraction from the biomass of *C. versicolor*. Also an increase in treatment time (72 h) did not lead to an increase in colour release from *C. versicolor*. The short contact times used between biomass and the decolourisation medium during shake flask experiment would preclude biodegradation as a mechanism of colour removal. Therefore, in the case of *C. versicolor* alkali treatment could not be used as a reliable means to quantify the fate of colour removal from the medium during cultivation.

GEL PERMEATION ANALYSIS

Gel permeation chromatographs of effluent before and after treatment in RBC are shown in Figs. 4.26 to 4.28. Treatment by *C. versicolor* resulted in a change of the apparent molecular mass distribution profile indicating that high molecular mass compounds were degraded (Fig. 4.27).

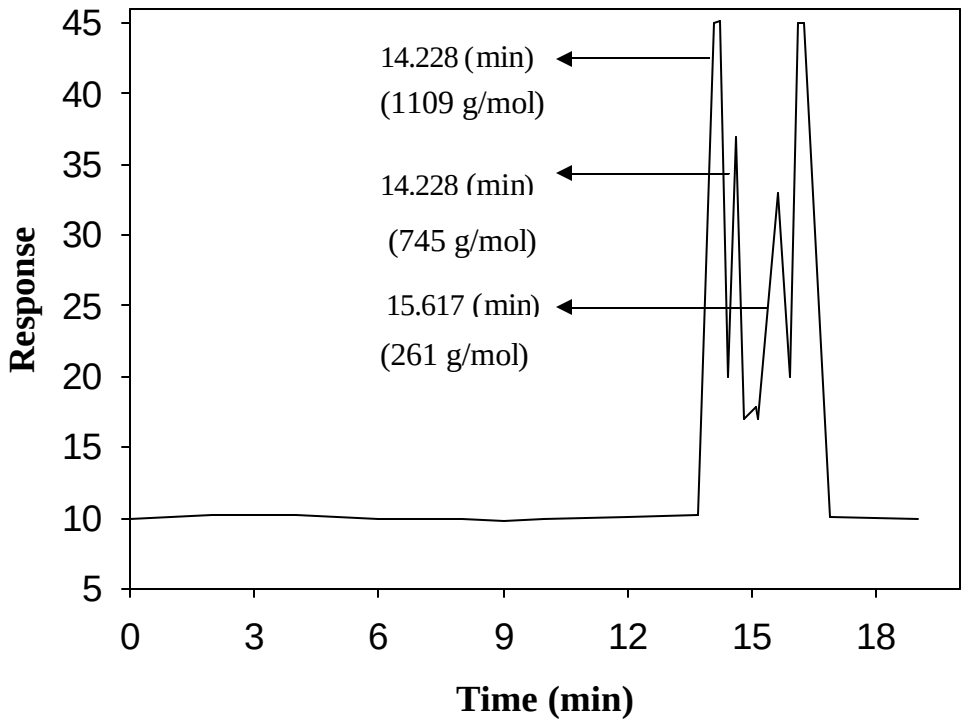


FIG. 4.26: Gel permeation chromatogram of decolourisation medium before fungal treatment in RBC (Retention times of peaks were used to estimate apparent molecular mass).

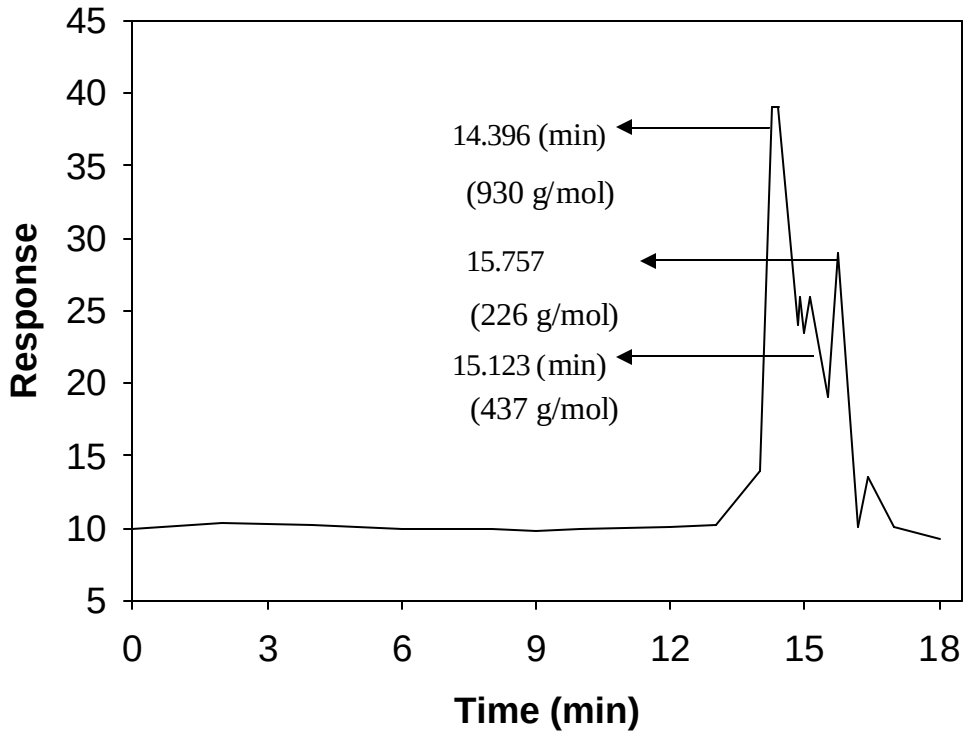


FIG. 4.27: Gel permeation chromatogram of decolourisation meduim following treatment of decolourisation medium by *Coriolus versicolor* in RBC. (Retention times of peaks were used to estimate apparent molecular mass)

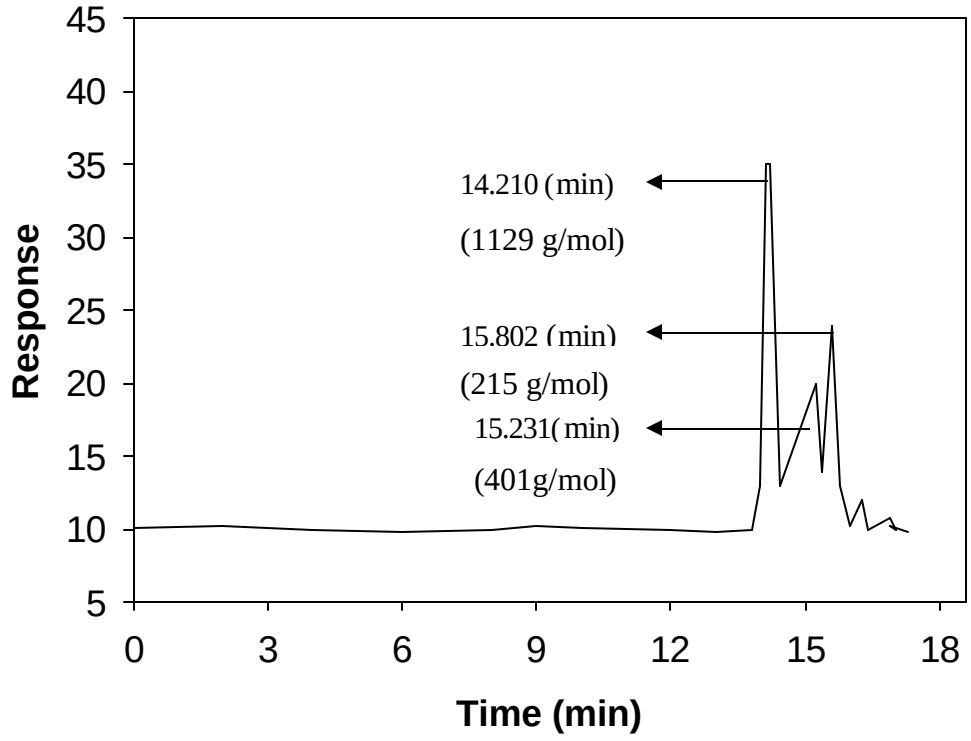


FIG. 4.28: Gel permeation chromatogram obtained following treatment of decolourisation medium in RBC by *Rhizomucor pusillus*. (Retention times of peaks were used to estimate apparent molecular mass).

In contrast to the GPC results obtained with *C. versicolor,* molecular mass profiles obtained with *R. pusillus* for the high molecular weight peaks (> 1 000 g/mol) were similar before and after treatment except that peak areas were reduced. This could indicate that the mechanism of decolourisation in this case precluded degradation and conversion of high molecular mass chloroorganic compounds to lower mass compounds. This provided evidence that the mechanism of colour removal by *C. versicolor* proceeded

by biodegradation as well as adsorption, whereas colour reduction by *R. pusillus* can be ascribed mainly to adsorption.

LIGNINOLYTIC ENZYME ACTIVITIES RECORDED DURING TREATMENT IN RBC

No ligninolytic enzyme activities could be found following treatment of decolourisation medium by *R. pusillus* in the RBC. *Coriolus versicolor* produced both laccase and manganese dependent peroxidase activities, but no lignin peroxidase activity was detected during cultivation in the RBC (Fig. 4.29).

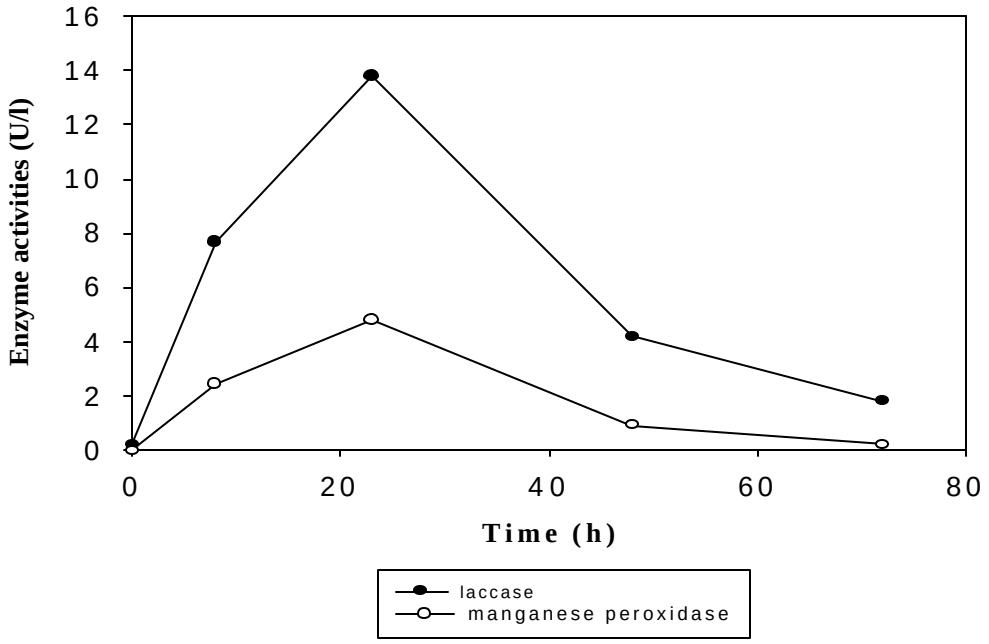


FIG. 4.29: Time profiles of laccase and manganese peroxidase activities obtained during cultivation of *Coriolus versicolor* in RBC (Enzyme activities were recorded in samples taken from the outflow of the reactor).

Ligninolytic enzymes are associated with lignin and lignin derivative depolymerisation in white-rot fungi (Nagarathnamma and Bajpai, 1999). The presence of these enzymes in *C. vericolor* treated effluent suggests that enzymatic action could be responsible for part of colour removal. Laccase and manganese peroxidase activities decreased after 23 h of treatment. Enzyme activities were also measured over time in samples taken from the reservoir, these activities were much lower than that detected at the reactor out flow (data not shown). During shake flask cultivation of *C. versicolor* it was noted that enzyme activities were suppressed in the presence of Eo-effluent. Ligninolytic enzyme activities were much higher when decolourisation medium without effluent were used (data not shown). Various strengths of effluent were added to shake flask cultures of *C. versicolor* pre-grown in decolourisation medium without effluent. This was done to determine the effect of effluent strength on ligninolytic enzyme activities during cultivation in shake flasks. The control contained only decolourisation medium, without effluent. The results of these experiments are shown in Fig. 4.30.

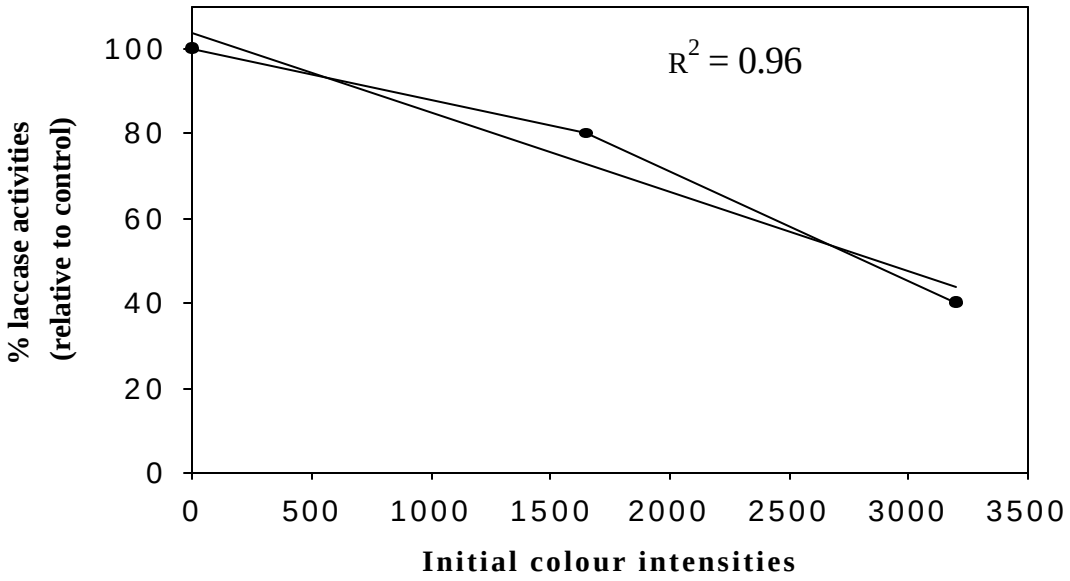


FIG. 4.30: Laccase activity as a function of colour intensities. (Enzyme activities determined after 20 d of treatment in shake flasks)

The pH of the effluents added was adjusted to that of the shake flasks just before addition so that cultivations were conducted at the same pH level (3.3). After pH adjustments effluents were filter sterilised to maintain aseptic conditions. During these experiment only laccase activities were detected. According to the results shown in Fig. 4.30 there was an inverse relationship between laccase activity and the initial colour intensities. At a colour intensity of 3 200 PCU only 40% of the laccase activity remained as compared to the control. These data indicate that the presence of effluent had a significant effect on laccase activities.

Biomass obtained after treatment of decolourisation medium for 23 h by *C. versicolor* in the RBC (when maximum decolourisation of 63% was attained) was treated with NaCl to extract enzyme activities associated with the biomass. Results are presented in Table 4.6.

Table 4.6: Ligninolytic enzymes associated with biomass of *C. versicolor* in RBC

Procedures used	Enzyme activities (U/l)	
	laccase	**MnP**
Wash[a]	0.8	0.3
Extracted	0.3	1.9

[a] Biomass was washed with distilled water before extraction and enzyme activities determined in the wash water

Results indicate that some of the manganese peroxidase (MnP) activity was cell-associated in contrast to laccase activities. The highest enzyme activities were present in the tube containing the immobilised fungus, therefore decolourisation activity was mainly restricted to the tube.

LONG-TERM DECOLOURISATION STUDIES

The results of long-term decolourisation experiments are shown in Fig. 4.31. Best decolourisation observed during these investigations were plotted over time. *Coriolus versicolor* exhibited an average decolourisation activity of 55% during a 34 d period. Thereafter the decolourisation level dropped to 39%. At day 30 it was observed that green conidiospores accumulated in the reservoir. Cultures isolated from the reservoir showed that these conidiospores were from a fungus tentatively identified as belonging to the genus *Penicillium*. *Rhizomucor pusillus* decolourised the effluent containing medium by a average of 65% during a 54 d period. However, at day 56 biomass loss from the reactor became evident and this process became so extensive that operation of the RBC was terminated.

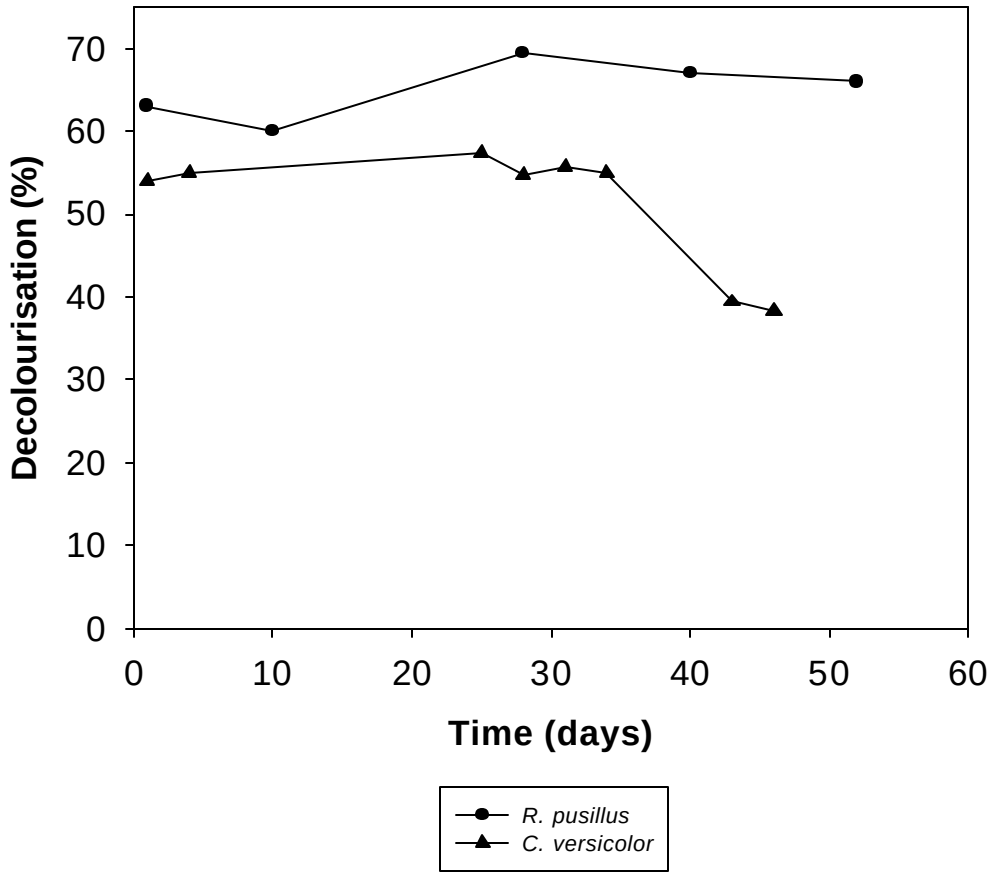


Fig. 4.31: Decolourisation time profiles of *Rizomucor pusillus* and *Coriolus versicolor* in RBC.

TREATMENT OF EFFLUENT ORIGINATING FROM ROTATING BIOLOGICAL CONTACTOR IN ACTIVATED SLUDGE REACTOR

Effluent from the RBC was treated in the AS system to further improve the quality of the wastewater. RBC treated wastewater had to be diluted before treatment in the AS could be attempted. This was necessary to obtain the correct loading conditions for the AS system to function optimally. The COD at the onset of the experiment was 921 mg/l for the *R. pusillus* treated effluent and 1116 mg/l for the *C. versicolor* treated wastewater. Feed influent pH was adjusted to 7.3. Chemical analysis of the effluents obtained after treated in the RBC was used to estimate the nutrients that were required for subsequent treatment in the AS. Results, showing effluent quality improvements after treatment in the RBC was followed by treatment in the AS, are presented in Table 4.7.

Table 4.7: Improvements in effluent quality following treatment of RBC wastewater in AS

	Removal (%)	
	Fungus used to pretreat effluent in RBC	
Parameter	***C. versicolor***	***R. pusillus***
AOX	9	9
COD	93	84
2,4-dichlorophenol	79	41

COD was almost completely removed when *C. versicolor* treated wastewater from the RBC was used as influent to the AS reactor therefore most of the recalcitrant compounds were removed during RBC treatment. However, only modest levels of AOX were removed in the AS under these conditions. This might indicate that AOX reduction is concentration dependent, with lower levels being removed when low concentration are

present during treatment. Chlorophenol reduction in the AS was higher in effluent pre-treated by *C. versicolor* in the RBC as compared to *R. pusillus* pre-treated wastewater. Media originating from the RBC showed no toxic effect (as defined by the test, see material and methods) when tested by the BGIT method, before or after treatment in the AS. However, a decrease in the stimulation of bacterial growth was recorded when influent samples were compared to effluent samples taken from the AS following treatment. This phenomenon could be due to a decrease of nutrient quality of effluent following treatment in the AS, since bacterial growth response are measured in minimal medium (data not shown).

TREATMENT OF EFFLUENT FROM ACTIVATED SLUDGE REACTOR IN ROTATING BIOLOGICAL CONTACTOR

Effluent from the AS was used as an influent to the RBC. Prior to treatment in the RBC the AS treated effluent was concentrated by evaporation at 50ºC, to increase the colour intensity to a final value of 5 700 PCU. Effluent was supplemented with nutrients, the pH adjusted to 3.5 and heat sterilised before treatment in the RBC commenced. The levels of nutrients added were estimated following chemical analysis of the AS effluent processed as described in the text above. Table 4.8 shows improvements in effluent quality following treatment of AS pre-treated wastewater in the RBC, using *C. versicolor* and *R. pusillus* respectively.

Table 4.8: Improvements in effluent quality following treatment of wastewater originating from AS in RBC

	Removal (%)	
	Fungus employed during RBC treatment	
Parameter	***C. versicolor***	***R. pusillus***
AOX	60	49
COD	78	79
colour	72	85
2,4-dichlorophenol	ND[a]	ND

[a] Not detected

Coriolus versicolor removed the most AOX from AS pre-treated effluent, however COD reduction by both fungi, in general, was similar. Colour removal was improved by a margin of about 11% when compared to values obtained without AS pre-treated effluent being used during RBC treatment. Overall, pre-treatment of effluent in AS, followed by RBC treatment, resulted in better effluent quality improvements being obtained than when RBC treatment was conducted without pre-treatment. As was noted in the section above, media originating from the AS showed no toxic effect (as defined by the test, see material and methods) when tested by the BGIT, before or after treatment in the RBC. However, bacterial growth was less stimulated following treatment, indicating a possible decrease in nutrient quality following treatment in the RBC (data not shown).

5. CONCLUSIONS

In general, biological treatment methods significant improved bleach plant effluent quality. Treatment in an activated sludge reactor reduced COD, chlorophenol levels, and left the effluent non-toxic, however, colour was not significantly reduced. This can be ascribed to the high molecular nature of coloured compounds in the effluent that cannot pass through the cell membrane of microorganisms.

Coriolus versicolor exhibited the highest decolouring activity (61%) during experiments using trickling filters. This fungus required a co-substrate to efficiently decolourise the effluent. Based on the trickling filter results *C. versicolor* was selected for studies using the RBC system.

In a RBC system, colour, chloropenol and COD levels were reduced. Furthermore, the effluent was rendered non-toxic following treatment. It seems that adsorption could be one of the first steps of decolourisation. *C. versicolor* required a carbon source to decolourise bleach plant effluent effectively, in contrast *R. pusillus* did not. Colour intensity levels did not affect decolourisation significantly except at the lowest intensity values (1 550 PCU) tested. This could indicate that reduced colour intensities resulted in

lower initial adsorption and thus the interaction between chromophoric material and mycelium could have been diminished. Therefore the biodegradation probably was not effectively initialised. Initial colour levels were directly proportional to the specific decolourisation rate expressed as change in colour/g.d in both fungi. The decrease in effluent toxicity demonstrated in this work correlated well with the AOX removal: *R. pusillus* removed the highest AOX levels and also showed the greatest improvement in toxicity reduction. Decolourisation by *R. pusillus* appeared to be due to physical adsorption. However, with *C. versicolor*, only part of the colour removed could be ascribed to adsorption, suggesting the presence of two mechanisms of colour removal in this fungus: biodegradation and bioadsorption. Ligninolytic enzymes were detected in the *C. versicolor* treated effluent containing medium, however, no ligninolytic enzymes activities were found in effluent treated by *R. pusillus*. It could be demonstrated that the presence of bleach plant effluent inhibited laccase activities. Some of the manganese peroxidase activity produced during treatment in the RBC was cell-associated. The highest enzyme activities were present in the tube that contained the immobilised fungus, therefore decolourisation activity was mainly restricted to the reactor tube. It has been demonstrated in the present work that using a laboratory RBC reactor both *R. pusillus* and *C. versicolor* were able to decolourise and detoxify the effluent within three days of continuous treatment and could in principle be used for bioremediation of industrial wastewaters. Decolourisation activities was sustained up to 34 d during long-term decolourisation studies when *C. versicolor* was used whereas with *R. pusillus* up to 54 d of colour removal was possible. Therefore, long-term decolourisation were feasible in the RBC.

Further improvements in effluent quality were possible when effluent from AS was treated in the RBC or visa versa. Almost all the COD was removed when *C. versicolor* pre-treated effluent was used in the AS. Chlorophenol levels were also significantly reduced. However, only modest improvements in AOX reductions were observed. When effluent from the AS was used as an influent to the RBC, AOX, colour and COD were significantly reduced when compared to levels found during RBC treatment conducted without AS pre-treatment.

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CHAPTER V

GENERAL CONCLUSIONS

1.1. Bleach plant effluents comprise complex solutes containing high colour intensities and COD contents and with compounds of various molecular mass also being present. Nitrogen, phosphate and sugar concentrations found in these effluents in general are low or non-existent. Therefore, mainly, these nutrients had to be supplemented before effective biological treatment could be implemented.

1.2. Commercial adsorbents such as activated carbon proved highly effective in decolourisation of the effluent. Unconventional adsorbents such as biomass of *R. pusillus* compared well with other physico-chemical adsorbents and could be regenerated using alkali. In contract, only partial regeneration was possible using the commercial physico-chemical adsorbents, suggesting chemisorption of coloured compounds onto the sorbent material occurred during treatment. Effluent decolourisation was significantly higher at lower pH levels for all the adsorbents tested, except in the case of activated carbon. This might in part be due to the contribution made by acid catalysis during nucleophilic addition reactions, during which amino groups of chitin/chitosan react with carbonyl compounds present in Eo-effluent. Isotherm data, obtained at an effluent pH of 7.3, indicated that activated carbon was the best adsorbent of colour followed by chitosan, chitin and lastly *R. pusillus* biomass.

1.3. Cell wall fractions extracted from the biomass of *R. pusillus* (alkali-resistant, residual and chitosan fractions) appeared to be principally responsible for the decolouring of Eo-effluent by this fungus. The residual and chitosan fractions were able to remove more colour from the bleach plant effluent (41%) than the intact biomass (39%).

The major compound(s) of the cell wall fractions could be either chitosan and/or chitin, based on the release of glucosamine upon acid hydrolysis of these fractions. However, minor components also occur in some of the cell wall hydrolysates. Attempts to identify these compounds were unsuccessful and therefore their significance in the adsorption process could not be determined. Furthermore, adsorption in the presence of different ionic strengths indicated that non-ionic interactions prevail during adsorption using *R. pusillus*. In contrast, both chitosan and to a lesser extent chitin, exhibited coloumbic interactions during adsorption of effluent colour. Thus, the adsorption attributes of *R. pusillus* biomass cannot be ascribed solely to the existence of chitin/chitosan in the cell wall because of the complexity and heterogeneity of the wall structure. A complex mechanism of colour removal involving chemical and hydrophobic interactions between the effluent chromophores and cell wall material of *R. pusillus* might exist.

1.4. Flocculation of coloured materials using chitosan dissolved in acetic acid was possible. A lower charge of chitosan was required as coagulant in order to obtain decolourisation activities comparable to that observed when chitosan was used as adsorbent of colour from bleach plant effluent.

1.5. Generally, biological treatment methods significant improved bleach plant effluent quality. Treatment in an activated sludge reactor reduced COD, chlorophenol levels, and left the effluent non-toxic, however, colour was not significantly reduced. This can be ascribed to the high molecular mass of coloured compounds in the effluent. Bacteria do not have extracellular enzyme systems to degrade these high molecular mass compounds and therefore cannot decolourise the effluent.

1.6. In a RBC system, colour, chloropenol and COD levels were reduced. Furthermore, the effluent was rendered non-toxic following treatment. It seems that adsorption could be one of the first steps of decolourisation. *Coriolus versicolor* required additional nutrients to decolourise bleach plant effluent effectively, in contrast *R. pusillus* did not. Decolourisation was decreased when low colour intensity

(1 550 PCU) effluent was used during treatment. This could indicate that reduced colour intensities resulted in lower adsorption initially and thus the interaction between chromophoric material and mycelium could have been be diminished. Therefore, the biodegradation probably was not initialised effectively. Initial colour levels were directly proportional to the decolourisation rate in both fungi. The decrease in effluent toxicity (BGIT) demonstrated in this work correlated well with the AOX removal: *R. pusillus* removed the highest AOX levels and also showed the greatest improvement in toxicity reduction. Decolourisation by *R. pusillus* appeared to be due to physical adsorption. However, with *C. versicolor*, only part of the colour removed could be ascribed to adsorption, suggesting the presence of two mechanisms of colour removal in this fungus: biodegradation and bioadsorption.

1.7. Ligninolytic enzymes were detected in the *C. versicolor* treated effluent containing medium, however, no ligninolytic enzymes activities were found in effluent treated by *R. pusillus* It could be demonstrated that the presence of bleach plant effluent inhibited laccase activities. Some of the manganese peroxidase activity was cell associated. The highest enzyme activities were present in the tube containing the immobilised fungus, therefore decolourisation activity was mainly restricted to the tube.

1.8. It has been demonstrated in the present work that using a laboratory RBC reactor both *R. pusillus* and *C. versicolor* were able to decolourise and detoxify the effluent within three days of continuous treatment and could in principle be used for bioremediation of industrial wastewaters. Long-term treatments were also viable since *C. versicolor* could decolourise the effluent by an average of 55% over a 34 d period and *R. pusillus* decolourised the effluent by an average of 65% during 54 d of treatment.

1.9. An empirically derived model described the kinetics of decolourisation by both fungi reasonably well. Statistical evaluation indicated that the r^2 values were 0.89 in

the case of *C. versicolor* and 0.94 for the curve fitted to the experimental results obtained with *R. pusillus*.

1.10. Further improvements in effluent quality were possible when effluent from AS was treated in the RBC or visa versa. This might be attributable to toxicity reduction gained during the pre-treatment steps. Almost all the COD was removed when *C. versicolor* pre-treated effluent was used in the AS. Chlorophenol levels were also significantly reduced. However, only modest improvements in AOX reductions were observed. When effluent from the AS was used as an influent to the RBC, AOX, colour and COD were significantly reduced to levels superior to RBC treatment conducted without an AS pre-treatment step.

CHAPTER VI

SUMMARY

Keywords: Chloroorganics, bleach effluent, bioremediation, adsorption, decolourisation, chitosan, chitin, white-rot fungi, biodegradation mechanisms

Bleach plant effluent was characterised by physico-chemical methods. The chemistry of the bleach plant effluent was examined to devise effective treatment methods. Effluent contained trace amounts of nitrogen as well as carbohydrates and no ortho phosphate could be detected in the wastewater.

The best decolourisation activities were obtained using adsorption as treatment method, with activated carbon removing > 99% colour from effluent. Chitosan (81%) and chitin (77%) could remove appreciable levels of colour from bleach plant effluent, followed by biomass from *Rhizomucor pusillus*, a mucoraelean fungus (71%). Chitosan and chitin from the cell wall of *R. pusillus* might be involved in the fungus decolourisation ability. Effluent pH was inversely related to effluent decolourisation when *R. pusillus*, chitosan or chitin was used as adsorbents. This might in part be due to acid catalysis during nucleophilic addition reactions, where amino groups of chitin/chitosan react with carbonyl groups in Eo-effluent. Also, chitin and chitosan amino groups can be protonated under acidic conditions and acquire positive charges that can interact with the chromophores found in Eo-effluent. However, pH exerted no significant effect on decolourisation when activated carbon was employed as adsorbent of effluent colour. Decolourisation employing commercial adsorbents seemed to be mainly due to chemisorption. Adsorption experiments conducted at various ionic strengths indicated that coulombic interactions are responsible for a fraction of the decolourisation activity of chitosan and chitin. Nevertheless, decolourisation obtained with RM7 and activated carbon was unaffected by the ionic strength. Flocculation of coloured compounds from Eo-effluent by chitosan containing solutions resulted in a maximum decolourisation of

75%. Anion-exchange treatment removed 96% colour from Eo-effluent. Ultraviolet irradiation could decolourise the Eo-effluent by about 42 to 43%. Decolourisation using organic solvent extraction proved ineffective with a highest colour removal efficiency of only 21% being achieved.

Biological methods used for effluent remediation were: 1) Trickling filters, 2) Activated sludge reactors and 3) Rotating biological contactor reactors (RBC). Treatment using one biological system was followed by treatment in another system

With trickling filters containing immobilised white-rot fungi, the highest decolourisation (61%) was obtained with *Coriolus versicolor*. This fungus required a co-substrate to efficiently decolourise the effluent.

Effluent treatment in an activated sludge reactor reduced toxicity, COD and chlorophenol levels. However, colour and high molecular mass compounds were not affected significantly by this method of treatment.

Decolourisation was studied in a RBC using immobilised *C. versicolor* and *R. pusillus*, respectively. The decolourisation rate by both fungi was proportional to initial colour intensities. Decolourisation was not adversely affected by colour intensity, except at the lowest level tested. Decolourisation of 53 to 74% could be attained using a hydraulic retention time of 23 h. *Rhizomucor pusillus*, removed 55% of AOX compared to a 40% AOX reduction by *C. versicolor*. Treatment employing *R. pusillus* and *C. versicolor*, respectively, rendered the effluent essentially non-toxic. Addition of nutrients to the decolourisation media stimulated colour removal by *C. versicolor*, but not significantly in the case of *R. pusillus*. Ligninolytic enzymes (manganese peroxidase and laccase) were only detected in effluent treated by *C. versicolor*. Decolourisation mechanisms were investigated using gel permeation chromatography. *Rhizomucor pusillus* decolourised the effluent by adsorption and *C. versicolor* removed effluent colour by adsorption as well as by biodegradation. *Coriolus versicolor* could decolourise the effluent for a period of 34 d whereas *R. pusillus* decolourised the effluent up to 54 d. Further improvements in effluent

quality could be attained when treatment using one system was followed by treatment in another system, possibly because of toxicity reduction in the pre-treatment steps.

CHAPTER VII

OPSOMMING

Sleutelwoorde: Chloroorganiese verbindings, bleik-effluent, bioremediëring, ontkleuring, chitosan, chitin, witvrot-fungi, biodegraderings meganismes

Fisiese en chemiese metodes was toegepas om bleik-effluent vanaf ? sulfiet pulp meul te karakteriseer. Die chemiese aard van die effluent moes bepaal word om effektiewe behandelings metodes te kon saamstel. Lae vlakke van stiksof en koolhidraat was aanwesig in die effluent. Orto-fosfaat was afwesig.

Naastenby al die effluent kleur was verwyder gedurende adsorpsie eksperimente uitgevoer met geaktiveerde koolstof. Chitosan en chitin kon ook die effluent kleur tot ? beduidende mate verwyder. *Rhizomucor pusillus* biomassa was die volgende beste adsorbeermiddel van kleur na chitosan en chitin. Chitosan/chitin kan moontlik hoof komponent(e) wees van die selwand van *R. pusillus* en daarom ? belangrike bydrae lewer tot die kleur adsorpsie vermoë van die fungus. Ontkleuring deur chitosan, chitin en *R. pusillus* was omgekeerd eweredig aan die effluent pH. Dit kan gedeeltelik toegeskryf word aan suurkatalise gedurende nukleofiliese addisie reaksies waartydens aminogroepe van chitin en chitosan reageer met karbonielgroepe teenwoordig in die effluent. Verder, by lae pH vlakke kan die aminogroepe van chitosan en chitin positiewe ladings verkry en reageer met kleurgroepe in die effluent. Die pH het egter geen wesenlike effek getoon op die onkleuring veroorsaak deur geaktiveerde koolstof nie. Adsorpsie eksperimente uitgevoer in die teenwoordigheid van verskillende ioniese-sterktes het aangetoon dat ? gedeelte van die ontkleuring deur chitosan en chitin deur ioniese interaksies veroorsaak word. Hierteenoor, het die ioniese sterkte geen invloed getoon op die ontkleuring verkry met of geaktiveerde koolstof of *R. pusillus* nie. Flokkulasie van gekleurde verbindings in Eo-effluent het gelei tot ? maksimum ontkleuring van 75%. Anioon-uitruilings behandeling was effektief en 96% van die kleur kon daarmee verwyder word. Ultraviolet

bestraling het 42 tot 43% ontkleuring veroorsaak. Ontkleuring deur ekstraksie van effluent met organiese oplosmiddels was oneffektief en die grootste ontkleuring verkry was slegs 21%.

Biologiese behandelings is uitgevoer in: 1) biologiese filters (BF), 2) geaktiveerde slyk (AS) en 3) roterende biologiese kontaktor (RBK) reaktors. Behandeling in een reaktor is ook in sekere gevalle gevolg deur behandeling in ? ander reaktor.

Behandeling in BF met *Coriolus versicolor* het maksimale ontkleuring (61%) veroorsaak. Die fungus het ? ko-substraat nodig gehad vir effektiewe ontkleuring .

Behandeling in AS het tot verlaagde toksisiteits, COD en chlorofenool vlakke gelei. Tog kon die effluent kleur en die vlakke van hoë molekulêre massa verbindings nie noemenswaardig verlaag word nie.

Ontkleuring was ondersoek in die RBK met geimmobiliseerde *C. versicolor* en *Rhizomucor pusillus*, onderskeidelik. Kleur verwyderings tempo's met beide fungi was eweredig aan die inisiële kleur intensiteite gebruik. Vlakke van ontkleuring was laer wanneer effluent met kleur intensiteite < 2000 PCU behandel was. Ontkleurings van tussen 61 tot 74% was moontlik by ? hidroliese retensie tyd van 23 uur. *Rhizomucor pusillus* het 55% van die AOX verwyder in vergelyking met die 40% verwyder deur *C. versicolor*. Fungale behandeling met beide organismes, onderskeidelik het die effluent nie-toksies gelaat. Byvoeging van voedingstowwe tot die ontkleurings media van *C. versicolor* het ontkleuring gestimuleer, maar ontkleuring deur *R. pusillus is* nie daardeur beinvloed nie. Ligninolitiese ensiem aktiwiteite (mangaan peroksidase en lakasse) was slegs aanwesig in effluent behandel met *C. versicolor.* Ontkleuring meganismes is ondersoek deur gel permease chromatografie. Ontkleuring veroorsaak deur *R. pusillus* kon toegeskryf word aan adsorbsie, in teenstelling, *C. versicolor* het effluent kleur verwyder deur adsorpsie sowel as biodegradering. *Coriolus versicolor* kon die effluent ontkleur vir ? periode van 34 dae terwyl *R. pusillus* ontkleuring van die effluent vir 54 dae kon volhou. Verdere verbeterings in effluent kwaliteit kon verkry

word indien een behandeling gevolg is deur ? ander biologies behandeling, moontlik as gevolg van toksisiteits verlaging teweeg gebring in die eerste behangdelings stap.