DECOLOURIZATION OF TEXTILE DYES AND TEXTILE INDUSTRY EFFLUENT IN A FIXED BED BIOFILM REACTOR USING NATIVE MICROORGANISMS

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Degree of Doctor of Philosophy

Department of Chemical and Process Engineering

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DECLARATION OF THE CANDIDATE AND SUPERVISOR

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Abstract

Textile and apparel industry produces huge quantities of wastewater with unfixed dyes, which generate colour and toxicity in discharged water, creating environmental pollution. Physical and chemical effluent decolourization techniques are widely used at present to remove colour in effluents in textile industries, however, they have several drawbacks and therefore not productive. Compared to physical and chemical methods, biological treatments have gained much attention globally as environmental-friendly and cost-effective techniques to decolourize textile industry effluent. Hence, in this work, decolourization potential of textile dyes by microbial strains, which were isolated from local-environment, and their applicability in industrial wastewater decolourization were investigated.

Five bacterial strains, with dye decolourizing potential were isolated from an effluent treatment facility of a local textile industry and identified using 16S rRNA gene sequencing analysis. Ability of these strains to decolourize selected textile dyes as individual strains and in a bacterial consortium was investigated using free bacterial cells cultured in 250 ml Erlenmeyer flasks containing 100 ml of decolourization media. Out of the isolated bacteria, *Proteus mirabilis* showed the highest capability to decolourize all dyes and was able to decolourize 50 ppm dye solutions of Yellow EXF, Red EXF, Blue EXF, Black WNN and Rhodamine under static conditions at 35 °C. Colour removal of 96, 94, 83, 95 and 30% respectively was observed after 72 h of treatment when decolourization media was inoculated with 2% (v/v) of bacterial culture. The developed bacterial consortium composed of *Proteus mirabilis*, *Morganella morganii* and *Enterobacter cloacae*, decolourized more than 90% of all four reactive dyes and 36% of Rhodamine dye after 72 h of incubation. Furthermore, the developed bacterial consortium was able to decolourize more than 83% of the synthetic dye mixture and 60% of the textile industry effluent, respectively after 46 h and 138 h of incubation at 35 ^{o}C temperature under static condition.

Effects of physico-chemical parameters (pH, temperature, concentration of dye, agitation and sources of carbon) for biological decolourization of dyes were studied in batch cultures with free cells. It was observed that dye decolourization was more effective under oxygen-limited, static conditions than shaking conditions and the maximum decolourization of dyes was observed at 40 ^{o}C and pH 7-8 in the media containing yeast extract as the carbon source.

Dye decolourization was further investigated in a fixed bed biofilm reactor where the biofilm was composed with the developed bacterial consortium. Decolourization of the synthetic dye mixture was done with three different concentrations of yeast extract in the feed and more than 90% decolourization of the synthetic dye mixture was observed when the concentration was 2 and 1 g/l in batch operation of the reactor. However, even when the concentration was reduced to 0.25 g/l, 75% decolourization of synthetic dye mixture was achieved in both batch and continuous operation of the reactor. Results showed that dye decolourization was more effective with attached cells (bacterial consortium) in the reactor than with free cells (used in flasks). Stability of the dense microbial communities in biofilms and their ability to survive and degrade dyes at extreme conditions could be the reason for observed high colour removals in the decolourization studies conducted in the reactor. Structural changes occurred in dyes due to biological treatments were studied using ultraviolet-visible spectral and high-performance liquid chromatography analyses. Metabolites formed due to biological degradation were analyzed using gas chromatography-mass spectrophotometry and found to be non-toxic and benign.

A maximum of 45% colour removal was observed when the diluted textile effluent was treated in the fixed bed biofilm reactor operated in continuous mode whereas 70% colour removal was achieved in 48 h with undiluted textile wastewater treated in batch mode. This shows the ability of the developed bacterial consortium to endure in highly complex and toxic environment in the fixed bed biofilm reactor and the potential application in textile industry wastewater treatment.

Key words: biological, decolourization, dyes, fixed bed biofilm reactor, textile effluent

DEDICATION

Dedicated to my parents and husband for their unconditional love, endless support and encouragement.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF FIGURES IN APPENDICES

LIST OF ABBREVIATIONS

AEBR	Anaerobic expanded bed reactor
AQDS	Anthraquinone-2, 6-disulfonate
AQS	Anthraquinone-2-sulfonate
BLAST	Basic local alignment search tool
BOD	Biochemical oxygen demand
CEA	Central environmental authority
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPL	Environmental protection license
EPS	Extracellular polymeric substances
EtBr	Ethidium bromide
FAD	Flavin adenide dinucleotide
FADH	Reduced form of flavin adenide dinucleotide
FBBR	Fixed bed biofilm reactor
FBR	Fluidized bed reactor
FMN	Flavin adenide mononucleotide
FTIR	Fourier-transform infrared spectroscopy
GCMS	Gas chromatography–mass spectroscopy
GOTS	Global organic textile standards
HDPS	High density polystyrene
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
IV	Intravenous tubing
LB	Luria–Bertani
MBBR	Moving bed biofilm reactor
MRSL	Manufacturing restricted substances list
MSDS	Material safety data sheets

MTBE	Methyl tertiary butyl ether
MTP	Microtiter plate
NADH	Reduced form of nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NCBI	National center for biotechnology information
NIST	National institute of standards and technology
OD	Optical density
PCR	Polymerase chain reaction
PE	Polyethylene
PMMA	Poly (methyl methacrylate)
PP	Polypropylene
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PVC	Polyvinyl chloride
RBC	Rotating biological contactors
rRNA	Ribosomal ribonucleic acid
SAMBR	Submerged anaerobic membrane bioreactor
SBR	Sequencing batch reactor
SEM	Scanning electron microscope
TAE	Tris-acetate-EDTA
UASB	Up-flow anaerobic sludge blanket
US EPA	United States environmental protection agency
UV	Ultraviolet
ZDHC	Zero discharge of hazardous chemicals

CHAPTER 1

1 INTRODUCTION

1.1 Background to the Research Problem

Polluted water bodies are a major global concern because, polluted water creates adverse health effects on human and aquatic life and disturbs the ecological balance of nature. Water pollution is growing in an accelerated phase due to urbanization and industrialization in the modern world.

Industries all over the world produce huge quantities of wastewater composed of various types of pollutants. Out of these industries, textile dyeing and finishing industry is considered as the second largest polluter of clean water globally [1] and contaminate water bodies with large varieties of chemicals, such as surfactants, soaps, salts, dyes, softeners, organic solvents, resins, waxes, organic stabilizers and sizing agents [2].

Textile wet processing includes singeing, desizing, kiering, bleaching, mercerizing, and dyeing where the dyeing individually contributes to 15-20% of wastewater generated in the total production flow [1]. Dyeing is the aqueous application of organic dyes on textile substrates to impart color [3] and during the process, part of dyes which remains unfixed to fibres will be washed out, producing coloured effluent. According to the United States' environmental protection agency (US EPA), 50-60% of reactive dyes, 10-20% acid dyes, 30% direct dyes and 5-25% amount of disperse dyes remain unfixed [4].

Even at a lower concentration, dyes are highly visible in water and create problems when released to the aqueous ecosystem without proper treatment. Industrial effluents containing synthetic dyes reduce light penetration in rivers and consequently affect the photosynthetic activities of aquatic flora, severely affecting the food sources of aquatic organisms. Dyes are toxic to flora, fauna and humans and degradation of dyes may deplete dissolved oxygen levels in water affecting the survival of aquatic organisms [5] as it increases the biochemical oxygen demand (BOD) in water. Further, depending on the length of exposure and the concentration of dye, aquatic organisms may subject to acute or chronic toxicity.

Therefore, stringent government regulations have been imposed in most countries to avoid such negative consequences generated by the release of textile dyeing effluent to natural water bodies. Enforcement of law ensures textile industries to treat dye-containing effluents up to the discharge standards before releasing to the environment. Hence, to achieve the regulated discharge standards, textile-dyeing companies use different techniques to decolourize textile dyes.

These effluent decolourization techniques can be broadly categorized as physical, chemical and biological treatments. Membrane-filtration (nanofiltration, reverse osmosis, electrodialysis), adsorption and irradiation are some examples of physical treatment techniques. Coagulation, flocculation, chemical oxidation (ozone or H_2O_2), advanced oxidation processes and electrochemical processes (electrokinetic coagulation, electro-oxidation) are some chemical treatment techniques used for effluent decolourization [5, 6, 7]. In biological treatment, living and non-living microorganisms and their enzymes [5, 6] are used for colour removal.

Even though chemical and physical dye decolourization techniques are widely applied in textile industries, these methods have several drawbacks such as high cost (for chemicals, electricity, ultra-violet irradiation), difficulties associated with dewatering and disposing of generated sludge, and ineffective decolourization of some types of dyes [5, 8]. Conversely, biological dye decolourization techniques are identified as environmentally friendly and economical methods that produce less sludge compared to physical and chemical treatments [9].

Various categories of microorganisms such as filamentous fungi [10, 11], yeasts [12], bacteria [13, 14] and algae [15] have exhibited potential in decolourization of synthetic dyes. Most of these biological dye decolourization studies have been conducted for model dyes with known structures such as Methyl red [16], Indigo and Congo red [17] and Malachite green [13]. Application of such dyes in current industrial dyeing processes is limited, and recently developed dyes with enhanced properties are mostly used instead. Biological decolourization of these dyes and analysis of their degraded compounds are rarely reported. The main reason for this may be the unavailability of dye structures due to trade secrets.

Dye decolourization conducted in different types of reactors such as moving bed biofilm reactors (MBBR) [18, 19, 20], up-flow anaerobic sludge blanket reactors

(UASB) [21, 22], rotating biological contactors (RBC) [23, 24] and packed bed biofilm reactors are reported in literature. Efficiency of dye decolourization varies depending on the type of reactor, operating conditions of the reactor, concentration of dyes and the microbial species utilized.

Textile and apparel industry plays a huge role in the Sri Lankan industrial sector accounting for a large share of export earnings in the country. In the first quarter of the year 2019, textile and apparel industry earned US$ 2,761.4 million which accounts for 58% of total industrial export earnings in Sri Lanka [25]. Despite the economic importance of textile industry to the country, a very low number of local research studies are reported on biological decolourization of textile industry effluents. Hence, this study mainly focuses on decolourization of modern textile dye effluent by indigenous microbial strains.

1.2 Problem Statement

Textile dye decolourization using different microorganisms in lab scale and pilot scale reactors are reported in literature and most of these bioreactors had been seeded with anaerobic granular/activated sludge where the microorganisms responsible for dye decolourization were unknown. The limited numbers of reported work of dye decolourization with isolated microorganisms (in packed bed reactors) are difficult to implement in industrial scale due to limitations in methods used (requirement of high dosages of external carbon sources and instability of biofilm support material etc.)

Hence, a comprehensive study is needed to investigate the dye decolourization potential of isolated microorganisms supported on a suitable material in a reactor which can be upgraded to industrial scale.

1.3 Research Objectives

The overall objective of this research is to isolate indigenous microorganisms with textile dye decolourization potential and to investigate their applicability in textile wastewater decolourization.

Specific objectives

1. Isolate indigenous microbial strains for efficient dye decolorization.
2. Determine optimal conditions required for maximum decolorization of selected dyes using isolates.
3. Investigate decolourization of each selected dye and dye mixture using a mixed culture of microorganisms.
4. Employ the developed bacterial consortium to decolourize dye mixture and textile industry effluent in a lab scale reactor.

CHAPTER 2

2 LITERATURE REVIEW

In this chapter, a comprehensive review on textile dyes, hazards generated by improper discharge of dye containing effluent, chemical, physical and biological textile effluent decolourization methods are reported. Further, dye decolourization in different bioreactors are reviewed.

2.1 Textile Industry

Textile industry is one of the major industries in the world, which satisfies the basic human requirement of textile products. Throughout the centuries, this industry has been gradually developing and is currently employed with modern technologies to achieve high efficiency in the production processes.

Textile manufacturing process consists of a large number of production stages where the process is initiated with the manufacturing of fibres, which could originate either from natural materials (cotton, wool, etc.) or synthetic materials **(**polyester, nylon, etc.) [26]. Prepared fibres will then undergo spinning process, which will convert fibres in to yarn. Then, the yarn will be converted in to fabrics using different techniques such as weaving and knitting. In order to enhance the strength of yarn and to reduce the breakage during processing, sizing chemicals such as starch and polyvinyl alcohol (PVA) will be added. Then desizing process will be carried out to remove sizing chemicals from the yarns prior to fabric preparation. Scouring is a process step which will be used to remove impurities from fibres. During this step, alkali solution will be used mainly to breakdown waxes, natural oils, surfactants and fats in fibers and to suspend impurities in the scouring bath. To eliminate unwanted colour from fibres, bleaching chemicals such as sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) will be used. Finally, dyeing process will be carried out to provide required colours to fibres or fabrics [27].

2.2 Textile Dyes

Dyes are coloured substances that absorb light in the visible range of the spectrum at a certain wavelength. The major structural element responsible for light absorption in dye molecules is the chromophore group. In addition, auxochromes such as $-NH_3$, -COOH, HSO_3 and –OH will enhance the colour of dyes [5]. Annually, more than one million tons of dyes are produced globally, of which 50% are textile dyes [6]. Dyes can be mainly classified into two categories depending on the application and dye structure.

Based on the application/ usage, dyes can be classified as reactive, acid, direct, basic, mordant, disperse, sulphur and vat [7]. Dyes with their most compatible fiber types are indicated in Table 2.1.

Table 2.1: Classification of dyes according to usage [28]

Dye class	Major substrates
Acid	nylon, wool, silk, paper, inks and leather
Azoic dyes	cotton, rayon, cellulose acetate and polyester
Basic	acrylic, modified nylon, polyester paper and inks
Direct	cotton, rayon, paper, leather and nylon
Disperse	polyester, polyamide, cellulose acetate, acrylic and plastics
Reactive	cotton, rayon, wool, silk and nylon
Sulfur	cotton and rayon
Vat	cotton, rayon and wool

Considering the chemical structure, the most widely used dye categories in the industry are azo, anthraquinone, indigoid, xanthene, arylmethane and phthalocyanine derivatives. According to the estimations, azo dyes represent about 70% by weight of the dyes produced in the world [27]. Dyes that are representing azo category, should at least contain a single azo (-N=N-) bond in their structure (Figure 2.1).

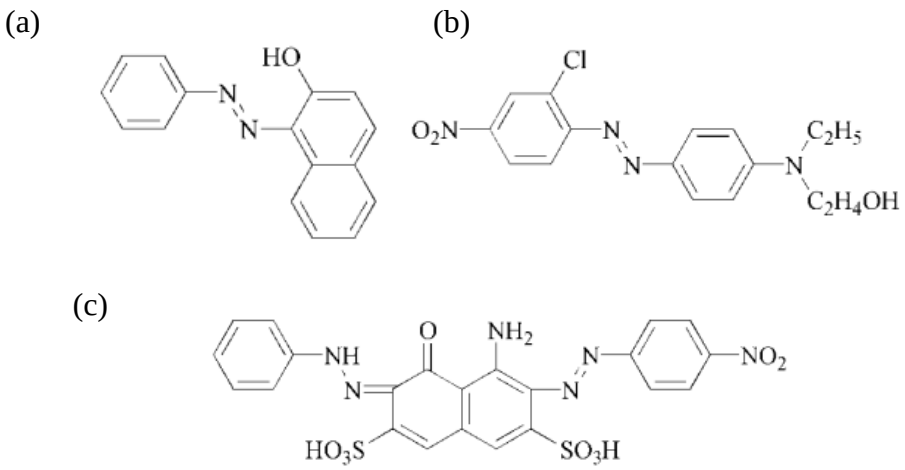


Figure 2.1: Examples for azo dyes [29] (a) C.I. Solvent Yellow 14 (b) C.I. Disperse Red 13 and (c) C.I. Acid Black 1

Anthraquinone dyes are the second most important group of dyes and are based on 9,10-anthraquinone structure (Figure 2.2).

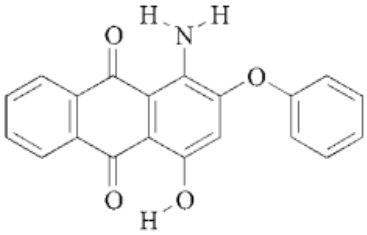


Figure 2.2:C.I. Disperse Red 60 dye with anthraquinone structure [29]

Indigoid dyes are one of the oldest groups of organic dyes and indigo dye is the most important and widely used dye in this category (Figure 2.3).

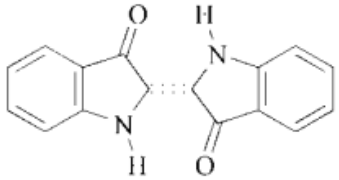


Figure 2.3: Indigo dye with indigoid structure [29]

Xanthene dyes contain xanthylium or dibenzo-γ -pryan nucleus (xanthene) as the chromophore of the dye structure. Due to rigid chromophoric nucleus, xanthenes are often fluorescent [30]. An example for xanthene dye is shown in Figure 2.4.

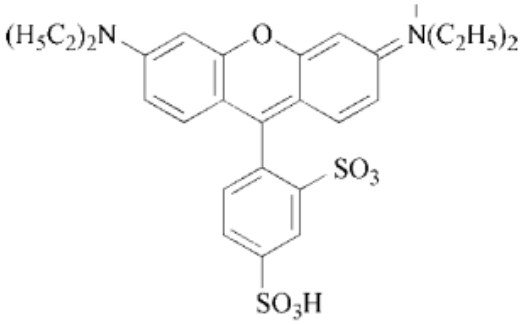


Figure 2.4: Xanthene dye C.I. Acid Red 52 [29]

Chromophoric system of triarylmethane dyes are consist of a central carbon atom joined to three aromatic rings [31] as shown in Figure 2.5.

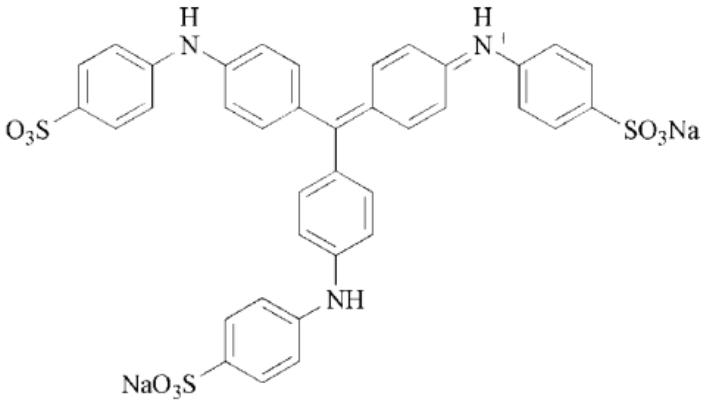


Figure 2.5: Triarylmethane dye C.I. Acid Blue 93 [29]

2.3 Environmental Hazards and Health Issues Caused by Textile Dyes

2.3.1 Impact on aquatic environment

The presence of even trace quantities of dyes reduces the transparency and quality of water bodies. Dyes in water bodies may absorb or reflect sunlight which will lower the amount of sunlight obtained by the water hence affect the photosynthetic activities of aquatic vegetation and algae [32]. A study conducted for toxicity evaluation of Optilan yellow, Drimarene blue and Lanasyn brown dyes using a green alga *Chlorella*

vulgaris have revealed a significant reduction of algal pigment, chlorophyll, with the increase of dye concentration in water. Further, the reduction of specific growth rate, increase in generation time of algae was observed with increasing concentrations of the three dyes [33].

Different types of bioassays can be used to evaluate the toxic effect of textile dyes and effluents on aquatic organisms whereas conventional acute toxicity tests with fish and *Daphnia* are the most common. An acute toxicity study conducted using *Daphnia magna* as an aquatic experimental animal model has indicated minor acute toxicity in both dyes and in five textile and textile dyeing effluents (out of six effluents) used for the study [34]. Toxicity of textile dyes (reactive, vat and direct types) on zebrafish (Danio rerio) embryos has been studied and induction of malformations during embryonic and larval development of zebrafish have been observed [35]. Fish bioassay on textile effluents and their selected constituents conducted to evaluate mortality and erythrocyte disorders on a freshwater fish *Gambusia affinis* is reported [36]. According the study textile effluents were toxic to fish and cause several internal and external injuries such as dyes deposition over the external (gills) and internal organs (lateral line and digestive system) and extensive mucous secretion.

2.3.2 Impact on vegetation

Generally water effluents produced in textile dyeing industries are released to natural water bodies with or without proper treatment. After entering the natural water bodies these dye containing effluents may be introduced to irrigation systems so ultimately utilized in agricultural activities. Some textile dyes have been proven to have toxic effect on agricultural crops [37]. Large number of phytotoxicity analyses conducted on evaluating the toxicity effect of textile dyes and dye containing effluents on various types of plants are reported in literature.

Phytotoxicity analysis on *Allium sativum L., Vicia faba L.* and *Lactuca sativa L.*, conducted with wastewater obtained from the region Fez-Boulmane, Morocco has shown different levels of phytoxicity on selected three plant types [38]. In another study conducted on phytotoxicity of textile effluent collected from industrial cluster in Tamil Nadu, South India, using two agriculturally important plants, *Vigna*

unguiculata (Cow pea) and *Cicer arietinum* (Bengal gram) showed that the untreated textile effluent has negative effect on seed germination and plant growth, however, plants were unhealthy and died after 15 days of growth due to toxicity of dyes [39].

2.3.3 Impact on human health

Effect of textile dyes on human health can be identified under three categories as dermatological effect, toxicological effect and respiratory effect [40]. Dermatological effects such as allergies, urticaria and/or dermatitis can cause when dyes migrate and penetrate the skin [41, 42, 43]. Employees in dye manufacturing and processing plants are in direct contact with dyes and hence they can be considered as the highest risk group. Studies conducted on textile dyeing industry workers suggest the potential to develop acute and chronic respiratory diseases in workers due to the exposure to dyes [44, 45]. Further, research revealed that some textile dyes can migrate from fabrics to the human skin during perspiration as clothes are directly in contact with human body [40] and hence all the wearers of such textiles are at risk.

Some of the textile dyes are known carcinogens and mutagens hence have ability to change DNA structure of cells and to cause diseases such as cancer [40, 46, 47]. Studies conducted to evaluate genotoxicity or mutagenicity of textile dyes are reported in literature. Ames *Salmonella* reversion assay conducted for 12 randomly selected textile dyes showed that 11 dyes were positively mutagenic [48]. Ames test conducted using five "his" *Salmonella typhimurium* strains to evaluate the mutagenic effect of eight textile dyes has revealed that almost all dyes tested were mutagenic to all the strains [49]. Textile effluent obtained from Fez-Boulmane region in Morocco has been used for genotoxicity evaluation and reported that it showed cytotoxic and genotoxic effects on *S. cerevisiae* D7 strain [38].

Considering the aforementioned negative effects, utilization, handling and disposing of textile dyes should be done with extreme care to mitigate possible threat on humans and the environment.

To control the usage and discharge of toxic dyes by textile industries, environmental legislations have been adopted by countries all over the world. The European Union has banned the usage of azo dyes which produce 22 amines listed in the legislation, in

a concentration higher than 30 ppm, by the directive 2002/61/EC, and reformulated by the directive 2004/21/CE [50, 28]. To comply with the legislations, textile dyeing and finishing industries obtain written confirmations from dye suppliers to assure the purchased dyes are free of banned aromatic compounds. Global organic textile standards (GOTS) approval and zero discharge of hazardous chemicals (ZDHC) manufacturing restricted substances list (MRSL) conformance certificates are examples for documents which are provided by dye manufacturers to assure the quality of their dyes.

2.4 Effluent Decolourization Techniques

2.4.1 Chemical and physical treatments

Discharge of dye-containing effluents into the environment may result in several environmental pollution problems in addition to health concerns. Therefore, dye-containing textile effluents should be properly treated (decolourize) until they reach the regulatory discharge limits.

To meet the standard discharge limits, textile dyeing and finishing companies are using different decolourization techniques to eliminate the unwanted colour from effluents. These dye decolourization techniques can be mainly classified in to three categories as physical (membrane-filtration, sorption techniques, etc.) chemical (coagulation or flocculation, electro flotation, electrokinetic coagulation, conventional oxidation methods, irradiation, etc.) and biological methods (using microorganisms or their enzymes) [5]. Disadvantages of widely used chemical and physical dye decolourization methods in industry are given in Table 2.2.

Membrane filtration is considered as an important dye decolourization technique due to its positive features such as resistance to temperature and removal of all types of dyes [51]. However, the drawbacks such as residue disposal problems, high capital cost and the possibility of clogging limits its application in the industry [52].

Table 2.2: Chemical and physical dye decolourization methods widely used in industry

Treatment method	Disadvantages
Adsorption (Physical)	High cost Requirement of regeneration of adsorbent after each cycle Low-cost adsorbents, shows less effectiveness in dye removal and therefore requires long retention times
Membrane filtration (Physical)	Residue disposal problems High capital cost Possibility of membrane clogging
Coagulation and flocculation (Chemical)	Not efficient with effluents that contain acid, direct, reactive and vat dyes Generation of large quantities of secondary sludge and difficulties in disposing the generated sludge
Chemical oxidation with ozone (Chemical)	Short half-life of oxygen Ineffectiveness in removing water insoluble disperse dyes High cost
Fenton method (Chemical)	Generation of excess sludge which require disposal

Although coagulation and flocculation technique has shown efficient colour removal in certain dyes, generation of large quantities of sludge has been identified as the main drawback of this method [51]. Oxidative processes such as the usage of Fenton reagent and ozonation are reported as the most commonly used methods of effluent decolourization by chemical means. However, Fenton method is incorporated with issues in excess sludge generation where further disposal of sludge is required. Ozonation method is highly expensive due to the short half-life of ozone typically being 20 min, which results in increased cost of wastewater treatment process [51].

Compared to chemical and physical methods, biological techniques are considered as an attractive solution for textile effluent decolourization due to its reputation as a low-cost, sustainable and publicly acceptable technology [5].

2.4.2 Biological treatments

Microorganisms such as bacteria, filamentous fungi, algae and yeast or their enzymes have indicated potential in the decolourization of textile dyes (Tables 2.3 and 2.4). Decolourization of textile dyes using these living organisms or substances produced by them is termed as biological dye decolourization. Biological decolourization of dyes can be resulted either from biosorption or biodegradation or both. In biosorption, the original structure of the dye particle may not be destroyed but it will be entrapped into the living or dead microbial cells [5]. The potential of a microorganism for biosorption depends on the heteropolysaccharide and lipid components of the cell wall, which contain different functional groups, including amino, carboxyl, hydroxyl, phosphate and other charged groups, resulting in strong attractive forces between the azo dye and the cell wall [9]. Other than that, the functional groups present in the dye molecule also affect the biosorption. Hydroxyl, nitro and azo groups present in the dye molecule have the potential to enhance the biosorption whereas sulfonic acid groups result in the reduction of biosorption [53].

When compared to biosorption, biodegradation can be considered as a more environmental friendly technique as it mineralizes toxic dye compounds into non-toxic substances such as CO_2 and H_2O. Biodegradation of textile dyes has been reported under aerobic, anaerobic or facultative anaerobic conditions by different microbial species. Dye degradation mechanisms can be mainly identified as oxidative or reductive degradation [9].

Bacterial dye degradation under aerobic conditions due to mono and di-oxygenase enzymes is quite difficult, especially when dyes contain nitro and sulfonic groups. However, some bacterial strains such as *Pseudomonas* species have been reported to degrade textile dyes under aerobic conditions due to aerobic azo reeducates enzymes [27, 54].

Fungal dye degradation is more efficient in aerobic conditions than anaerobic conditions and found to be attributed to the functions of exoenzymes such as peroxidases and phenoloxidases. Lignin and manganese peroxidases degrade textile dyes starting from enzyme oxidation in the presence of H_2O_2 where azo dyes then reduce the enzymes into their original form. Tyrosinases and laccases are the two types

of phenoloxidases which are oxidoreductase enzymes that can catalyze the oxidation of aromatic compounds without the use of cofactors [9, 27]. Fungal dye degradation is mostly reported with wood- rot fungi (*Phanerochaete chrysosporium, Pleurotus ostreatus,* etc.) whereas biosorption of dyes are commonly reported with fungal species other than wood-rot fungi (*Aspergillus niger, Rhizopus stolonifer,* etc.) [55].
Anaerobic or oxygen-limited conditions are reported to be more favorable for the degradation of azo dyes by bacteria. Generally, anaerobic degradation of azo dyes occurs in two steps involving the transfer of four electrons with the help of azoreductase enzymes. During the initial step, hydrazo intermediate is formed by the transfer of two electrons to the azo dye (1). Then this intermediate will accept two more electrons and be reductively cleaved into the respective aromatic amines (2). In both steps, the dye behaves as the final electron acceptor [27]. Due to the breakage of the azo bond, colour will be removed from the solution. Some of the aromatic amines formed by the breakage of azo dyes are reported to be toxic. However, these aromatic amines can be further mineralized into non-toxic products under aerobic or anaerobic biological treatments [56].

$$R_1\text{-}N{=}N\text{-}R_2 + 2e^- + 2H^+ \longrightarrow R_1\text{-}NH\text{-}NH\text{-}R_2 \quad (1)$$

$$R_1\text{-}NH\text{-}NH\text{-}R_2 + 2e^- + 2H^+ \longrightarrow R_1\text{-}NH_2 + R_2\text{-}NH_2 \quad (2)$$

As reported, the transfer of electrons between the extracellular dye and the intracellular reductases are accelerated by the redox mediator, which acts as an electron shuttle during the anaerobic dye degradation. The presence of reducing equivalents such as FADH (reduced form of flavin adenine dinucleotide), NADH (reduced form of nicotinamide adenine dinucleotide) and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) are essential for azoreductases to catalyze this reaction [9]. Quinone-based compounds (riboflavin (vitamin B2), anthraquinone-2,6-disulfonate (AQDS), cyanocobalamin (vitamin B12), anthraquinone-2-sulfonate (AQS), lawsone (2-hydroxy-1,4-naphthoquinone), etc.) and flavin-based compounds (flavin adenide mononucleotide (FMN), flavin adenide dinucleotide (FAD), etc.) have been reported as redox mediators [56].

Some species of algae that have the ability to decolourize textile dyes either by biodegradation or biosorption are reported in literature [6]. Similar to bacterial dye degradation, some algal species have been able to cleave azo dyes reductively via azo reductase enzymes forming aromatic amines. In addition, some algal species have indicated an ability to further degrade the formed aromatic amines resulting in the complete mineralization of dyes [57].

Other than the use of microorganisms, dye decolourization studies conducted using enzymes isolated from microorganisms are also reported in literature. Peroxidase, laccase, polyphenol oxidase, lignin peroxidase, catalase, veratryl alcohol oxidase, NADH–DCIP reductase and azoreductase are some of the enzymes produced by different plant and microbial species which have the ability to decolourize textile dyes [58]. Utilization of isolated enzymes in dye decolourization has some benefits over the usage of whole microbial cells such as the ability to withstand shock loads, no issues in biomass disposal and ease of process control and standardization. When isolated enzymes are used for dye decolourization, they should be delivered in immobilized form to enhance the stability, reusability and localization. However, in industrial effluent decolourization, various chemicals present in effluent may result in the deactivation of these enzymes. Further, high cost of isolation, purification and production of enzymes have constrained the direct usage of enzymes in textile effluent decolourization [5].

Biological dye decolourization techniques have captured the attention of researchers all over the world and have become an emerging research area. However, this area of study was not popular among the local researchers until the recent past. Hence, the literatures on the isolated microbial strains, which have the ability to decolourize textile dyes, have not been reported from Sri Lanka. Recently, Wijetunga et al. [59] reported the decolourization of Acid Red 131, Acid Yellow 79 and Acid Blue 204 dyes using mixed anaerobic granular sludge under anaerobic conditions. Decolourization of Direct Blue 201 by several *Pseudomonas, Bacillus* and *Micrococcus* species isolated from Sri Lankan textile dyeing industrial effluent was recently reported by Ekanayake and Manage [60]. Same authors reported the decolourization of Direct Blue 201 by locally isolated bacteria *Alcaligenes faecalis, Micrococcus luteus* and *Staphylococcus warneri* [61].

Table 2.3: Decolourization of textile dyes using bacteria and algae

Organism	**Dyes**	**% decolourization**	**Comments**	**Reference**
Enterococcus gallinarum	Direct Black 38	53- 63% within 24 h	25, 50, 75, and 100 mg/l dye concentrations in minimal medium	[62]
		71–85% within 24 h	20, 50, 100, 200, and 250 mg/l dye concentrations in minimal medium	
Bacillus cereus	Acid Blue 25	96 % within 6 h	100 mM concentration	[13]
	Malachite Green	96 % within 4 h	55 mM concentration	
	Basic Blue X- GRRL	98 % within 2 h	750 mM concentration	
Paenibacillus polymyxa, *Micrococcus luteus* and *Micrococcus* sp.	Reactive Violet 5R	93% within 38 h	100 mg/l concentration	[63]
Bacillus sp.	Metanil Yellow	100% within 27 h	200 mg/l concentration	[64]
Lysinibacillus sp.		100% within 12 h	200 mg/l concentration	
Kerstersia sp.	Amaranth, Fast Red E, Congo Red and Ponceau S	100% within 24 h	100 mg/l concentration	[65]
	Orange II	84% within 24 h		
	Acid Orange 12	73% within 24 h		
	Acid Red 151	44% within 24 h		
Oscillatoria curviceps	Acid Black 1	84% within 8 days	100 mg/l concentration, marine cyanobacteria	[66]

Chlorella vulgaris, *Lyngbya lagerlerimi*, *Nostoc lincki*, *Oscillatoria rubescens*, *Elkatothrix viridis* and *Volvox aureus*	Methyl Red, Orange II, G-Red (FN-3G), Basic Cationic and Basic Fuchsin	~4 to 95% within 7 days (individual dye decolourization with individual microorganisms)	Some green algae and cyanobacteria	[57]
Moraxella osloensis	Mordant Black 17	87 % within 48 h	100 mg/l concentration, shake condition	[67]
		92 % within 48 h	100 mg/l concentration, stationary condition	
Micrococcus glutamicus	Reactive Green 19A	100% within 42 h	50 mg/l concentration	[68]
Galactomyces geotrichum and *Bacillus* sp.	Brilliant Blue G	100% within 5 h	50 mg/l concentration, stationary condition	[69]

Table 2.4: Decolourization of textile dyes using fungi and yeast

Organism	**Dyes**	**% decolourization**	**Comments**	**Reference**
Saccharomyces cerevisiae	Methyl Red	100 % within 16 min	100 mg /l dye concentration	[12]
Aspergillus alliaceus	Indigo	98.6% within 9 days	150 mg/l dye concentration	[17]
	Congo Red	98% within 9 days	150 mg/l dye concentration	
Phanerochaete chrysosporium	Reactive Yellow MERL	~98% within 11 days	10 mg/l dye concentration, wood rot fungi	[70]
	Reactive Red ME4BL			
Phanerochaete chrysosporium	Amido Black 10B	98 % within 72 h	white-rot fungi	[10]
Candida albicans	Direct Violet 51	73.2% in 96 h non-autoclaved conditions	100 mg/l dye concentration decolourized by yeast cells by biosorption and biodegradation	[71]
		87.26% in 96 h autoclaved conditions		
Thermomucor indicae-seudaticae	Azure B, Congo Red, Trypan Blue and Remazol Brilliant Blue R	90.42%	100 mg/l concentration (dye mixture)	[11]
		67.99%	1000 mg/l concentration (dye mixture)	

As a country that gains a significant amount of socio-economic benefits through the textile industry, it was required to investigate further on this sustainable method of effluent decolourization. Hence, during this study, investigations were conducted to evaluate the potential of locally isolated bacterial species to decolourize textile dyes and effluents.

2.5 Reactor Studies

2.5.1 Decolourization of textile dyes in reactors

Even though dye decolourization ability of isolated microorganisms are frequently reported in the literature, the majority of those studies have been limited to batch processes having volumes less than 150 ml. Only a few researchers have taken attempts on decolourizing textile dyes in laboratory-scale reactors.

Although conventional aerobic treatment systems such as trickling filters, stabilization ponds, activated sludge and aerated lagoons have been effectively used to degrade biodegradable components in industrial effluents, they are not effective for textile dye decolourization [72].

Table 2.5 indicates the details of some dye decolourization studies conducted using various types of biological reactors such as rotating biological contactor (RBC), air-lift bioreactor, submerged anaerobic membrane bioreactors (SAMBRs), sequencing batch reactor (SBR), moving-bed biofilm reactors (MBBR) and upflow anaerobic sludge blanket (UASB). Some types of reactors such as rotating biological contactor and air-lift bioreactor have been successfully operated under aerobic conditions mainly utilizing fungal strains for the decolourization of textile dyes (Table 2.5).

Out of the different anaerobic reactor systems, UASB reactors with anaerobic granular sludge have been utilized in most of the studies (Table 2.5). In a UASB reactor, wastewater enters from the bottom of the reactor and move upward through the activated granular sludge. These granules are generally composed of different layers of bacteria such as acidogenic bacteria, methanothrix-like bacteria and H_2-producing and H_2-utilizing bacteria [73]. Activity of microorganisms in sludge bed allows the treatment of wastewater. Due to the high stability of granular sludge, it will not be washed-out even at the continuous operation of the reactor [74]. Gas produced within

the reactor will be collected from the top of the reactor. Treated water will be moved through a three phase separator (air, liquid and solid) and taken out from the top of the UASB.

Most of the reactors employed for dye decolourization have been seeded with anaerobic or aerobic sludge directly obtained from existing wastewater treatment plants (Table 2.5). Therefore, precise identification of the microorganisms involved in dye decolourization was not possible in those studies. However, the reactors that containing attached growth (biofilm) systems have been mostly utilized in dye decolourization studies conducted using isolated microorganisms (Table 2.5).

When considering the anaerobic attached growth processes, anaerobic up-flow packed/fixed bed reactor, anaerobic expanded bed reactor (AEBR) and anaerobic fluidized bed reactors (FBR) are the most commonly utilized processes [75].

Upflow anaerobic packed bed reactors are in cylindrical or rectangular shapes and packing material could be filled in entire depth or in the upper 50-70% of the reactor. Corrugated plastic cross-flow or tubular modules and plastic pall rings are the most commonly used packing materials in industrial applications. Biomass will be retained in the void spaces of the packing materials and attached to the packing surfaces. Low up-flow velocities are generally maintained to prevent the wash-out of biomass from the reactors [75].

Up-flow anaerobic expanded bed reactors are generally utilize silica sand as the packing material. Bed expansion obtains by controlling the up-flow liquid velocity. Smaller packing materials provide high surface area per unit volume and thereby enhance the growth of biomass.

Anaerobic fluidized bed reactors are similar to up-flow anaerobic expanded bed reactors in physical design and utilize sand as the packing material. However, FBR reactors operate at higher up-flow liquid velocities to provide typically 100% bed expansion. Besides sand, other packing materials such as anion and cation exchange resins, diatomaceous earth packing and activated carbon have been utilized in FBR reactors [75].

Among these different anaerobic attached growth reactor systems, fixed/packed bed reactors are most widely used in laboratory-scale dye decolourization studies due to its operational simplicity.

Table 2.5: Dye decolourization studies conducted using different biological reactors

Reactor type	**Dyes**	**Microorganisms**	**Reactor working volume (l)**	**Hydraulic retention time (HRT)**	**Mode of operation**	**Decolourization (%) and remarks**	**Reference**
RBC	Diluted textile effluent	*Phanerochaete chrysosporium* (white-rot fungus)	3	48 h	Continuous	64% decolourization when 50% diluted with media containing glucose	[23]
UASB	Acid Red 131 Acid Yellow 79 Acid Blue 204	Anaerobic granular sludge	1.25	24 h	Continuous	Over 85% of decolourization with all dye concentrations of 10, 25, 50, 100, 150, 300 mg/L	[76]
SAMBR	Reactive Orange 16	Anaerobic granular sludge	11.4	2.5 d	Continuous	Over 99% of decolourization	[77]
Air-lift bioreactor	Acid dyes and reactive dyes	*Bjerkandera adusta* (white-rot fungus)	2	72 h	Batch	Decolorized 91–99% of 200 mg/l of each dye (except acid orange 7)	[78]
Anaerobic reactors	Reactive Red 2	Anaerobic sludge	NA	96 h	Semi-continuous	Above 76% decolourization	[79]
SBR	Remazol Brilliant Violet 5	Activated sludge	1	Cycle time 24 h	Sequencing batch	90% color removal, aerated reaction phase of 10 h	[80]
	Remazol Black B					75% color, aerated reaction phase of 10 h	
RBC	Direct Red-80 Mordant Blue-9	*Phanerochaete chrysosporium*	3	24 h	Batch	77-89 % decolourization with 200 ppm dye concentration	[81]

MBBR	Remazol Brilliant Violet 5R	Bacteria enriched with anaerobic sludge	4	Cycle time 8 h	Sequencing batch	94.4% colour removal but color removal rate decreased after first 5 h of anaerobic stages	[20]
MBBR	Remazol Black 5	Mixed culture	5	24-6 h	Continuous	76-81% colour removal at 24-6 h retention times	[19]
UASB	Textile wastewater	Anaerobic granular sludge	1.25	24 h	Continuous	Over 92% colour removal	[21]
UASB-CSTR	Direct Black 38	Anaerobic sludge	1.8 (UASB)	3.6 d	Continuous	81% in UASB and overall 94 % colour removal	[82]
		Activated sludge	9 CSTR)	18 d			
Up-flow packed bed bioreactor	Textile wastewater	*Aspergillus carbonarius* and *Penicillium glabrum*	0.4	40 min	Continuous 3 d operation time	78.8% colour removal. Macro porous polymeric support has been used for the growth of filamentous fungi.	[83]
UASB	Congo Red	Anaerobic sludge	5.2	24 h	Continuous	95-98% colour removal	[84]
	Textile wastewater					57% colour removal	
Anaerobic packed bed reactor	Dye mixture	Anaerobic digesting domestic sewage sludge	15	5 d	Continuous	98% colour removal with 250 mg/l dye concentration. Pall rings have been used as the packing material	[85]
Fixed-bed biofilm reactor	Acid Red 18	Anaerobic granular sludge and activated sludge	14	Cycle time 24 h	Sequencing batch	92% COD and 95% dye removal has achieved using integrated anaerobic/aerobic treatments. Pumice stones and plastic media made of polyethylene have been used as the packing material	[86]

2.5.2 Biofilm

Biofilm can be defined as an assemblage of microbial cells that are irreversibly attached to a surface and surrounded by a matrix of primarily polysaccharide material [87]. Naturally grown biofilms may contain a single species of a microorganism or extensively a combination of several types. During the formation of biofilms, microorganisms may change from the planktonic stage to sessile growth conditions with limited mobility. Formation of biofilm structures allows microorganisms to survive at extreme conditions such as nutrient deprivation, pH changes and the presence of oxygen radicals and withstand physical forces such as shear stresses created by liquid flow [88].

Formation of a biofilm on a surface is a process which can be expressed in few steps. Initially, inorganic and organic molecules will be secreted by microorganisms on to a surface to make it more favourable for cell settlement. Then the planktonic cells will interact with the conditioned surface followed by the attachment of cells to the surface reversibly and later on irreversibly. Thereafter, attached cells will be multiplied and simultaneously secrete extracellular polymeric substances (EPS) which attach cells to the surface as well as to each other. EPS are mainly composed of polysaccharides, nucleic acids, lipids and proteins which will contribute to the mechanical stability of biofilms [89]. Biofilm structure is then gradually developed (maturation) and forms a cohesive, three-dimensional polymeric network that interconnects microbial cells. Finally, the matured biofilms get dispersed either by shedding of daughter cells from actively growing cells, detachment due to quorum sensing or nutrient levels, or shearing of biofilm aggregates due to flow effects [87]. As matured biofilm structures are exposed to strong mechanical and hydrodynamic forces, their potential for detachment is higher. Detached biofilm sections can re-adhere to the substratum to form new biofilms or can be washout from the system [90].

The formation of biofilms on a surface mainly depends on several factors such as surface properties, microbial cell properties and environmental conditions [91]. Microbial cell components such as flagella, fimbriae and type IV pili-mediated motilities are important in the initial attachment stage of biofilm formation [92]. Flagella are significant for initial interactions between cells and surface while the

aggregation of cells for the formation of microcolonies is enhanced by pili-mediated twitching motilities [93]. Further, the surface properties such as roughness, wettability, or hydrophobicity influence the ability of microorganisms to attach to the particular surface [94]. Different types of natural materials (pumice, woodchips, etc.) and synthetic materials (polyurethane (PU), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), nylon, glass, stainless steel, etc.) have been studied and proven to have biofilm formation ability [91, 94]. As reported by Morgan [91], some researchers have observed higher microbial activity rates and better biomass attachment with relatively more hydrophobic materials such as PP, PVC and high density polystyrene (HDPS) [95]. Contradictorily, another group of studies have reported higher biofilm formation in less hydrophobic materials such as nylon and melamine [96, 97]. Hence, it can be assumed that the biofilm formation ability on different surfaces is moreover microbial strain specific and rely on the other conditions such as external environment and electrostatic interactions of the surfaces [95].

2.5.3 Decolourization of textile dyes in fixed (packed) bed biofilm reactors

According to the literature, most of the dye decolourization studies conducted in packed bed reactors have utilized natural packing materials such as brick pieces [98], sheep bone chips [99], gravel [100], granular activated carbon [101], laterite pebbles [102], volcanic rocks [103] and seashells [104]. As shown in Table 2.6, most of these studies have been conducted in reactors of volumes less than 1L to evaluate the biological decolourization ability of individual dye solutions.

Although these natural carrier materials support the biofilm formation, their usages in industrial-scale reactors are challenging. The weight of heavy packing materials such as brick pieces and gravel may result in tightly packed columns, which will ultimately create reactor clogging, and high-pressure effluent pumping requirements. Further, possible degradation of these natural carrier materials can interfere the reactor operation. For successful reactor operation, biofilm support material should be chemically inert, should not be easily degradable and should have a high surface to volume ratio, low cost and lightweight [91, 105]. Considering the above requirements, lightweight plastic carriers developed with high surface to volume ratio could be

considered as more beneficial over natural support materials in large-scale biofilm reactor operations.

Table 2.6: Dye decolourization studies conducted in packed bed biofilm reactors with natural support materials

Microorganisms	**Dyes**	**Biofilm support material**	**Reactor volume**	**Reference**
Bacterial consortium	Amaranth dye	Sheep bone chips	65 ml	[99]
Bacterial consortium	Remazol Black B	Gravel	125 ml	[100]
Bacterial consortium	Acid Blue-15	Brick pieces	148 ml	[98]
Bacterial consortium	Acid Violet-17	Seashells	138 ml	[104]
Microbial consortium	Dye mixture	Laterite pebbles	850 ml	[102]
Halomonas sp.	Reactive Black 5, Remazol Brilliant Violet 5R, Reactive Orange 16	Volcanic rocks	235 ml	[103]
Dye degrading bacteria	Acid Orange 7	Granular activated carbon	2.3 l	[101]

CHAPTER 3

3 MATERIALS AND METHODS

This chapter describes materials and methodologies used for the investigation of textile dye decolourization by locally isolated microorganisms. Dye decolourization in flasks using free bacterial cells, test methods and analytical techniques used, fabrication and investigation of dye and textile effluent decolourization in a fixed bed biofilm reactor are explained in detail.

3.1 Industrial Survey Questionnaire

An industrial survey was conducted to gather information about the currently used dyes and effluent decolourization techniques in Sri Lankan textile industry. For that, a questionnaire composed of 13 questions (Appendix A) was distributed to 15 textile-processing companies. In order to identify the most widely used dyes in local textile dyeing facilities, data was collected about the types of dyes used in each company and their monthly consumption. Additionally, information was gathered on the major quality parameters of textile effluent namely; pH, BOD (mg/l), chemical oxygen demand (COD) (mg/l), temperature (^{o}C) and colour. Moreover, questions were formed to obtain information regarding wastewater treatment and special dye-decolourization techniques used in local textile dyeing companies.

3.2 Chemicals and Reagents

3.2.1 Dyes used

Commercial grade Sumifix Supra Yellow EXF (Yellow EXF), Sumifix Supra Red EXF (Red EXF), Sumifix Supra Blue EXF (Blue EXF) and Cibacron Black WNN (Black WNN) dyes were obtained from a local textile processing facility located in Colombo, Sri Lanka (company G). Each of these dyes is a mixture of reactive dyes and their structures (except Black WNN) are not available due to trade secrets. According to material safety data sheet, 60-70 % of Black WNN dye is composed of Sodium 4-Amino-5-hydroxy-3,6-bis [[4-[[2-(sulfooxy)ethyl]sulfonyl]phenyl]azo]-

2,7-napthalene disufonate (chemical abstracts service (CAS) number-17095-24-8) and its structure is given in Figure 3.1. Even though the structures of Sumifix Supra dyes are not available, they are reported as bifunctional reactive dyes containing Aminochlorotriazine-sulphatoethylsulphone groups [106]. Nylosan-Rhodamine-EB (Rhodamine) dye was obtained from a textile processing facility located in Kurunegala, Sri Lanka (company C)**.**

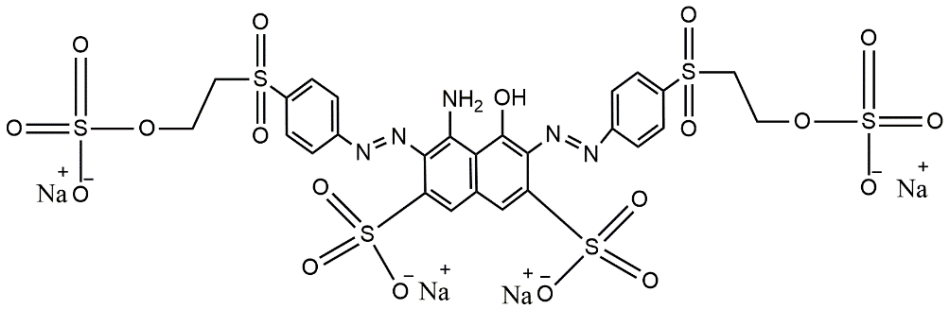


Figure 3.1: Structure of major dye in Black WNN

3.2.2 Chemicals used

All the chemicals used for media preparation were from Hardy Diagnostics (USA) and MP Biomedicals (USA). Chemicals used for polymerase chain reaction (PCR) analysis were of molecular grade (Promega, USA). Ethyl acetate used for extraction purposes was of high performance liquid chromatography (HPLC) grade.

3.3 Dye Stock Solution Preparation

Dye stock solutions were prepared by dissolving 1 g of each dye in 1 l of distilled water. Prepared dye stock solutions were filter sterilized using 0.22 µm syringe filters. Filtered dye solutions were stored in sterilized glass bottles until used. Synthetic dye mixture was prepared mixing equal volumes of 1 g/l stock solutions of Yellow EXF, Red EXF, Blue EXF and Black WNN dyes.

3.4 Luria–Bertani Medium (LB) Preparation

Given quantities of (Table 3.1) bacto-tryptone, yeast extracts and NaCl were added to an Erlenmeyer flask and the volume of the solution was adjusted to 1 l using distilled water. Flask was hand-shaken until all the nutrients were dissolved and then sterilized by autoclaving at 121 °C for 20 min.

Table 3.1: Composition of LB medium [107]

Chemical	**Concentration (g/l)**
Bacto-tryptone	10
Yeast extracts	5
NaCl	10

3.5 Agar Plate Preparation

To LB broth prepared, agar 16 g/l was added (Table 3.2) and then sterilized by autoclaving at 121 °C for 20 min. The agar medium was allowed to cool down to roughly 40 ^{o}C temperature. Then 15 ml portions were poured into sterilized Petri dishes that were placed in the biological safety cabinet and allowed to be solidified. Solidified agar containing plates were then wrapped with aluminium foil and stored in the bottle cooler at 4 ^{o}C temperature until used.

Table 3.2: Composition of agar medium

Chemical	**Concentration (g/l)**
Bacto-tryptone	10
Yeast extracts	5
NaCl	10
Agar	16

3.6 Decolourization Media Preparation

Chemicals given in Table 3.3 were added to an Erlenmeyer flask and the volume of the solution was adjusted to 1 l using distilled water. The flask was thoroughly shaken to dissolve the contents, pH was adjusted to 7 (OHAUS Starter 2100) and then sterilized by autoclaving at 121 °C for 20 min. This medium was used in all decolourization studies unless otherwise specifically mentioned.

Table 3.3: Composition of decolourization medium [13]

Chemical	**Concentration (g/l)**
Yeast extracts	5.0
$Na_2HPO_4 .7H_2O$	12.8
KH_2PO_4	3.0
NaCl	0.5
NH_4Cl	1.0

3.7 Isolation of Indigenous Microbial Strains that have Potential in Dye Decolourization

3.7.1 Effluent and sludge collection

Sludge containing effluent sample was obtained from the oxidation ditch of company G. The samples were brought to the laboratory on an ice pack in a cooler box and stored at 4 ^oC. The physiochemical parameters such as pH, temperature (measured at the site) and BOD of the sample was determined as soon as the sample was brought to the laboratory.

3.7.2 Co-incubation of effluent samples in the nutrient medium

Nutrient media (LB-9 ml) with 100 mg/l of each dye was added into test tubes separately and were inoculated with 1 ml of textile effluent samples. Caps of the test tubes were tightened and tubes were then incubated at 35 ^oC for 24 h.

3.7.3 Isolation of bacterial species

After incubation for 24 h, 1 ml samples were withdrawn from each test tube and serial dilutions of 10^{-1} to 10^{-8} were prepared. Samples of each dilutions were spread on each dye containing agar plates and incubated at 35 ^{o}C for 24 h. During the preparation of agar plates (section 3.5), concentrated dye solutions were added to the molten agar medium to obtain final 100 mg/l concentration of dye. After 24 and 48 h incubation, agar plates were observed and morphologically different colonies were sub cultured by streak plate technique.

3.8 Nutrient Broth Screening for Dye Decolourizing Isolates

Isolated microorganisms were then screened to check their potential to decolourize textile dyes in test tubes containing LB media and 100 mg/l of each dye. Out of the screened microorganisms, the isolates that exhibited visible colour removal were selected for further studies.

3.9 Preservation of Bacterial Strains

Twenty-four hour grown Cultures of the selected bacterial isolates were prepared in LB broths. 600 µl of each culture and 400 µl of 50 % glycerol solution were added into 1.5 ml Eppendorf tubes. Tubes were then dipped in liquid nitrogen to freeze and were stored in a low-temperature freezer at -25 ^{o} C.

3.10 Identification of Isolated Dye Decolourizing Strains

Bacterial strains that indicated dye decolourization potential were characterized by several biochemical tests such as gram staining [108], capsule staining [109], endospore staining [110] and catalase test and identified by sequencing analysis of 16S rRNA gene.

3.10.1 Gram staining

A bacterial smear was prepared on a glass slide by placing a small amount of an isolated colony using a sterile inoculating needle and the smear was fixed to the slide

by passing the slide over the flame (heat fixed) to make the microorganisms stick to the glass. Then the smear was stained with crystal violet and after 1 min the smear was covered with Gram's iodine and left for 1 min. Next, excess crystal violet-iodine complex was drained out from the slide and the slide was washed with 95% ethanol for 10-30 s to decolourize the smear. After that, the slide was immediately rinsed with water. Finally, it was counterstained with safranin for 30 s. After the slide was air dried, microscopic observations of the smear were done using oil immersion objective.

3.10.2 Capsule staining

A bacterial smear was prepared on the glass slide (as in section 3.10.1), air-dried and flooded with the primary stain, crystal violet for 5-7 min. Then the smear was washed with 20% copper sulphate solution. After the slide was dried, microscopic observations were made using oil immersion objective.

3.10.3 Endospore staining

Bacterial smear was prepared on a glass slide. A small piece of bibulous paper was placed over the smear and the paper was saturated with 0.5% (wt/vol) malachite green. The slide was gently heated over the Bunsen burner for 5 min while keeping the bibulous paper saturated with malachite green. The bibulous paper was removed and the slide was gently rinsed with water. Finally the smear was counterstained with safranin for 2 min and rinsed gently with water. When the slide was dried, microscopic observations were performed using oil immersion objective.

3.10.4 Catalase test

Bacterial cells were taken into the inoculation loop from the agar plate and placed on a clean glass slide. One drop of 3% H_2O_2 was added to the bacteria and the suspension was observed for bubble formation.

3.10.5 Polymerase chain reaction (PCR)

3. 10. 5. 1 Template DNA preparation

Template deoxyribonucleic acid (DNA) samples were prepared by colony PCR method [65]. A trace amount of particular bacterial colony grown on an agar plate for 24 h was taken using a pipette tip and mixed with 20 µl of nuclease-free water in a sterile Eppendorf tube. Next, this tube was heated at 95 ^{o}C for 20 min in a water bath and then, centrifuged for 5 min in microcentrifuge (Eppendorf 5424R) at 10000 g at 4 ^{o}C to separate cell pellets. The supernatant was taken as the template DNA for PCR analysis.

3. 10. 5. 2 Master mix preparation

Reagents (Table 3.4) taken out from the freezer were thawed in an ice bucket. Measured volumes of each reagent were added in a sterile 1.5 ml Eppendorf tube in the order given in Table 3.4. After adding all the reagents, it was gently mixed by pipetting up and down. Universal primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' were used for the amplification of 16S rRNA gene.

Table 3.4: Reagents used for PCR master mix preparation

	Reagent	**Working concentration**	**Volume (µl)**	**5X Master mix volume (µl)**
I	PCR Buffer	5X	10	50
II	$MgCl_2$	25 mM	3	15
III	Forward Primer	20 µM = 20 pmol/µl	1	5
IV	Reverse Primer	20 µM = 20 pmol/µl	1	5
V	dNTP mix	10 mM	1	5
VI	Nuclease free water	-	32.75	163.75
VII	Taq DNA Polymerase	5 Units/µl	0.25	1.25

3. 10. 5. 3 Preparation of PCR samples and sample loading

Sterilized PCR tubes (0.5 ml) were taken and labeled appropriately. 49 µl of the prepared master mix was added into each tube while tubes were kept in PCR stand dipped in an ice bucket. The volume of the negative control was adjusted up to 50 µl by adding nuclease-free water. The volumes of other samples were adjusted to 50 µl by adding 1 µl of each DNA template separately to each tube. Tubes were tapped gently to mix the components properly and then placed in the thermal cycler (T100TM thermal cycler- Bio Rad). PCR was programmed to provide the cycling conditions given in Table 3.5.

Table 3.5: PCR cycling conditions

Cycle step	**Temperature (**°C)	**Time (min)**	**Number of cycles**
Initial Denaturation	95	2	1
Denaturation	95	0.5	30
Annealing	53.1	0.5	30
Extension	72	1	30
Final Extension	72	5	1

3.10.6 Agarose gel electrophoresis

3. 10. 6. 1 Preparation of the gel

Agarose gel (1% w/v) was prepared by dissolving 0.45 g of agarose in 45 ml of TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. The mixture was melted by heating in a microwave until the agarose had dissolved. When the agarose gel was cooled down to 50-60 ^{o}C, 3.5 µl of Ethidium bromide (EtBr) was added.

Subsequently, the gel tray was placed in the casting apparatus and the melted agarose was poured into the tray. A comb was placed on the gel tray and allowed the agarose to be set at room temperature. Once the gel was solidified, the comb was removed and the gel tray was placed in the gel electrophoresis unit. The gel box was then filled with a running buffer (TAE) to cover the surface of the gel.

After PCR cycles were completed, the tubes were taken out from the thermal cycler and 8 µl of loading dye was added to each sample. 50 µl of each sample was taken and introduced into the wells of the gel. In addition, a 5 µl of 1kb ladder mixed with 1 µl of loading dye was introduced into a well as the reference. Then, the electrophoresis unit was switched on and the samples were allowed to migrate through the gel.

3. 10. 6. 2 Observing separated DNA fragments and extraction of amplified PCR product

When the samples migrated up to two-third of the gel length, the power supply to the electrophoresis unit was turned off. The gel was taken out, the excess buffer was drained off and then placed on the gel documentation system. Bands were observed under the ultraviolet (UV) light and pictures of the gel were taken.

Next, the gel was placed on the UV-trans illuminator to observe bands. A scalpel blade was used to cut the DNA fragment of interest in a minimal volume of Agarose. Weight of the gel slice was measured following standard procedure.

Membrane binding solution was added at a ratio of 10 µl of solution per 10 mg of Agarose gel slice. The mixture was vortexed and incubated at 50-65 ^{o}C for 10 min until the gel slice completely dissolved.

Wizard SV mini-column was placed in a collection tube (mini-column assembly). The dissolved gel mixture was transferred to the mini-column assembly and then microcentrifuged at 14,000 rpm for 1 min. Mini-column was then removed from the column assembly and the liquid in the collection tube was discarded. Then the mini-column was re-located on the collection tube. Next, the column was washed by adding 700 µl of membrane wash solution and the mini-column assembly was centrifuged for 1 min at 14,000 rpm. Liquid in the collection tube was discarded and mini-column was placed back in the collection tube. Washing was repeated with 500 µl of membrane wash solution and then centrifuged for 5 min at 14,000 rpm. Collection tube was emptied and column assembly was re-centrifuged for 1 min while the lid of the microcentrifuge was opened to allow evaporation of any residual ethanol.

Mini-column was then transferred to a clean microcentrifuge tube and 50 µl of nuclease-free water was directly added to the center of the column. Then the column was incubated at room temperature for 1 min and centrifuged at 14,000 rpm for 1 min. Finally the mini-column was discarded and the eluted DNA containing microcentrifuge tube was sent for 16S rRNA gene sequencing.

3.10.7 Identification of microbial isolates

Purified PCR products were sequenced by Macrogen, Korea. The alignment of the sequences were done using CLUSTALW program at European Bioinformatics site (www.ebi.ac.uk/clustalw). Aligned sequences were analyzed using the basic local alignment search tool (BLAST) at BLAST-n site in national center for biotechnology information (NCBI) server (www.ncbi.nih.gov/BLAST). The sequences were deposited in the NCBI GenBank, PubMed database and accession numbers were obtained.

3.11 Dye Decolourization

A 100 ml sample of the decolourization medium (section 3.6) was transferred to a 250 ml Erlenmeyer flask and sterilized at 121 ^{o}C for 20 min. Dye solutions were filter sterilized with 0.22 µm membrane filters and mixed with the medium to obtain a final dye concentration of 50 mg/l. This dye concentration was selected considering the unfixed dye concentrations of textile effluents and dye concentrations used in similar studies [68, 111].

Bacterial isolates were pre-cultured in LB medium and incubated at 35 ^{o}C for 24 h. The sterilized media containing each dye were inoculated with 2% (v/v) (optical density (OD) adjusted to 0.3) of the inoculum and incubated at 35 °C under static (anoxic) conditions.

Samples of 5 ml were withdrawn from dye containing medium at defined time intervals and centrifuged at 10,000 g (12000 rpm) at 28 ^{o}C for 10 min. The supernatant was scanned at maximum absorbance wavelengths (λ_{max}) of each dye under visible light in a spectrophotometer (UV-1800 Shimadzu spectrophotometer). The

uninoculated dye free medium was used as the blank. All assays were performed in duplicates.

Percentage dye decolourization was calculated using equation 3.

$$\text{Percentage decolorization} = \frac{(A_{initial} - A_{final})*100\%}{A_{initial}} \quad (3)$$

Where $A_{initial}$ is the absorbance before decolorization and A_{final} is the absorbance of the corresponding inoculated samples at a specific time.

Spectral absorption coefficient, α (λ) of the dye and effluent samples was measured using equation 4 as given in ISO 7887 international standard.

$$\text{Spectral absorption coefficient, } \alpha(\lambda) = \frac{A*f}{d} \quad (4)$$

Where A is the absorbance of the water sample at wavelength λ, d is the optical path length (in millimeters) of the cell and f is a factor to give the spectral absorption coefficient in reciprocal meters (f=1000).

3.12 Determination of Cell Growth

Bacterial cell growth in the decolorization media was estimated using optical density values of the samples at 600 nm wavelength. As the samples contained both bacterial cells and dyes, light scattered by bacterial cells in the sample ($OD^{X}_{600\ nm}$) was calculated using equations 5 and 6 as described by Chen et al. [112] and Silveira et al.[111]. For that, samples withdrawn from the flasks during decolourization studies were examined at 600 nm wavelength with and without bacterial cells (before and after the centrifugation) and OD values were measured. An uninoculated decolourization medium without dyes was used as the blank.

1) OD_{600nm} of the sample mixtures before centrifugation;
   $OD^{X+dye}_{600nm} = OD^{dye}_{600nm} + OD^{X}_{600nm}$ (5)
2) OD_{600nm} of sample supernatant (sup) after centrifugation for 10 min at 10,000g;
   $OD^{sup}_{600nm} = OD^{dye}_{600nm}$ (6)

3.13 Determination of Dye Category

Structures of Yellow EXF, Red EXF and Blue EXF dyes were not available due to trade secrets. Hence, Fourier-transform infrared spectroscopy (FTIR) analysis was conducted to identify the chromophore group present in each of these dyes. Accordingly, each dye was analysed using the KBr pellet method [14, 71] in Bruker Alpha II FTIR at the region of 500 - 4000 cm^{-1} with a scan speed of 24 and at 10^4 resolution.

3.14 Dye Decolourization by Bacterial Consortium

Ability of a bacterial consortium to decolourize Yellow EXF, Red EXF, Blue EXF, Black WNN, Rhodamine and the mixture of dyes (section 3.3) were investigated separately by conducting batch decolourization studies in Erlenmeyer flasks as described in 3.11.

Bacterial strains that showed enhanced colour removal in previous studies (section 3.11) were selected for the preparation of bacterial consortium. The bacterial consortium was developed by mixing equal volumes (OD adjusted to 0.3) of 24 h grown cultures of each selected bacterial strain as described by Phugare et al. [113] and Lade et al. [98]. Each dye and nutrients containing flasks were inoculated with 2% (v/v) of the prepared bacterial consortium. Then these flasks were incubated at 35 °C under static (anoxic) conditions. Absorbance value of the samples obtained from each dye containing media was measured at defined time intervals and percentage decolourization was calculated as described in section 3.11.

3.15 Optimization of Decolourization Conditions

Dye decolourization was investigated at different temperatures, pH values, dye concentrations, agitation conditions and carbon sources to identify the most appropriate condition for the maximum decolourization. All assays were performed in duplicates.

3.15.1 Effect of agitation

The effect of agitation on dye decolorization was investigated by maintaining inoculated dye and nutrients containing flasks under both static and shaking (at 120 rpm in MaxQ SHKE8000 shaking incubator) conditions for Yellow EXF, Red EXF, Blue EXF, Black WNN and the mixture of dyes. Decolourization media that contained each dye was inoculated with the best decolourizing isolates that were screened during the dye decolourization studies (section 3.11). The bacterial consortium developed in 3.14 was used for the inoculation of the dye mixture. Percentage decolourization was calculated as described in 3.11, by measuring the absorbance of samples.

3.15.2 Effect of temperature

Temperature and pH optimization tests were conducted for Yellow EXF and Red EXF dyes with the most effective dye decolourizing strains that were screened during the decolourization tests (section 3.11). Further, these two optimization studies were conducted for the decolourization of a mixture of dyes, using the bacterial consortium. Cultures were incubated at varying temperatures of 25, 30, 35, 40, 45 ^{o}C and the percentage decolourization at each temperature was calculated as in 3.11.

3.15.3 Effect of pH

Dye containing media were prepared as in section 3.11 and the initial pH levels were adjusted to 5 to 10 in steps of 1 using 0.1 N NaOH and HCl. Colour removal with the most effective dye decolourizing strains that were screened during the decolourization tests (section 3.11) was determined at predetermined times (section 3.11).

3.15.4 Effect of initial dye concentration

To evaluate the effect of dye concentrations on the biological decolourization of textile dyes, Yellow EXF dye concentrations of 100, 200, 300, 400 and 500 mg/l were used and the percentage decolourization at each dye concentration was calculated. In typical industrial eluents, the concentration of the dye does not exceed 500 mg/l and hence this dye concentration range was selected.

3.15.5 Effect of carbon source

For the investigation of the effect of carbon source on decolourization of Yellow EXF, the same culture media (section 3.11) was used however; the carbon source was replaced with 5 g/l glucose and corn starch to compare with the medium containing yeast extract. An additional study was carried out with 2 g/l yeast extract in the medium.

Further, to investigate the effect of the quantity of yeast extract on colour removal of the dye mixture by the bacterial consortium, decolourization studies were conducted in media containing 0.25, 1 and 2 g/l yeast extract concentrations.

3.16 Decolourization of Malachite Green

Other than the previously considered dyes, Malachite green decolourization studies were conducted following the method described in 3.11 using 2% (v/v) of the constructed bacterial consortium as the inoculum. Decolourization studies were conducted in 5 g/l and 2 g/l yeast extract containing media and percentage decolourization was calculated as given in 3.11.

3.17 Decolourization of Textile Industry Effluent by the Bacterial Consortium

For this study, effluent samples were collected from three textile-dyeing companies (A, D and Z). Sample collection point was the inlet to the wastewater treatment plant, prior to any treatment. Samples were collected to plastic containers, brought to the laboratory and stored at 4 ^{o}C until used. pH of the effluent samples were adjusted to 7 and then sterilized by autoclaving at 121 ^{o}C for 20 min. Dissolved and sterilized yeast extract was added to 100 ml of effluent to make the final concentration of yeast extract to 5 g/l. Next, the samples were inoculated with 2% (v/v) bacterial consortium and were incubated at 35 ^{o}C. Absorbance values of samples withdrawn at predetermined times were measured and percentage decolourization was calculated (section 3.11).

3.18 Yellow EXF Degraded Compounds Analysis

3.18.1 HPLC analysis

Yellow EXF dye sample was decolourized as described in section 3.11. After 72 h of decolorization, the contents in the flask were centrifuged at 12,000 rpm for 10 min and the resulting supernatant was clarified by passing through a 0.45µm filter [62].

Filtered sample was analysed using HPLC. Prior to analysis, filtered sample was extracted using t-butyl methyl ether (MTBE) based on the ISO 14362 standard (which is used for the determination of certain aromatic amines derived from azo colourants) and concentrated. Then the extracted metabolites were analyzed in an HPLC (1260 infinity series using C18 reverse-phase column, at 32 ^{o}C using eluent methanol and potassium dihydrogen phosphate in water) to identify spectral changes of compounds due to biodegradation. The injection volume was 5 µl and the flow rate was 0.6 to 2.0 ml/min (gradient flow).

3.18.2 Gas chromatography–mass spectroscopy (GCMS) analysis

Two Yellow EXF dye samples were decolourized (in static conditions) as described in section 3.11. After 72 h of decolorization, the contents in one flask were centrifuged at 12,000 rpm for 10 min and the resulting supernatants were clarified by passing through a 0.45 µm filter.

Contents in the other flask were centrifuged at 12,000 rpm for 10 min and the supernatant was transferred to a sterile fresh flask under aseptic conditions. This flask was inoculated once again with 2% (v/v) of 24 h grown culture of *Proteus mirabilis* and placed in a shaking incubator at 35 ^{o}C, and 120 rpm rotational speed. After 7 days of incubation, contents in that flask was centrifuged and filtered through a 0.45 µm filter.

Both filtrates were extracted (as done for HPLC analysis) and concentrated metabolites were analyzed in a GCMS (5975C inert MSD, 7890A GC system) and the compounds were identified using national institute of standards and technology (NIST) library.

3.19 Decolourization of Textile Dyes and Effluent in a Reactor

3.19.1 Reactor seeding material preparation

3. 19. 1. 1 Cell immobilization with calcium alginate

Cell immobilized calcium alginate ($C_{12}H_{14}CaO_{12}$)n) beads were prepared as explained in [114], [115] with slight modifications. Four milliliters bacterial consortium ($1.5x10^8$/ml colony forming units) prepared as in section 3.14 was centrifuged at 5000 g for 5 min [116] at 4 ^oC, the supernatant was discarded and the separated cells were suspended in 10 ml of 3% (w/v) sterilized sodium alginate (($C_6H_7NaO_6$)n) solution. Next, 5 ml of prepared alginate solution was introduced dropwise into a 2% (w/v) $CaCl_2$ solution using a syringe in order to solidify each drop of alginate in to a soft bead. Then prepared alginate beads were washed with sterile water, added to a flask containing 100 ml decolourization medium (section 3.6) with 50 ppm concentration of synthetic dye mixture. Flasks were incubated as in section 3.11 and percentage colour reductions were measured in time intervals of 24 h.

3. 19. 1. 2 Incorporation of cells into activated sludge

Activated sludge obtained from industrial wastewater treatment facility (under operation) is generally used for seeding of new biological reactors in industry. Activated sludge may consist of organic fibres, inorganic particles, extracellular polymeric substances, filamentous bacteria, and ions (calcium, magnesium, etc.) [117]. Therefore, an in-house method was developed to eliminate living microorganisms and debris present in activated sludge sample (taken from the industrial wastewater treatment plant) and to incorporate the developed consortium cells (isolated bacteria) in to the sludge sample. First, activated sludge (20 g of wet weight) collected from an industrial wastewater treatment plant was sterilized in an autoclave at 121 ^oC for 20 min. Next, the autoclaved sample was centrifuged and the precipitated sludge was separated. Then, the sludge (20 g of wet weight) was suspended in 35 ml of nutrient medium (section 3.6) and the total volume was adjusted to 96 ml using sterile water. Four milliliters of bacterial consortium prepared as in section 3.14 was introduced to the flask containing sludge and incubated at 35 ^oC at 120 rpm for 30 days to incorporate consortium cells to the sludge. Thereafter, 20 ml

of the sludge was centrifuged at 2000 rpm for 10 minutes and the precipitated sludge was resuspended in 25 ml of sterile water. Two milliliters sample from sludge-suspended water ($1.1x10^8$/ml colony forming units) was introduced to a flask containing 100 ml of nutrient media (section 3.6) with 50 ppm concentration of dye mixture and colour reductions were measured as in section 3.11.

3. 19. 1. 3 Attached growth of cells

Microtiter plate (MTP) assay

Microtiter plate assay was carried out as in literature [118] with few modifications. 200 µl (1:100 diluted) of each 24 h culture (three bacterial isolates used for the bacterial consortium in 3.14) in LB medium was added to each well of the microtiter plate and incubated at 35 °C for 24 h. Wells were then stained with 200 µl of 0.001% crystal violet for 20 min, rinsed with water and the plate was air-dried. Then, 30% acetic acid was added to each well of the plate to solubilize crystal violet adhered to biofilms. Optical densities of the dissolved crystal violet in each well were read using an enzyme-linked immunosorbent assay (ELISA) reader at 595 nm wavelength.

Investigation of biofilm formation on plastic carrier materials

Carrier materials used in this study were kindly provided by Rex Industries (Pvt) Ltd, Negombo, Sri Lanka. These polypropylene carriers were corrugated cylindrical tubes with support interiors of 20 mm diameter and 13 mm height (Figure 3.2).

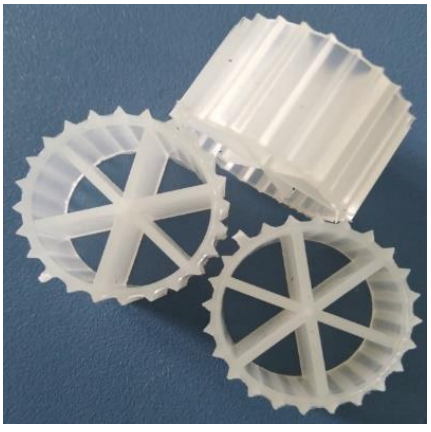


Figure 3.2: Biofilm support material

The biofilm formation ability of selected bacterial strains on these carrier materials was investigated in an experimental set-up made in house. In this apparatus, the substrate was allowed to flow through plastic carriers, to enhance the biofilm formation on the surfaces as reported by Sara Leifer [119] and Anjaneya et al [64] .
This laboratory set-up was consisted with a plastic box (20x13x10 cm) to hold carrier materials, feeding bottle and a bottle to collect the outflow stream (waste collector) (Figure 3.3). Sterile plastic tubes in intravenous tubing sets (IV set) were used to transfer nutrients and waste broth. Sterilized, 0.22 µm polytetrafluoroethylene (PTFE) syringe filters were connected to lids of each bottle for sterile venting.

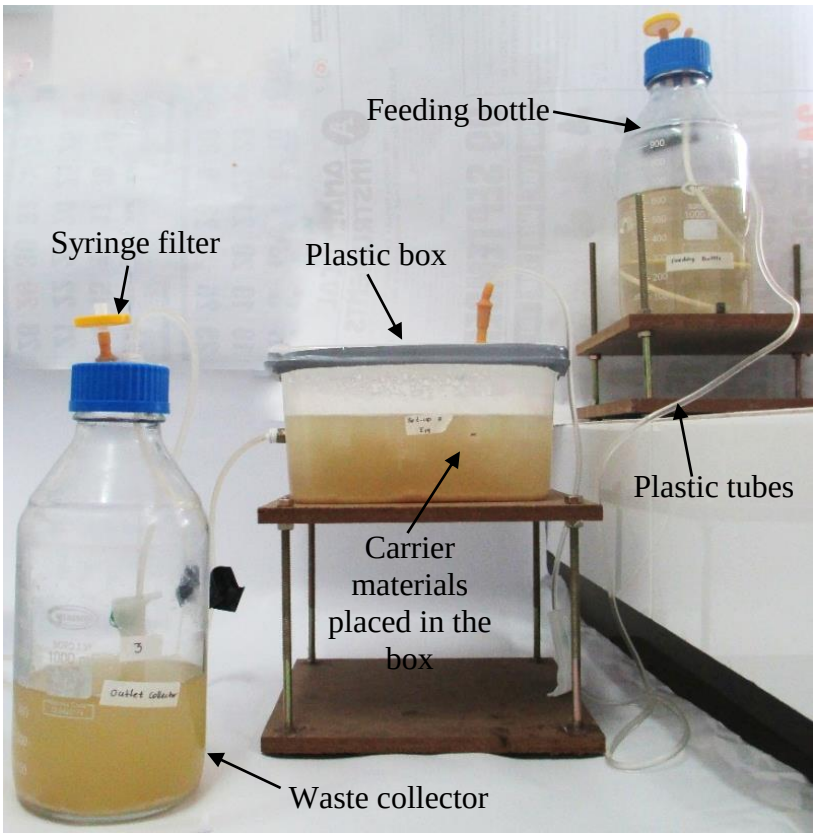


Figure 3.3: Laboratory set-up for biofilm growth

Three similar set-ups were separately used to investigate the biofilm formation ability of the three bacterial strains used for the bacterial consortium in section 3.14. Firstly, three layers of sterilized plastic carrier materials were placed at the bottom of the plastic boxes under sterile conditions. Then, 500 ml of LB medium was fed to each

box to completely cover the carrier material and thereafter, 200 ml of cell suspension (grown on LB broth) of each bacterial strain was added to respective container separately.

Feeding of nutrients and discharge of consumed media in each box were done using gravity flow (without using pumps). During initial 10 days, LB medium was fed at a rate of 10 ml/h and the flow rate was controlled using plastic roller clamps in IV set. Then, for next 45 days, bacteria in the plastic boxes were fed with nutrient media of composition given in Table 3.3 (concentration of yeast extract was reduced to 2 g/l) to develop biofilms on carrier materials. Consumed broth in waste collectors was autoclaved and discarded.

3.19.2 Reactor design and fabrication

Packed bed bioreactor (fixed bed biofilm reactor) with 5 l total volume and 3.8 l working volume was fabricated to continuously treat coloured wastewater. Three dimensional drawing (3D) of the reactor is shown in Figure 3.4.

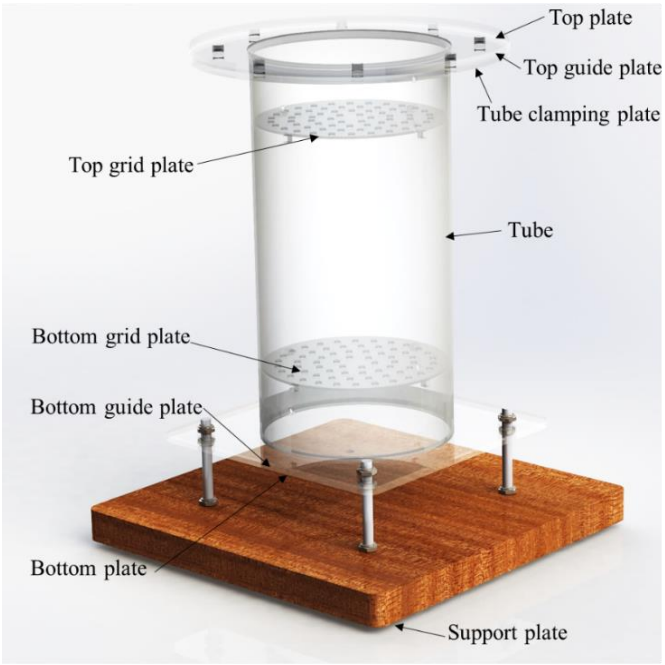


Figure 3.4: 3D drawing of the reactor

The main component of the bioreactor (the body) is a poly(methyl methacrylate) (PMMA) tube of 35 cm height and 15 cm of internal diameter. All sheets used to

construct the reactor (Table 3.6) were made out of PMMA whereas only support plate was wooden.

Table 3.6: Dimensions and details of the reactor components

Component	Dimensions
Top plate (P_1)	Diameter: 240 mm Thickness: 3 mm
Top guide plate (P_2)	Internal diameter:152 mm External diameter: 240 mm Thickness: 3 mm
Tube clamping plate (P_3)	Internal diameter:151 mm External diameter: 240 mm Thickness: 3 mm
Tube (P_4)	Diameter:150 mm Wall thickness: 3 mm
Grid plates (P_5, P_6)	Diameter: 140 mm Hole diameter : 5 mm Thickness: 3 mm
Bottom guide plate (P_7)	Length: 215 mm Width: 215 mm Hole diameter: 151 mm Thickness: 3 mm
Bottom plate (P_8)	Length: 215 mm Width: 215 mm Thickness: 3 mm
Support plate (P_9)	Length: 250 mm Width: 250 mm Thickness: 8 mm

Detailed description on fabrication of the reactor (initial assembling) is given in Appendix B.

3. 19. 2. 1 Final assembling of the reactor

Final assembling of the reactor was conducted under sterile conditions in the biosafety cabinet (Esco Airstream® Class II Biological Safety Cabinet). All the reactor components (pre-assembled) were washed with water, dried, then wiped with 70% ethanol and UV-sterilized in the biosafety cabinet. Sterilized male and female connectors (Figure 3.5) of port size 4 mm (purchased from B.E. Repairs and Maintenance, Piliyandala) were connected through the holes located on the reactor tube surface. These four connectors were used to attach inlet and outlet tubes (sterile plastic tubes in intravenous tubing set (IV set)) to the reactor.

Figure 3.5: Male female connectors

Next, bottom grid plate (P_6) was placed 5 cm above the bottom plate. Then, the reactor was packed with heat sterilized plastic carriers (section 3.19.1). Thereafter, the top grid plate (P_5) was placed on top of the carrier materials 23 cm apart from P_6. Then the reactor contents were UV-sterilized and reactor lid was fastened using nuts and bolts. When all the inlet and outlet connections were tightly sealed, the reactor was taken out from the biosafety cabinet. Finally, the reactor was placed in the pre-arranged location. Even though the full height of the reactor was 35 cm, it was filled only up to 30 cm height. Although total volume of the reactor was 5 l, loading volume (with packing material) was 3.8 l. Sterile plastic tubes in IV set were used to supply wastewater (influent) and to remove treated water (effluent). As shown in Figure 3.6, 2 l autoclavable glass bottles were used as feed reservoir (B_1) and treated dye (or wastewater) collector (B_2). Sterilized, 0.22 µm PTFE syringe filters were connected to the lid of each bottle for sterile venting. A peristaltic pump (12V Dosing pump) with sterilized tubes was used to transfer contents from feed reservoir (B_1) to the reactor. The feeding was done through the reactor inlet and influent was then moved upward through the carrier materials. The treated water was taken from the top of the reactor (reactor outlet) and collected to the treated dye collector (B_2) using gravity flow.

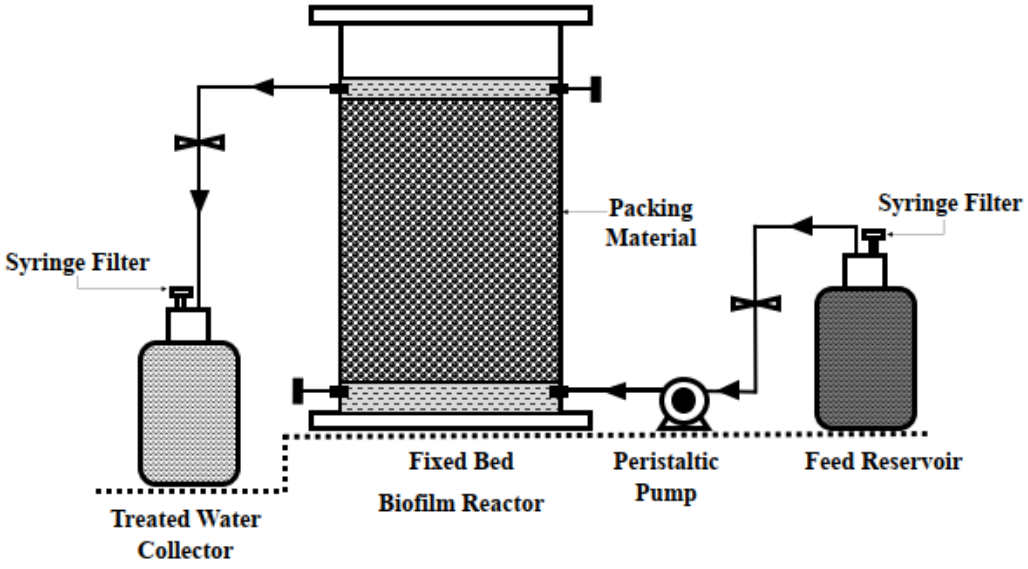


Figure 3.6: Schematic diagram of the reactor

3.19.3 Biofilm formation on carrier materials in the fixed bed biofilm reactor (FBBR)

Methods reported in Sharma et al. [104] and Anjaneya et al. [99] were followed with slight modifications for the development of biofilms on carrier materials. The reactor was filled with media containing 2.5 g/l yeast extract, 5 g/l NaCl and 5 g/l tryptone. 1 l of cell suspension (grown on LB) of the consortium was fed to the reactor as the inoculum and circulated for 7 days to allow the attachment of cells on plastic carrier material. Thereafter, the reactor was fed with a mineral salt medium composed of 2 g/l Na_2HPO_4 .$7H_2O$, 2 g/l KH_2PO_4, 0.5 g/l NaCl and 1 g/l NH_4Cl supplemented with 2 g/l of yeast extract and dye mixture at a flow rate of 40 ml/h for 2 months. This allowed the attached bacterial cells to grow initiating development of biofilms on carrier material. At the beginning, dye concentration of the reactor feed was 15 mg/l and then gradually increased to a final concentration of 50 mg/l within 2 months to acclimatize the biofilm bacteria to dye containing influent. The reactor was operated at room temperature.

3.19.4 Decolourization of synthetic dye mixture in batch FBBR

Sterilized medium containing dye mixture was fed to the FBBR from the feed reservoir using a peristaltic pump. Composition of the decolourization media (Table 3.3) used at the initial stage of reactor study was similar to that used for the previous studies (section 3.11) conducted with free bacterial cells (only the concentration of yeast extract was reduced to 2 g/l). However, then Na_2HPO_4 .$7H_2O$ and KH_2PO_4 concentrations in the decolourization media were gradually reduced until the percentage colour removal was not affected.

Thereafter, all the synthetic dye mixture decolourization studies were conducted using a medium composed of 2 g/l Na_2HPO_4 .$7H_2O$, 2 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl and (2, 1 and 0.25 g/l) yeast extract with 50 mg/l final concentration of dye mixture. Colour reduction in samples withdrawn from the reactor at 24 h intervals were measured by spectrophotometric method (section 3.11).

3.19.5 Decolourization of synthetic dye mixture in continuous FBBR

The FBBR was continuously fed with sterilized medium containing synthetic dye mixture using a peristaltic pump. Nutrient composition of the feed is similar to section 3.19.4 and dye mixture concentration was 50 and 25 mg/l. Dye decolourization in FBBR was studied under different conditions during the period of operation as given in Table 3.7.

Table 3.7: Different phases of synthetic dye mixture decolourization in continuous FBBR

Phase	**Time (day)**	**Feed concentration**	
		Yeast extract (g/l)	**Dye (mg/l)**
I	1 - 7	2	25
II	8 - 13	2	50
III	15 - 69	1	50
IV	70-73	0.25	50

Flow rate of the influent was initially maintained at 40 ml/h with 4 day retention time (phase 1) and then increased to 83 ml/h. At 24 h time intervals, samples were collected from the inlet and the outlet of the FBBR and the percentage decolourization was calculated using absorbance measurements at 554 nm wavelength as described in section 3.11.

Dilution rate of the reactor was calculated using equation 7.

$$\text{Dilution rate (D)} = \frac{\text{Flow rate of influent into the packed bed reactor}}{\text{Volume of influent in the packed bed reactor}} \quad (7)$$

3.19.6 Decolourization of textile industry effluent in batch and continuous FBBR

Wastewater samples were collected from a textile-dyeing mill (Company D), prior to any effluent treatment or addition of any other substance. When decolourization studies were conducted for effluent, initially, wastewater samples were diluted two-fold and pH was adjusted to 7.5 to ensure effective biological treatment. Then, wastewater samples were sterilized by autoclaving at 121 ^{o}C for 20 min and supplemented with 2 g/l of yeast extract (pre-sterilized). Wastewater samples were fed to the FBBR and decolourization was investigated in both batch and continuous modes.

Similarly, undiluted wastewater was fed to the FBBR during the batch mode of operation. Samples were collected from the FBBR inlet and outlet at 24 h time intervals and percentage decolourization was calculated using absorbance measurements at 401 nm wavelength as described in section 3.11.

3.20 Analysis of Biofilm on Carrier Materials in FBBR

3.20.1 Quantification of dry weight of biofilms

Five plastic carriers were randomly collected from different places of fixed bed reactor and washed to remove suspended cells. These carrier materials were then taken to a centrifuge tube and mixed thoroughly in the vortex mixture with a measured amount of water to separate the attached biofilms from the carrier surfaces. Thereafter, the biofilm containing water sample was vacuum filtered through 0.22 µm filter paper

(dried and pre weighed). Biomass containing filter paper was dried at 105 ^{o}C until constant weight. Dry weight of biofilm was calculated by subtracting the initial weight of filter paper from total weight (biomass containing filter paper) and expressed as mg/piece of carrier material.

3.20.2 Scanning electron microscopy analysis

Biofilm grown on the surface of carrier materials were examined through scanning electron microscope (SEM) (Carl Zeiss EVO 18 Research, Germany) following the method described by Anjaneya et al.[39]. First, biofilm containing carriers were washed twice with 50 mM phosphate buffer (pH 7.0) for 20 min. Washed plastic carriers were then dehydrated in a gradient of ethanol solutions (20, 40, 60, 80 and 100% ethanol) for 10 min at each concentration. Next, the plastic carriers were dried, coated with gold particles and examined through SEM. The control plastic carrier material was also prepared as described above.

3.20.3 FTIR analysis of biofilm

Carrier material that contains biofilm was removed from the FBBR (before starting the dye decolourization). The biofilm was scraped from the carrier surface and dried at room temperature. Dried biofilm was then ground in to a powder [120] and analyzed in FTIR according to KBr pellet method as described in section 3.13.

3.21 Determination of the Quality of Water Treated in FBBR

3.21.1 COD analysis

Chemical oxygen demand of reactor feed and treated water samples were measured using closed reflux titrimetric method (APHA Method 5220 C [121]). Prior to COD analysis, samples were centrifuged at 10,000 g for 10 min to remove bacterial cells and diluted fivefold with distilled water. Percentage COD reduction was calculated using equation 8.

$$\text{Percentage COD reduction} = \frac{(\text{COD}_{\text{feed}} - \text{COD}_{\text{treated water}})\text{X}100\%}{\text{COD}_{\text{feed}}} \quad (8)$$

3.21.2 Phytotoxicity studies

Phytotoxicity of textile effluent before and after biological treatment was investigated to assess the suitability of treated water to be released to the environment. 50% diluted textile industry wastewater and the same wastewater after biological treatment in continuous FBBR was used for assays and distilled water was used as the control. Phytotoxicity assays were conducted as described by Nachiyar et al. [39] with few modifications. Influent and effluent samples collected from the reactor were centrifuged twice at 10,000g for 10 min each to separate cells and the supernatant was used for the assays.

3. 21. 2. 1 Seed germination bioassay

Mature and healthy cowpea (*Vigna unguiculata*) seeds were taken to three 50 ml beakers (10 seeds per each). Five milliliters of textile wastewater, biologically treated wastewater and distilled water (control) samples were added to respective beaker separately at 24 h time intervals. Percentage germination of seeds, root (radicle) and shoot (plumule) lengths were daily monitored up to 4 days.
Seedling vigour index was calculated using equation 9 as described by Kumar et al.[122].

$$\text{Seedling vigor index} = \text{Germiniation (\%)} \times \text{mean seedling length} \quad (9)$$

Where, seedling lengths are the summation of root and shoot lengths of germinated seeds.

3. 21. 2. 2 Plant growth bioassay

Mature and healthy cowpea seeds were added to three soil-containing pots of 13 cm diameter and 8 cm depth (seven seeds per each pot). Seeds were watered with 10 ml of textile wastewater, biologically treated wastewater and distilled water (control) samples separately in time intervals of 24 h. Percentage seed germination and shoot lengths of the plants in each pot were daily monitored up to 6 days.

3.21.3 Analysis of the metabolites formed by biological degradation of dye mixture in FBBR

Reactor feed containing 0.25 g/l yeast extract and dye mixture and treated water from 68 h batch operation of FBBR (3.19.4) were centrifuged at 10,000 g for 10 min and the resulting supernatants were clarified by passing through a 0.45 µm filter. Filtered samples were extracted using t-butyl methyl ether (MTBE) based on the ISO 14362 standard and concentrated by rotary evaporation. Concentrated samples were then analysed using HPLC and GCMS techniques as described in section 3.18.

Summary of the major experiments conducted in this study is shown in Figure 3.7.

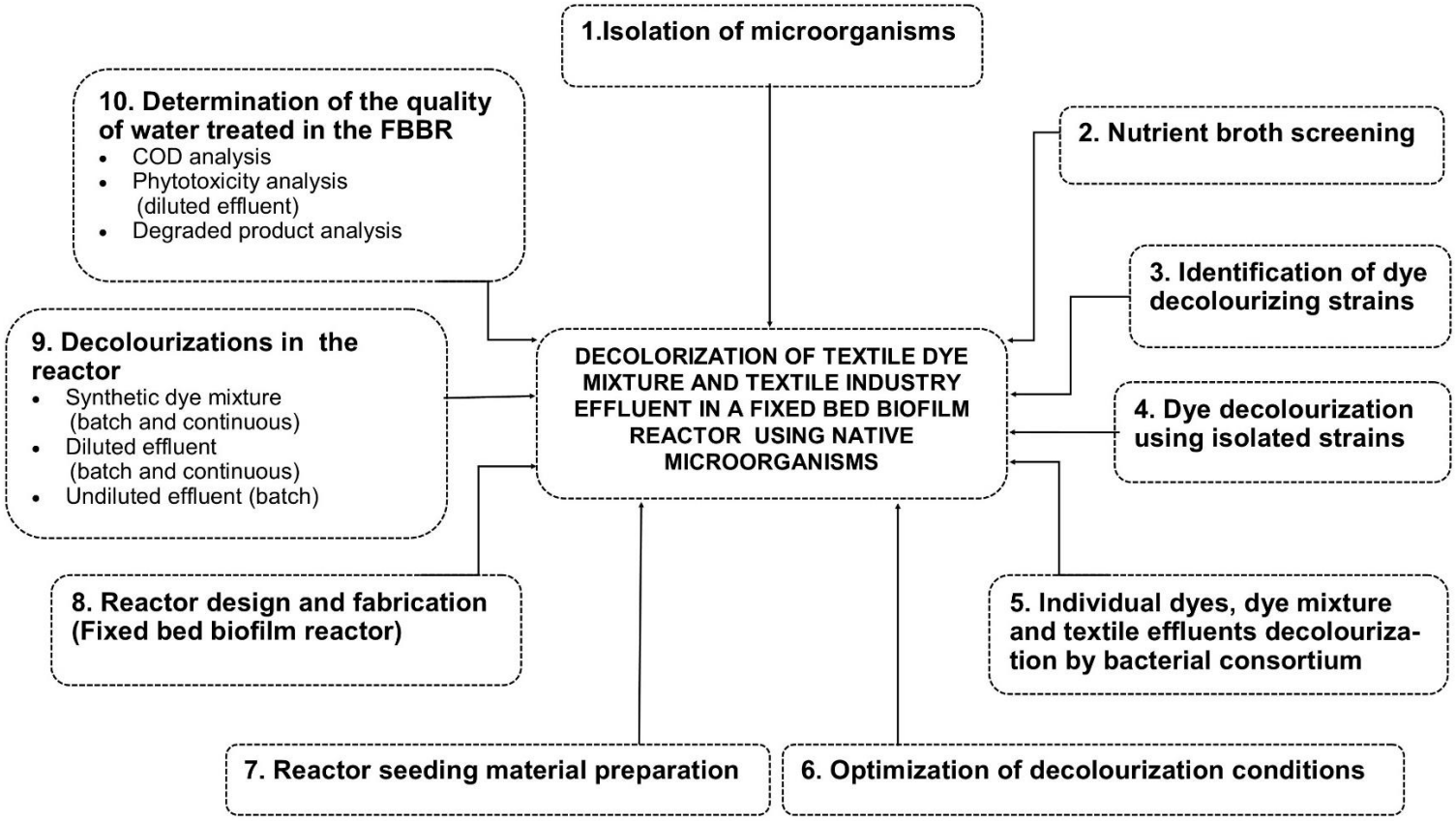


Figure 3.7: Conceptual diagram showing major experiments conducted in the study

CHAPTER 4

4 RESULTS AND DISCUSSION

Outcome of preliminary experiments conducted with isolated bacteria for decolourization of the selected textile dyes, optimum conditions required for maximum decolourization of selected dyes using the most effective dye decolourizing bacteria are investigated and presented. Furthermore, results of dye and effluent decolourization conducted in the fixed bed biofilm reactor and the quality of the treated water are presented and discussed.

4.1 Industrial Survey Questionnaire

4.1.1 Identification of the most widely used category of dyes in Sri Lankan textile industry

Industrial survey questionnaire was distributed among 15 textile-processing companies however, the feedback was received only from nine. Only five companies revealed the quantities of dyes used. Collected information revealed that local textile industries used, acid, solvent, vat, pigment, disperse and reactive dye categories in their dyeing process (Table 4.1).

Table 4.1: Consumption of dyes in textile processing companies in Sri Lanka (from industrial survey)

Company	Dye category	Quantity (kg/month)
B	Acid	1730
D	Reactive Disperse	39,181 1,114
F	Reactive Acid	500 1700
G	Reactive Pigment Direct	1000 1000 500
J	Acid	4000

Out of these, reactive dyes have been identified as the most widely used category of dyes in Sri Lanka Out of the nine, six companies have mentioned rhodamine as a type of dye that was difficult to eliminate with the available effluent treatment methods. Based on the information gathered, it was decided to evaluate the ability of biological methods for decolourization of reactive and rhodamine dyes.

4.1.2 Water consumption in textile dyeing and washing process

Textile industry is considered as one of the leading water consuming industries in the world. Quantity of water consumed in textile dyeing industry is highly dependent on the types of fiber used and the other parameters such as category of dyes, dyeing quantity, techniques used for the dyeing process. Hessel et al. [4] reported that reactive dyeing of 1 kg of cotton requires about 150 l of water, 0.6 kg of NaCl, and 40 g of reactive dyes. Rita Kant [1] reported that the daily water consumption of an average-sized textile mill having a production of about 8000 kg of fabric per day is about $1.6x10^6$ l. According to that report, approximately 200 l of water has been consumed for the fabrication of 1 kg of fabric.

Water consumption in Sri Lankan textile dyeing and washing industry was studied using Central Environmental Authority (CEA) data sources (Table 4.2). As shown in Table 4.2, daily wastewater generation in considered companies indicated huge variations in the range of 60-2800 m^3/day.

Information regarding the number of textile companies located in Sri Lanka with textile dyeing facilities was not available in literature. Therefore, distribution of textile dyeing facilities in the country was evaluated using the data available in CEA database.

According to the CEA database, industries and activities that require an environmental protection license (EPL) are classified into three lists as**,** "A", "B" and "C" depending on their pollution potential (Gazette notification No. 1533/16 published on 25/0/2008 under section 23A of the national environmental act, No. 47 of 1980). List "A" comprises of 80 significantly high polluting industrial activities. Most of the textile-related industries such as, Batik industries with 10 or more employees (A23) and textile processing (i.e. bleaching, dyeing, printing) industries, garment washing industries, textile sandblasting industries and commercial laundries with 10 or more

employees (A24) are included into the list A. Batik industries where less than 10 employees (B7) and garment industries where 25-200 workers per shift (B17) are included in list B.

Table 4.2: Water consumption and wastewater generation in textile processing companies in Sri Lanka

Company	**Product type/operation**	**Product quantity (kg/day)**	**Water consumption (m^3/day)**	**Water consumption (l/kg of product)**	**Wastewater (m^3/day)**
1	Textile dyeing	1,500	90	60	60
2	Polyester	3,000	105	35	100
3	NA	60,000	4000	67	2800
4	Washing (Denim, T-shirt)	NA	250	NA	245
5	Textile dyeing	NA	400	NA	350
6	NA	NA	3300	NA	2500
7	NA	NA	2450	NA	2275
8	NA	NA	113	NA	90
9	NA	NA	210	NA	120
10	NA	NA	1012	NA	1012

NA-Data not available

Distribution of selected categories of textile-related industries in Sri Lanka are shown in Table 4.3 [123].

Out of these different categories, textile dyeing, washing (A24) and batik industries (A23 and B7) are mainly responsible for coloured effluent generation. According to Table 4.3, 6, 26, and 160 textile-related industries are available under A23, B7 and A24 categories, respectively. However, the exact number of textile dyeing plants located in the country cannot be identified from this data source as sandblasting industries and commercial laundries are included in the same category, A24.

Table 4.3: Distribution of textile-related industries in Sri Lanka according to EPL listed classification

Industry catogery	Number of textile industries																								
	Western Province			Sothern Province			Central Province			Sabaragamuwa Province		Uva Province		Eastern Province			North Central Province		Nothern Province					North Western Province	
	Colombo	Kaluthara	Gampaha	Galle	Hambanthota	Mathara	Kandy	Matale	Nuwara Eliya	Kegalle	Rathnapura	Badulla	Monaragala	Ampara	Batticaloa	Trincomalee	Anuradhapura	Polonnaruwa	Jaffna	Mannar	Vavuniya	Mullativu	Kilinochchi	Kurunagala	Puttalam
A23	1	0	1	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A24	73	17	29	9	0	4	5	1	0	2	1	0	4	1	0	1	1	5	0	0	0	0	0	3	4
B7	1	1	3	4	0	3	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8
B13	73	43	64	6	8	10	38	6	0	9	2	8	0	4	1	0	0	1	0	0	2	0	0	31	6

Textile dyeing is a complex process that comprises of several intermediate steps and generates huge quantities of dye containing effluents. Special effluent treatment techniques are necessary for the decolourization of this coloured effluent. Due to these difficulties, most of the textile processing companies are prompted to use common textile dyeing facilities for dyeing process instead of an individual facility. Therefore, even though a large number of textile processing companies are located in Sri Lanka, textile dyeing is carried out only by a few major facilities. From the industry survey and CEA data sources, it was able to identify 15 dyeing facilities located in Sri Lanka.

4.1.3 Textile effluent decolourizing methods used in Sri Lanka

Based on the industrial survey results, effluent treatment techniques and dye decolourization techniques used in each textile company are shown in Table 4.4. Out of the nine considered companies, eight companies use chemical coagulation and flocculation technique for the decolourization of textile effluents while the other company uses electrocoagulation technique.

Table 4.4: Effluent treatment techniques used in textile dyeing facilities in Sri Lanka

Company	**Effluent treatment techniques**	**Effluent decolourization techniques**
A	Biological treatments (activated sludge system)	Coagulation and flocculation
	Coagulation and flocculation	
	Media filtration (activated carbon & anthracite)	
	Tertiary treatment for Recycling (ultra filtration & reverse osmosis)	
B	Equalization (pH adjusting)	Electrocoagulation
	High voltage charge	
	Dissolved air floatation tank	
	Mixed bed	
	Filter press	
C	Sedimentation	Coagulation and flocculation
	Screening	
	Coagulation and flocculation	
	Clarifier	
	Aerobic and anaerobic digestion	
D	Pre- Screening	Coagulation and flocculation
	Coagulation and flocculation	
E	Sedimentation	Coagulation and flocculation
	Coagulation and flocculation	
	Chemical treatments	
F	Activated sludge extended aeration system and Dissolved air floatation (DAF) type chemical treatments	Coagulation and flocculation
G	Biological treatment (Activated sludge system)	Coagulation and flocculation
	Coagulation and flocculation	
	Clarifier	
	Settling Tank	
I	Chemical treatments and filter press	Coagulation and flocculation
J	Chemical addition wastewater treatment plant (Coagulation and flocculation)	Coagulation and flocculation

However, coagulation and flocculation technique agglomerates dyes, and the secondary sludge produced should be further treated or disposed according to regulations. This dye-containing sludge produced in most Sri Lankan textile industrial wastewater treatment facilities are out-sourced for further treatments. The common treatment method employed is incineration, which is not economically feasible. Some companies dump this dye-containing solid sludge to separately allocated lands without proper treatments. Hence, there is a threat of re-dissolving dyes in rainwater, contaminating the soil and the natural water bodies.

4.1.4 Textile effluent characteristics

Most of the textile processing companies in Sri Lanka are located in industrial zones where the treatment of the generated effluent is generally carried out in common wastewater treatment plants. However, some companies that are located outside the industrial zones operate their own effluent treatment plants. Both these categories have effluent discharge tolerance limits depending on the location of discharge (Table 4.5). As shown in Table 4.5, the maximum allowable discharge limits for coloured effluent are equal for both categories despite the discharge location. Common wastewater treatment plants located in industrial zones do not have facilities for dye decolourization. Hence, the textile dyeing companies should reduce the colour of the effluent up to the permitted level within in the factory premises prior to discharge into common wastewater treatment plants.

Information gathered from industrial survey questionnaire and CEA data sources regarding the effluent quality characteristics of considered textile dyeing companies are shown in Figure 4.1-4.8. These figures indicate the temperature, pH, COD, BOD and colour of textile effluents before and after wastewater treatments.

Table 4.5: Tolerance limits for the textile industry effluents discharges (for the selected quality parameters) in Sri Lanka

Parameter	**Maximum tolerance limit[a]**		**Maximum tolerance limit[b]**	
BOD (5 days at 20 ^{o}C) (mg/l)	200		60	
COD (mg/l)	600		250	
pH	6.0-8.5		6.5-8.5	
Temperature (^{o}C)	40		40	
	Wavelength range	Maximum spectral absorption coefficient	Wavelength range	Maximum spectral absorption coefficient
	400 – 499 nm (Yellow range)	7 m^{-1}	436 nm (Yellow range)	7m^{-1}
	500 – 599 nm (Red range)	5 m^{-1}	525 nm (Red range)	5m^{-1}
	600 – 750 nm (Blue range)	3 m^{-1}	620 nm (Blue range)	3m^{-1}

a -Tolerance limits for industrial wastewater discharged into the common wastewater treatment plant [124]

b -Tolerance limits for wastewater from textile industry being discharged into inland surface waters (Gazette notification No. 1534/18 published on 01/02/2008 under Section 32 read with Section 23A and 23 B of the National Environmental Act, No. 47 of 1980)

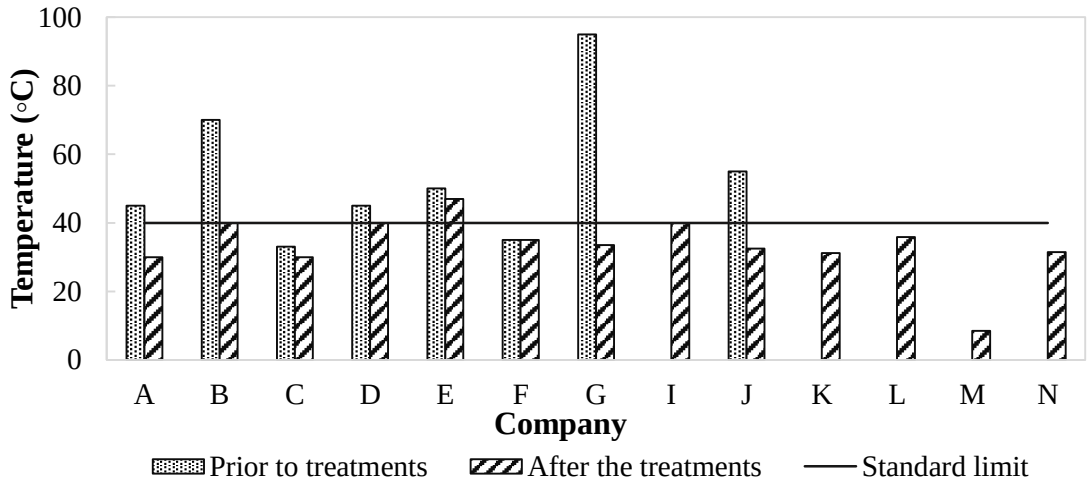


Figure 4.1: Temperature variation of textile industry effluent

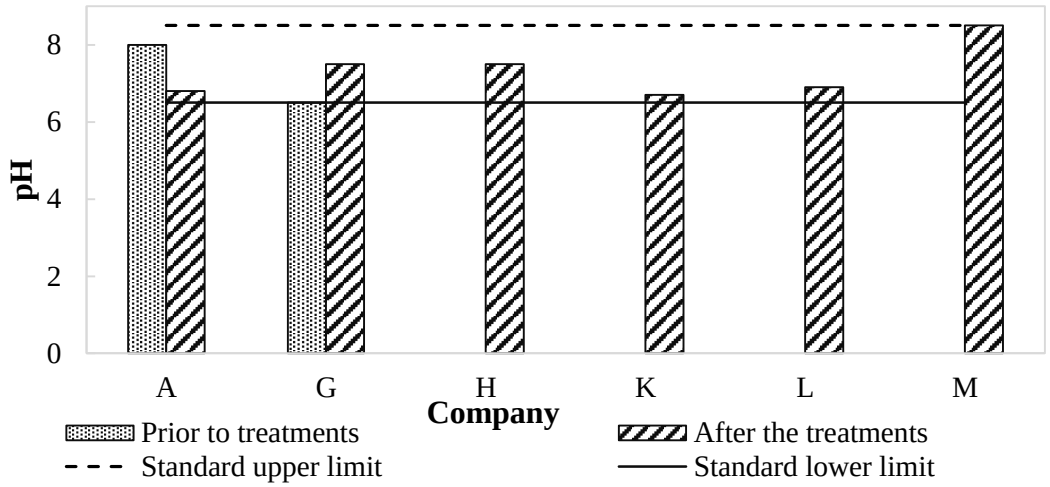


Figure 4.2: pH variation of textile industry effluent being discharged into the inland surface waters

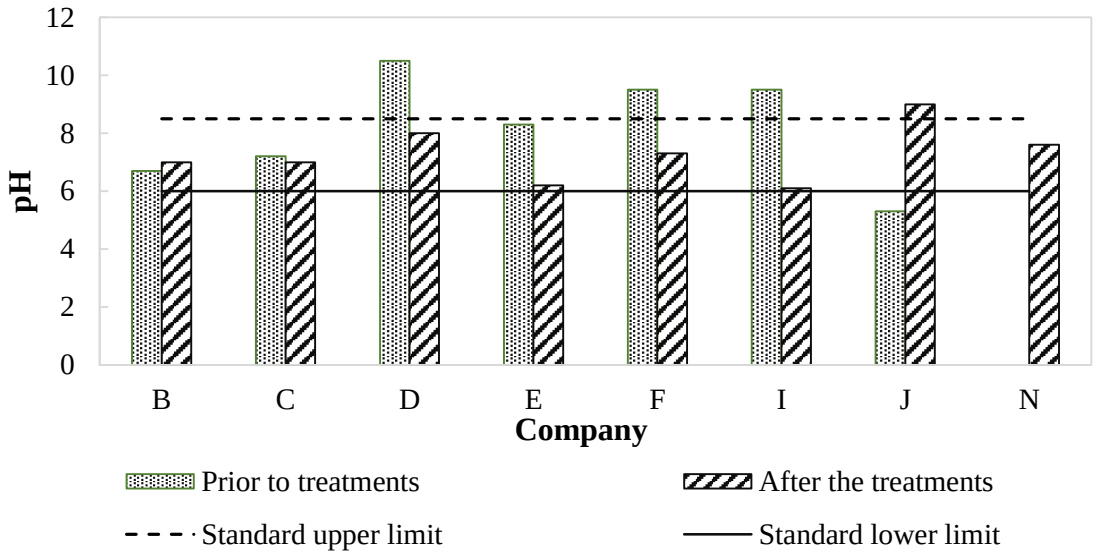


Figure 4.3: pH variation of textile industry effluent being discharged into the common wastewater treatment plant

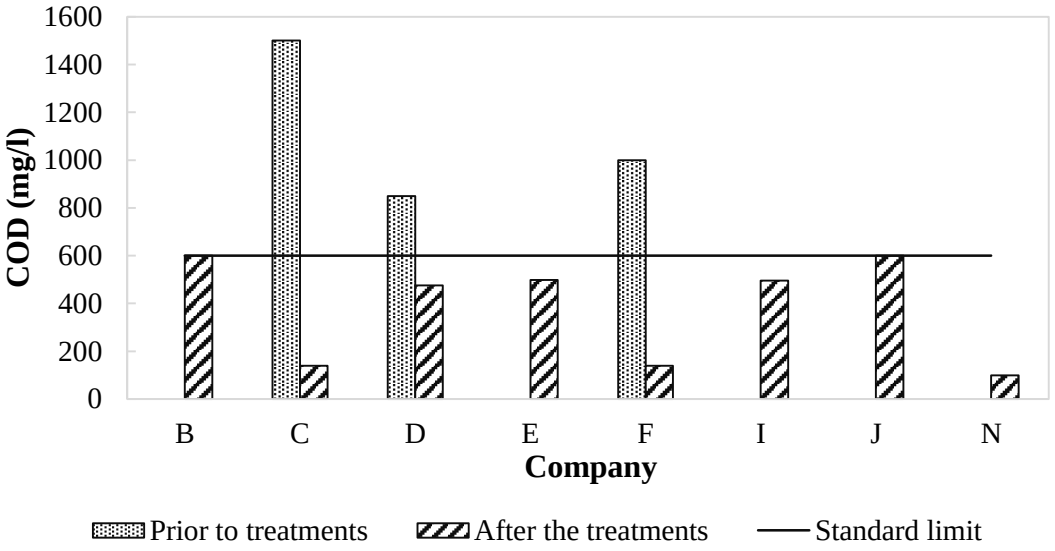


Figure 4.4: COD variation of textile industry effluent being discharged into the common wastewater treatment plant

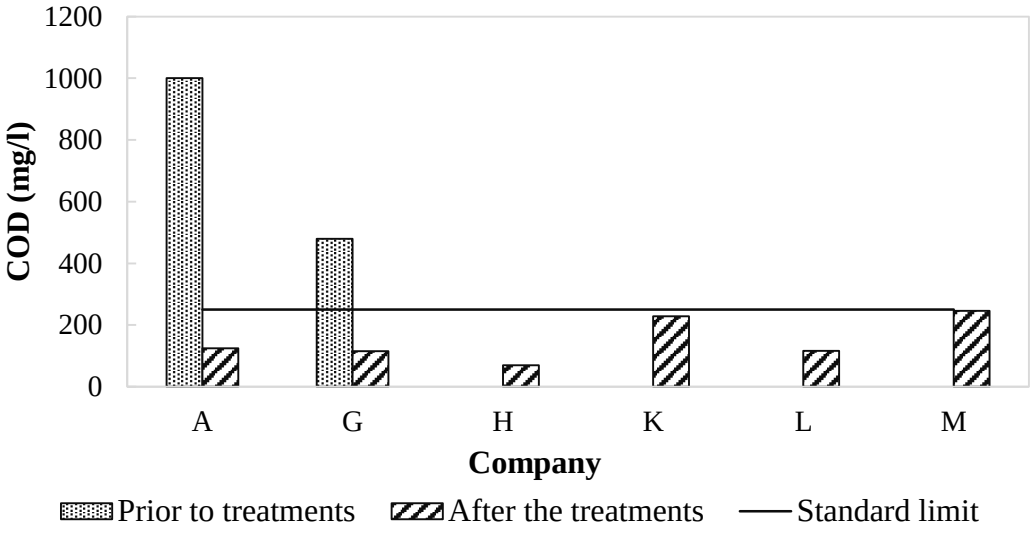


Figure 4.5: COD variation of textile industry effluent being discharged into the inland surface waters

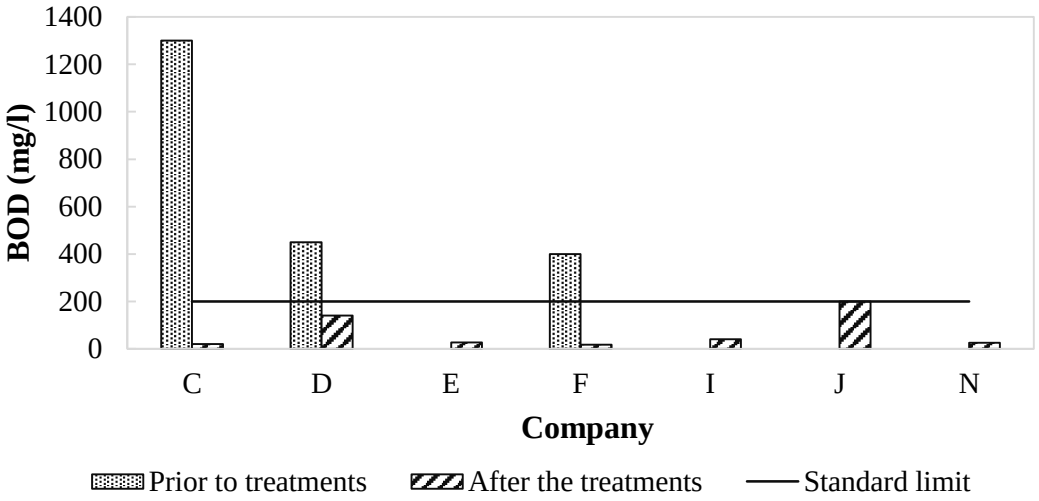


Figure 4.6: BOD variation of textile industry effluent being discharged into the common wastewater treatment plant

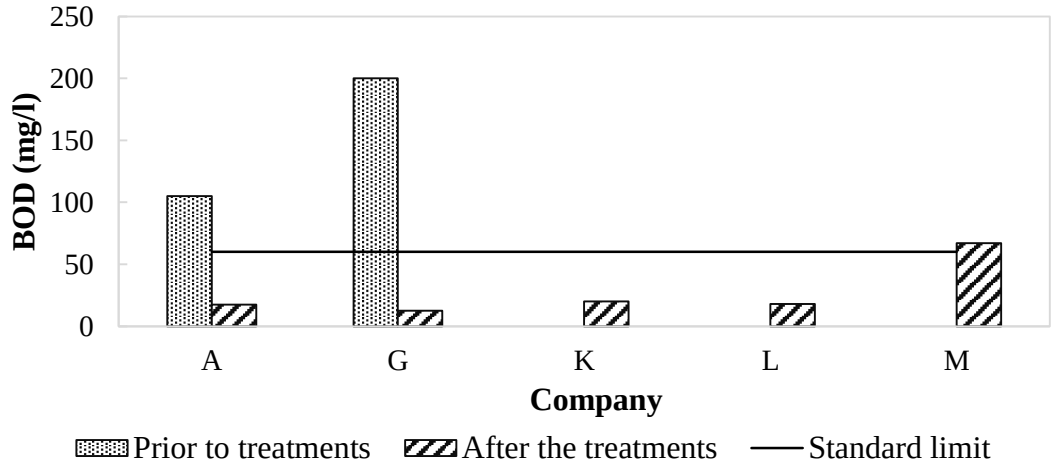


Figure 4.7: BOD variation of textile industry effluent being discharged into the inland surface waters

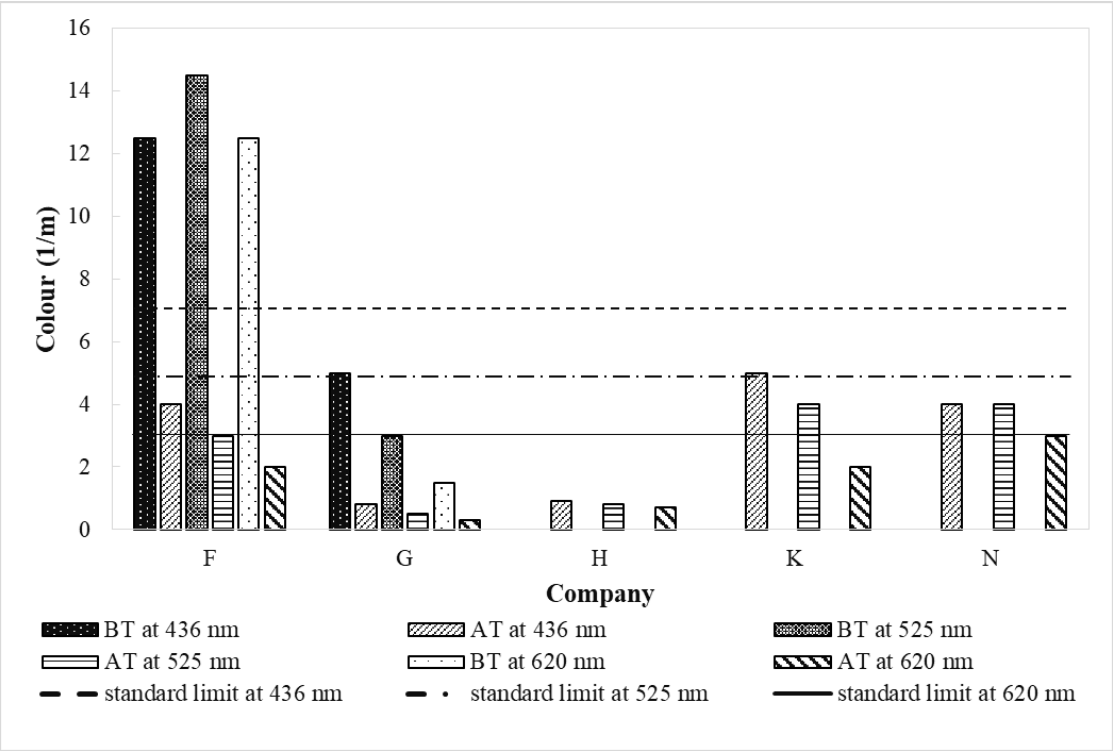


Figure 4.8: Colour variation of textile industry effluent (where, AT: after the treatments, BT: before the treatments)

Before any treatments, significant variations of effluent quality parameters can be observed in different companies (Figures 4.1- 4.8). According to the data provided, majority of the textile dyeing companies considered in this study treated their effluent up to permissible discharge limits. However, proper monitoring of these industrial discharges is necessary to maintain the environment sustainability.

4.2 Isolation of Indigenous Microbial Strains with Dye Decolourization Potential

4.2.1 Isolation of microorganisms by spread and streak plate techniques

Temperature of the sludge containing effluent sample at the time of collection was 39 ^{o}C and the pH and BOD values were 7.7 and 120 mg/l respectively.

Spread and streaked plates prepared during the isolation of microorganisms (section 3.7.3) are shown in Figure 4.9 and 4.10.

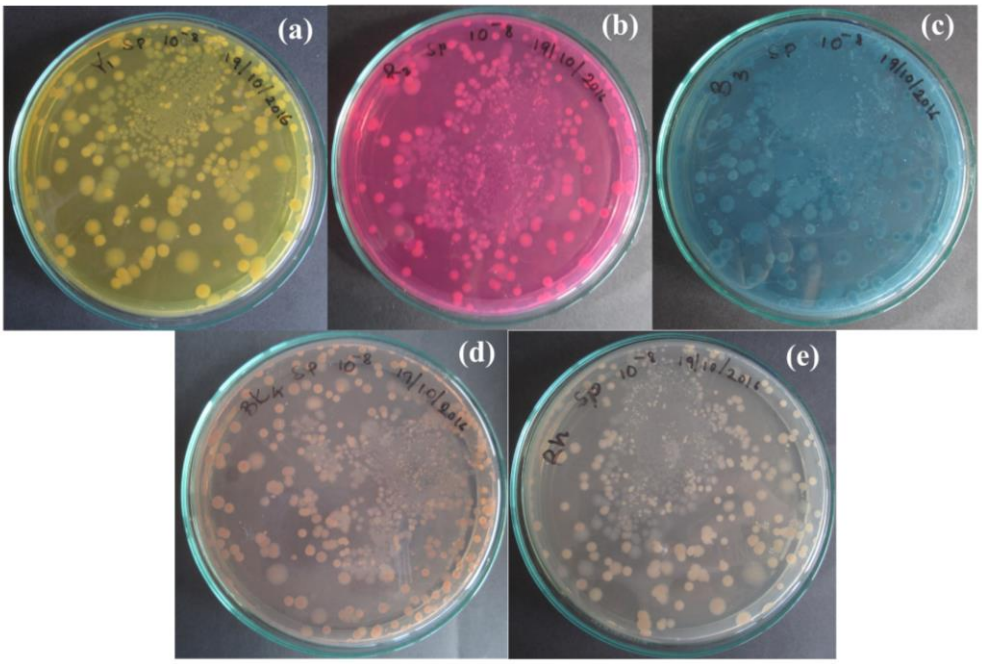


Figure 4.9: Bacterial growth on dye-amended nutrient agar medium (a) Yellow EXF (b) Red EXF (c) Blue EXF (d) Black WNN and (e) Rhodamine

Altogether 15 bacterial strains (Table 4.6) were isolated from Yellow EXF, Blue EXF, Black WNN and Rhodamine dye-containing plates and used for the nutrient broth screening.

Table 4.6: Bacteria isolated from each dye-amended medium with code numbers

Dye	Number of bacteria isolated	Assigned code numbers
Yellow EXF	7	1,2,3,4,18,19,20
Red EXF	5	5,6, 7, 18,19
Blue EXF	7	8,9,10,11,18,19,20
Black WNN	5	12,13,18, 19, 20
Rhodamine	6	14,15,16,17, 20

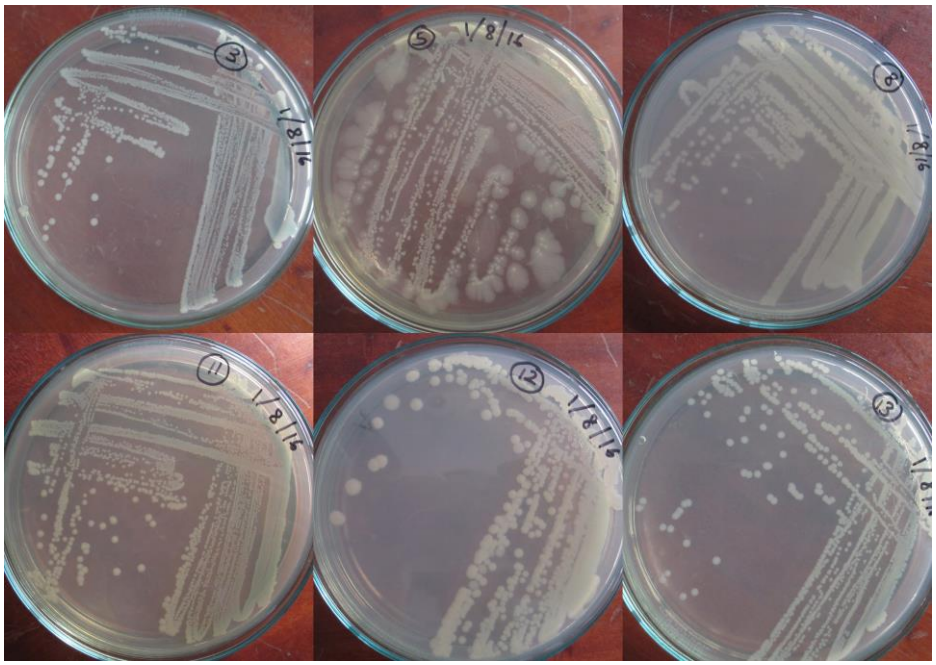


Figure 4.10: Agar plates streaked with some of the isolated bacteria

4.3 Nutrient Broth Screening for Dye Decolourizing Isolates

Figure 4.11 shows test tubes containing Yellow EXF dye decolourized by all the seven bacterial strains isolated from Yellow dye containing agar plates (isolates 1, 2, 3, 4, 18,19 and 20)

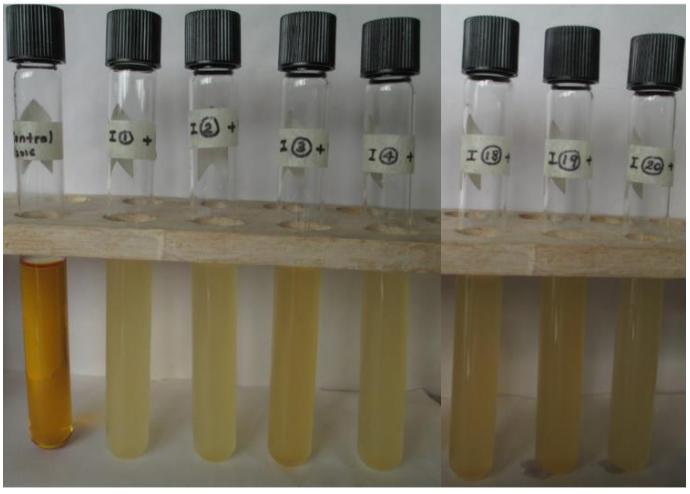


Figure 4.11: Yellow EXF dye-containing nutrient media inoculated with isolates 1, 2, 3, 4, 18, 19 and 20 after 4-days of incubation and control (first tube starting from left hand side)

Red EXF dye was completely decolourized by isolate 5 and slightly decolourized by isolate 19 (Figure 4.12).

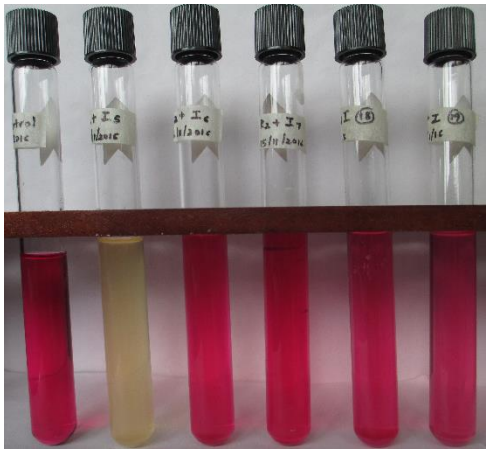


Figure 4.12: Red EXF dye-containing nutrient media inoculated with isolates 5, 6, 7, 18 and 19 after 8-days of incubation and control (first tube starting from left hand side)

As shown in Figure 4.13, Blue EXF dye was partially decolourized by isolates 10, 11 and 19.

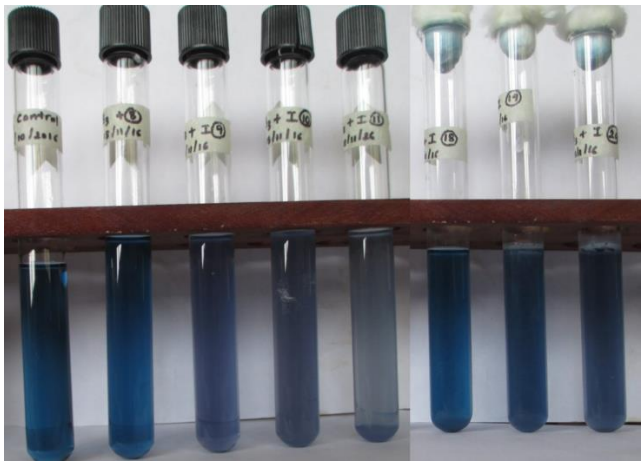


Figure 4.13: Blue EXF dye containing nutrient media inoculated with isolates 8, 9, 10, 11, 18, 19 and 20 after 10 days of incubation and control (first tube starting from left hand side)

Black WNN dye was decolourized by isolates 12, 13, 18 and 19 as shown in Figure 4.14.

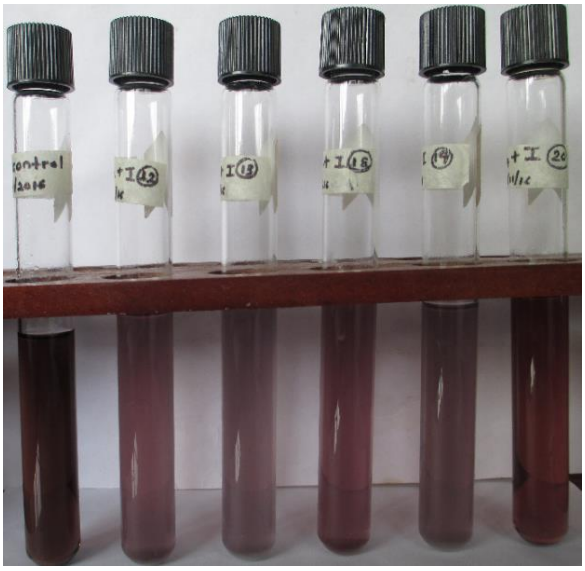


Figure 4.14: Nova Black WNN dye-containing nutrient media inoculated with isolates 12, 13, 18, 19 and 20 after 10-days of incubation and control (first tube starting from left hand side)

Rhodamine dye containing tubes did not indicate visible colour reduction in nutrient broth screening. Further, due to the pale colour of this dye, visual identification of colour reduction was difficult. However, the turbidity of the medium has increased due to bacterial growth and therefore, a colour change from pale pink to white was observed in some tubes (Figure 4.15).

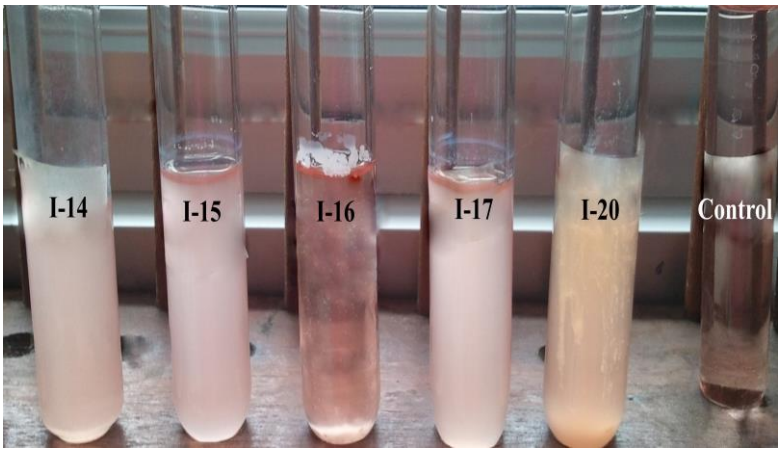


Figure 4.15: Rhodamine dye-containing nutrient media inoculated with isolates 14, 15, 16, 17 and 20 after 10-days of incubation and control (first tube starting from right hand side)

Fifteen bacterial isolates which indicated dye decolourizing potential were recognized by the nutrient broth screening (Table 4.7) and selected for the decolourization studies.

Table 4.7: Bacterial isolates capable of decolourizing each dye as per the nutrient broth screening

Dye	Isolates
Yellow EXF	1, 2, 3, 4, 18, 19, 20
Red EXF	5, 19
Blue EXF	10, 11, 19
Black WNN	12, 13, 18, 19
Rhodamine	14, 15, 17, 20

4.4 Identification of Dye Decolourizing Bacterial Strains

4.4.1 Biochemical tests and morphological characteristics

When these selected strains were investigated for colony morphology, there were two distinct groups. First group of bacteria indicated rapid growth on agar plates where colonies were observed after 24 h incubation. However, the second group (isolates 1,

2, 5, 10 and 15) took 48 h for the formation of colonies on agar plates. Colonies formed by this second group of bacteria were smooth, shiny and cream in colour and concentric rings were observed around the colony due to swarming mortality of these strains. Colonies of former group (isolates 3,4,11,12,13,14,17,18,19 and 20) were also smooth, shiny and cream in colour but concentric rings were not observed. Margins formed by isolate 19 were irregular in shape while others were round. Based on morphological and biochemical tests and nutrient broth screening results, the numbers of different bacterial strains isolated from all dye containing samples were reduced to seven (1, 4, 11, 13, 18, 19 and 20).

Cell morphologies of some of the isolates observed through the optical microscope after staining tests are shown in Figures 4.16-4.18.

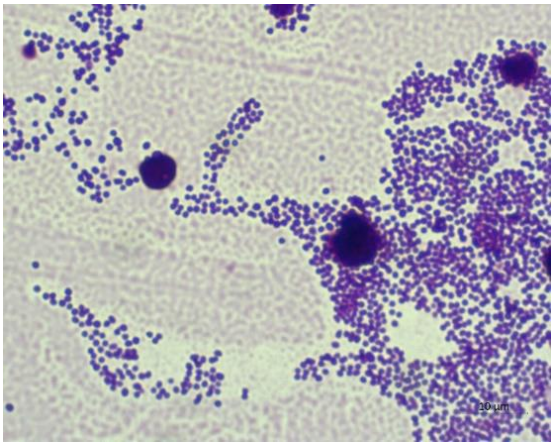


Figure 4.16: Capsule-staining image of isolate 20 (x100)

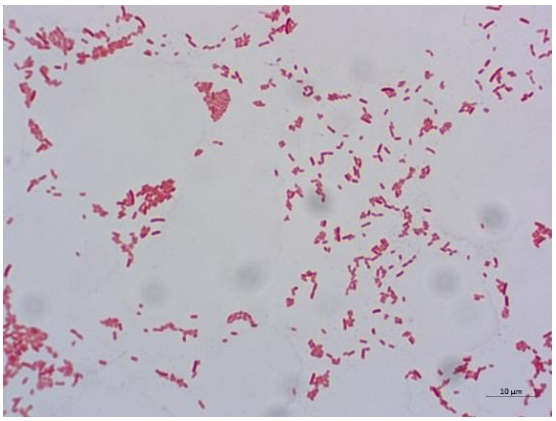


Figure 4.17: Gram-staining response of isolate 19 (x100)

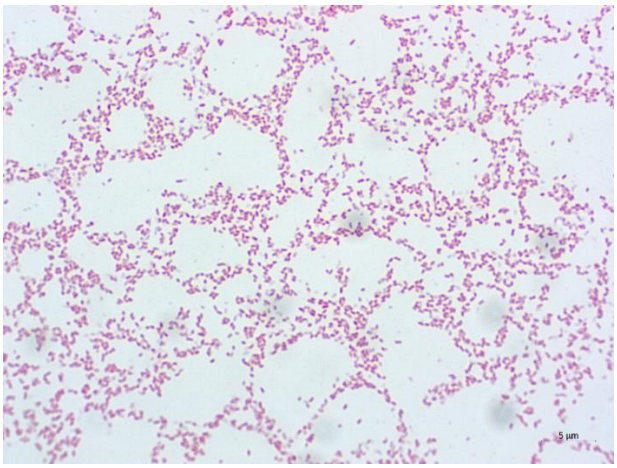


Figure 4.18: Endospore staining image of isolate 1 (x100)

4.4.2 16S rRNA gene sequencing analysis

Gel image of the amplified DNA samples of the seven bacterial species is shown in Figure 4.19. The size of the DNA samples was determined as 1500 base pairs compared to 1kb ladder.

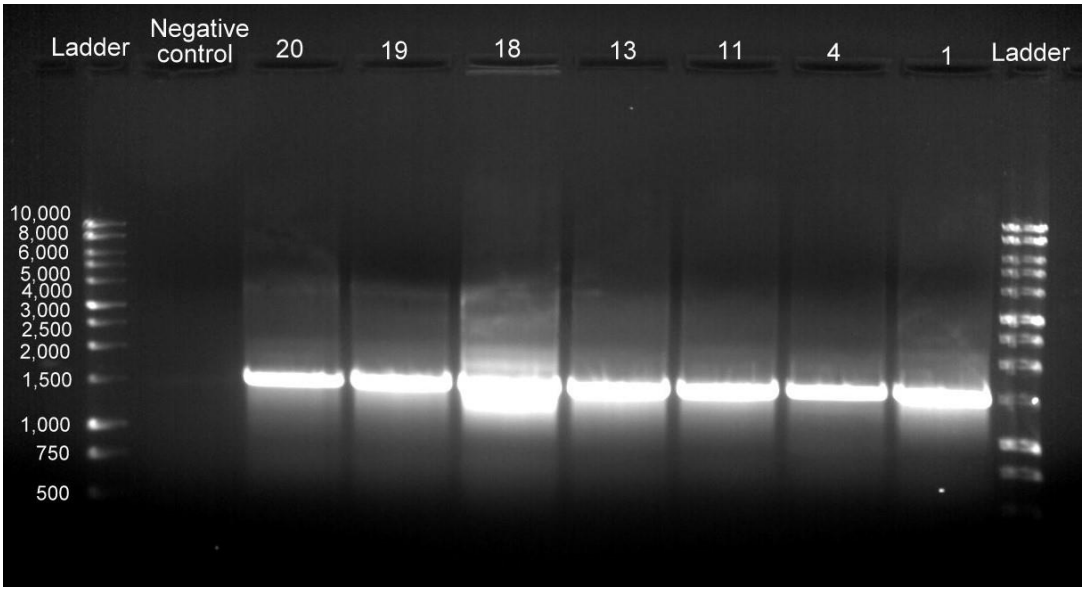


Figure 4.19: Gel image of the amplified DNA samples of the isolated bacteria

Blasting results showed similarities between isolates 4 and 13 and isolates 11 and 18. Ultimately, five different bacterial species were identified and their names are shown in Table 4.8.

Table 4.8: Description of the identified bacterial isolates

New Code	Previous code (isolates)	Bacterial isolates	% similarity	E value	Accession
I_1	1	*Proteus mirabilis*	99.92	0.0	MT138732
I_2	4, 13	*Escherichia fergusonii*	99.59	0.0	MT186253
I_3	11, 18	*Morganella morganii*	99.19	0.0	MT186254
I_4	19	*Enterobacter cloacae*	99.73	0.0	MT186255
I_5	20	*Acinetobacter baumannii*	99.00	0.0	MT186256

A summary of the biochemical test results is shown in Table 4.9.

Table 4.9: Biochemical test results of isolated bacterial strains

Bacterial isolate (Code)	Cell shape	Gram staining reaction	Catalase activity	Presence of a capsule	Formation of endospores
I_1	Rod	Negative	Positive	ND	ND
I_2	Rod	Negative	Positive	ND	ND
I_3	Rod	Negative	Positive	ND	ND
I_4	Rod	Negative	Positive	ND	ND
I_5	Short rod	Negative	Positive	Detected	ND

ND-Not detected

4.5 Dye Decolourization

4.5.1 Decolourization of individual textile dyes by isolated bacteria

Bacterial isolates that were selected from the nutrient broth screening (section 4.3) were used for the decolourization studies.

Yellow EXF decolourization was conducted with strains I_1, I_3, I_4 and I_5 and I_1 indicated the highest decolourization. I_1 and I_3 indicated more than 80 % colour reduction after 24 h incubation while, I_4 and I_5 exhibited less than 40% (Figure 4.20). Except I_5, all the other strains were able to decolourize Yellow EXF up to 95 % after 72 h incubation. Compared to other strains, I_5 indicated the lowest color removal of 73% after 72 h incubation.

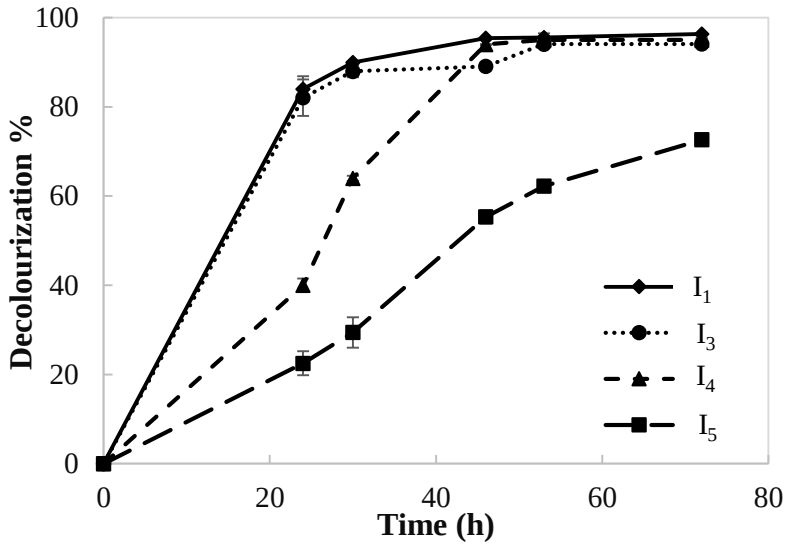


Figure 4.20: Effect of bacterial isolates and incubation time on decolorization of Yellow EXF dye

Out of the five isolated bacterial strains, *Acinetobacter baumannii* is an aerobic bacterium whereas all the other four strains are facultative anaerobes. During this study, dye decolourization investigations were conducted under static conditions where oxygen concentrations in the medium were low. This oxygen-limited condition may have negatively affected the growth of aerobic bacterium, *A. baumannii,* than the other bacterial strains. This could be the reason for observed low percentage color removals by *A. baumannii* in decolourization of Yellow EXF [125]. However, when

decolourization studies were conducted under shaking conditions *A. baumannii* indicated only 3% decolourization of Yellow EXF (results not shown). Even though high cell growth was observed, dye decolourization was not effective under shaking conditions. Conversely, Ning et al. [126] reported an effective decolourization of Congo red dye using this bacteria under aerobic conditions.

Red EXF decolourization studies were conducted with the isolated strains I_1 and I_4. I_1 indicated 65% and 94% colour removals after 24 h and 72 h incubation times respectively (Figure 4.21). During the first 24 h incubation, color removal by I_4 was low but, decolourization percentage increased with time and 89% reduction was observed after 72 h.

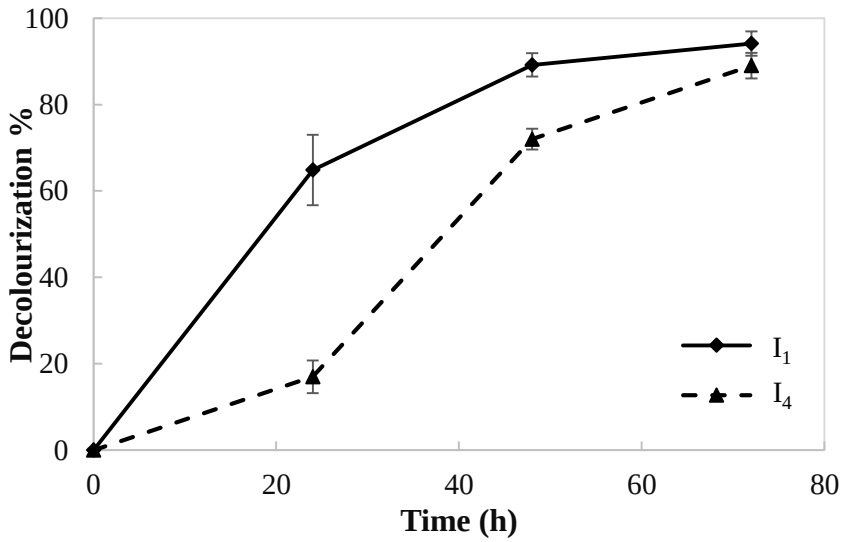


Figure 4.21: Effect of bacterial isolates and incubation time on decolorization of Red EXF dye

Time-dependent decolourization of Blue EXF was studied with the isolated strains I_1, I_3 and I_4. As shown in Figure 4.22, from the identified bacterial strains, I_1 indicated the highest color removal of 74% after 24 h. At the end of 72 h incubation 83%, 45% and 63% decolourization of Blue EXF were observed in the flasks inoculated with I_1, I_3 and I_4 respectively.

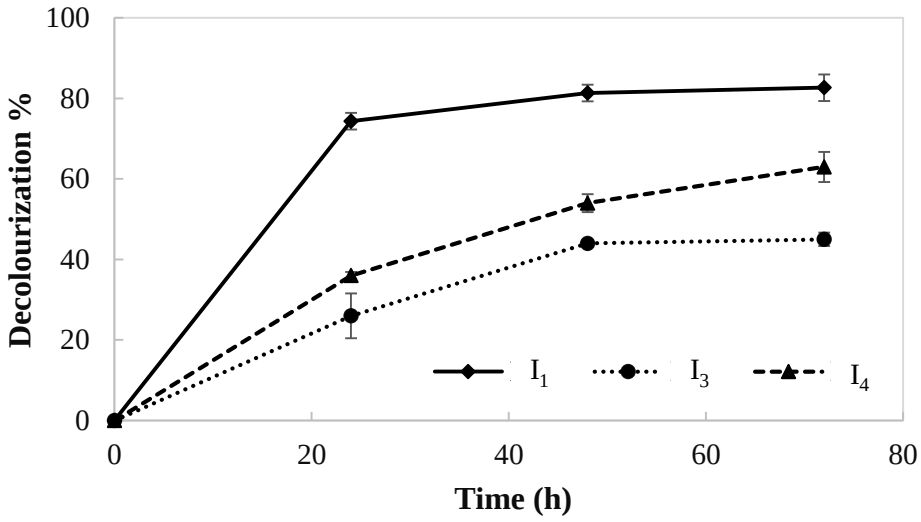


Figure 4.22: Effect of bacterial isolates and incubation time on decolorization of Blue EXF dye

Bacterial strains, I_1, I_2, I_3 and I_4 were used for the Black WNN decolourization studies and considerable removal of the dye was observed (Figure 4.23).

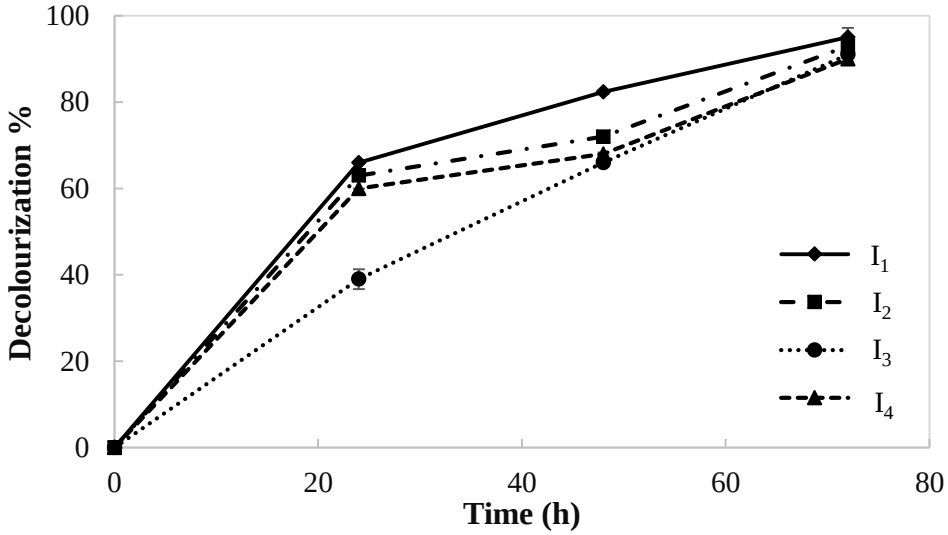


Figure 4.23: Effect of bacterial isolates and incubation time on decolorization of Nova Black WNN dye

Other than I_3, all the strains were able to decolourize more than 60% colour of the dye within 24 h. Black WNN was effectively decolourized by all the bacterial isolates and obtained more than 90% removal of color after 72 h incubation.

Based on the survey results, rhodamine has been identified as a group of dyes that is difficult to decolourize by conventional effluent treatment techniques (mainly coagulation and flocculation) used in Sri Lanka (section 4.1.1). Biological decolourization of rhodamine dyes are rarely reported in literature. However, Fulekar et al. [127] reported a maximum 56% decolourization of Rhodamine B dye by *Pseudomonas monteilii*.

Rhodamine used in this study was an acid dye [128] with xanthene structure. Bacterial strains I_1 and I_5 were used for Rhodamine decolourization studies. I_1 was able to decolourize 30% of Rhodamine dye, however, colour removal by I_5 was negligible and therefore not shown in Figure 4.24.

Maximum spectral absorption coefficient of the Rhodamine dye (50 mg/l), containing media after 72 h incubation with I_1 (calculated using equation 4) are shown in Table 4.10. Even though percentage removal of this dye was low, maximum spectral absorption coefficient of the biologically treated samples almost satisfies textile industry effluent discharge tolerance limits (Table 4.10).The UV-visible absorbance values or visible colour generated by this dye was comparably low to the other dyes considered at similar concentrations. Hence, the presence of trace quantities of this dye in effluent discharge locations cannot be identified by observation through naked eye.

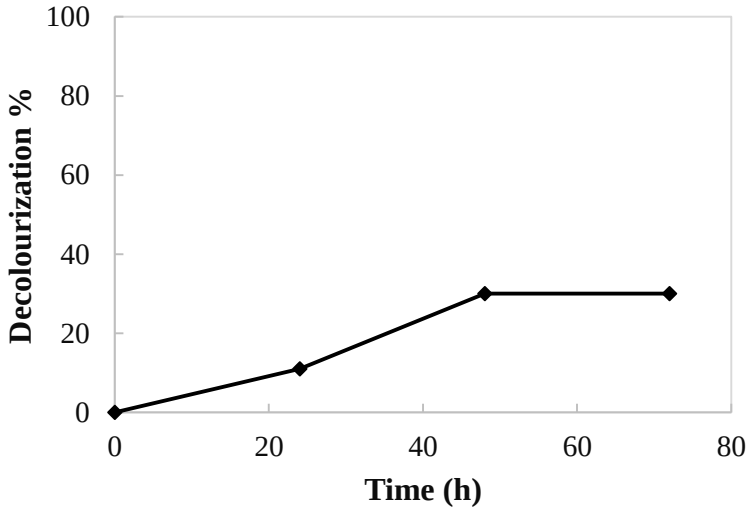


Figure 4.24: Effect of incubation time on decolorization of Rhodamine dye by *P. mirabilis*

Table 4.10: Comparison of Rhodamine dye decolourization results of *P. mirabilis* with effluent discharge limits

Sample	Wavelength	Maximum spectral absorption coefficient (m^{-1})		
		Before treatments	**After 72 h treatments**	**Tolerance limit**
Rhodamine (50 mg/l)	436 nm	11.8	3.3	7
	525 nm	14.8	8.0	5
	620 nm	6.9	1.7	3

Based on the results obtained in decolourization studies, I_1 has been identified as the strain with highest capability to decolourize dyes out of the five isolated bacteria. This bacterial species was able to decolourize more than 80% colour of all four reactive dyes used in the study after 72 h incubation.

Decolourization ability of different other dyes by *P. mirabilis* (I_1) have previously been reported in literature. Chen et al. [129] has reported *P. mirabilis* as a dye decolourizing microorganism considering its ability to decolourize Red RBN dye. Olukanni et al. [14] and Sonia et al. [130] reported the decolorization of Reactive Blue 13 and Light Red dyes by *P. mirabilis*. Further, Joshi et al. [131] reported the Acid Orange 7 and several other azo dye decolourization ability of a bacterial consortium with *P. mirabilis* as a member. Results obtained during this study and the reported literature proves the ability of *P. mirabilis* to decolourize different textile dyes effectively.

Ability of a bacterial consortium that containing *M. morganii* to decolourize Reactive Blue 59 [132] and Red HE3B and several other reactive dyes was reported by Patil et al. [133]. Dye decolourizing potential of *E. cloacae* was reported by Prasad and Aikat [134], considering its ability to decolourize Congo red. Further, Moawad et al. [135] reported the decolourization of reactive red dye by *E. cloacae.*

Textile dye decolourization ability of *E. fergusonii* is not widely reported in literature. However, Pan et al. [136] have detected this bacterial strain in a UASB reactor system which treated Brilliant red X-3B dye. In the current study, this strain was able to decolourize Black WNN dye effectively.

Even though the dye decolourization ability of these isolated bacteria was previously reported in literature, none of these species have been previously isolated from local

textile industrial wastewater treatment plants. Therefore, these bacteria have not been identified as potential dye decolourizing organisms in Sri Lanka. Hence, the results obtained from this study will be beneficial when implementing biological dye decolourization techniques in local effluent treatment facilities.

4.5.2 UV- visible spectrophotometric analysis of dye decolourization

Textile dyes are coloured substances which absorb light at the visible range of the spectrum at certain wavelengths [5]. Depending on the structural properties, each dye indicates the maximum absorbance peak at a specific wavelength of the visible spectrum. Biological decolourization may result in the reduction of absorbance values of the dyes at its maximum wavelength.

Biological decolourization of textile dyes can be due to biodegradation or biosorption or a combination of both [9]. In this study, when the supernatant was separated by centrifugation (24 h decolourized samples), it was clearly seen that the microbial cells in the flask containing blue dye were coloured blue. From this observation, it can be assumed that the colour of the Blue EXF has been initially adsorbed by the microbial cells (biosorption). However, microbial cells separated from the remaining flasks were not coloured and hence, it can be considered that the colour removal from the contents in those flasks were not due to biosorption. Hence, there is a strong possibility that the colour giving compounds have undergone a structural change due to microbial activity (biodegradation) and the degraded compounds do not show the original colour. Figures 4.25-4.28 show the UV-visible spectra of four dyes before and after decolourization with I_1, the isolate that showed the best performance. Yellow EXF indicated a peak at 422 nm before the treatment, however, after the biological treatment, this peak was no longer observed and a new peak formed at 364 nm (Figure 4.25). Red EXF displayed two peaks at 522 nm and 544 nm but these peaks have disappeared with incubation, forming a new peak at 439 nm (Figure 4.26). Blue EXF dye solution showed the maximum absorbance peak at 606.5 nm. However, with biological treatment, this peak gradually reduced and after 72 h incubation, a minor peak formed at 401 nm (Figure 4.27). Maximum absorbance peak of Black WNN solution can be seen at 598 nm and after decolourization with *P. mirabilis;* a slight

peak was observed at 405 nm (Figures 4.28). As the Rhodamine dye was decolourized partially (30%), a slight reduction in the absorbance peak at 563.5 nm was detected after 72 h incubation (Figure 4.29).

If decolourization was due to biosorption, absorption peaks decrease proportionately [137], whereas in biodegradation, either the major peak in the visible region completely disappears or gives rise to a new peak [138] as a result of formation of degraded compounds. In biosorption, dye structures do not change and hence formation of new compounds (peaks) is not visible in UV-visible spectrum. However, biosorption may lower the colour in the supernatant resulting in a reduction of absorbance values. Disappearance of major peaks and formation of new peaks in the visible region are observed with Yellow EXF, Red EXF, Blue EXF and Black WNN dyes showing biodegradation of these dyes. Proportional reduction of the peak, without forming new peaks within initial 24 h incubation, agrees with initial entrapment of dyes into the microbial cells of Blue EXF dye (Figure 4.31) [139].

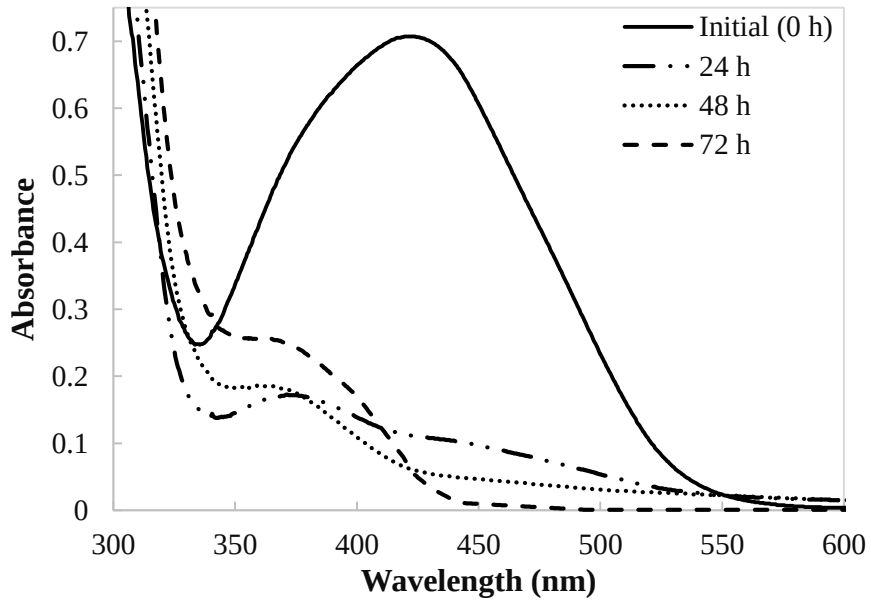


Figure 4.25: Variation of UV-visible spectra of Yellow EXF dye solutions treated with *P. mirabilis* for different time intervals under static conditions

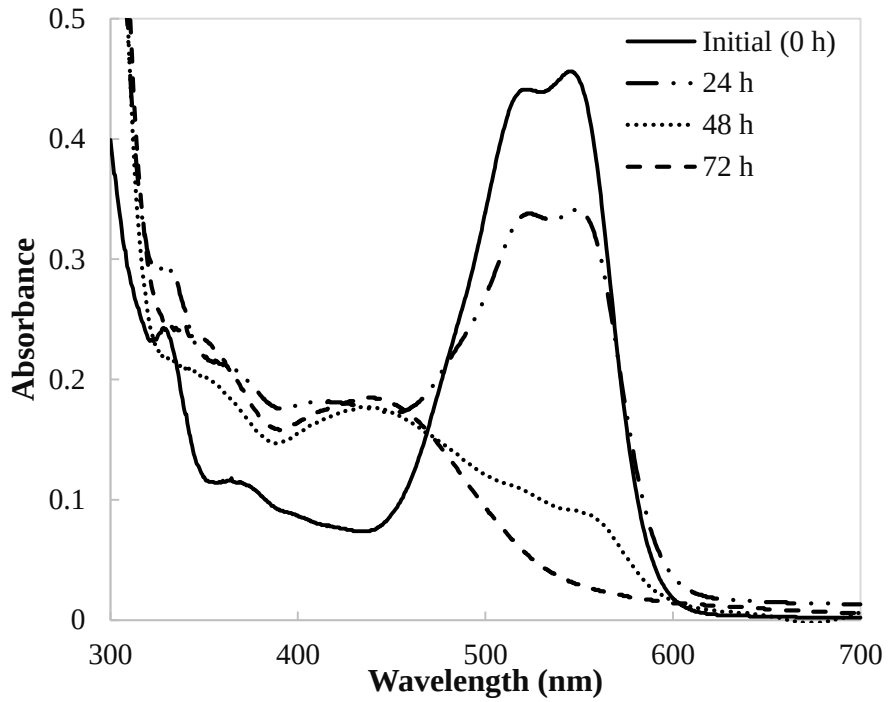


Figure 4.26: Variation of UV-visible spectra of Red EXF dye solutions treated with *P. mirabilis* for different time intervals under static conditions

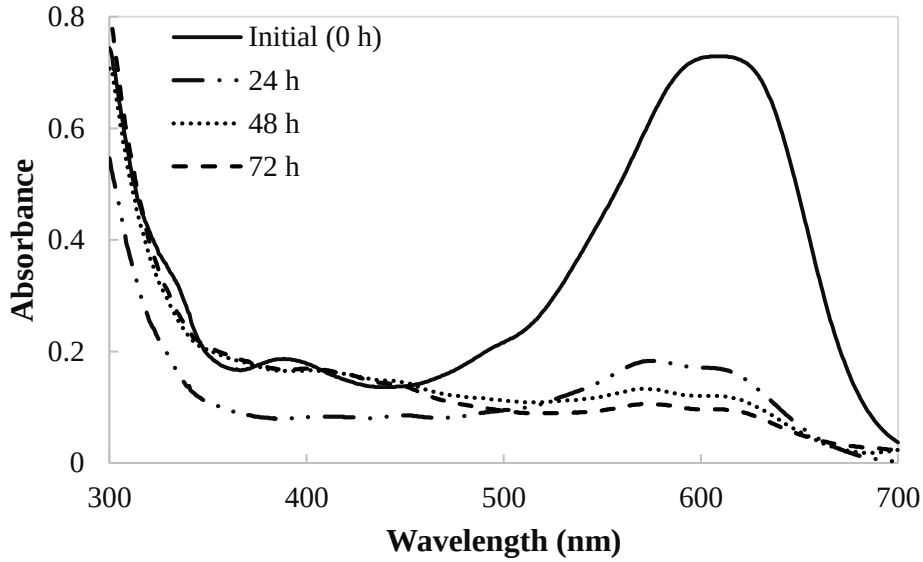


Figure 4.27: Variation of UV-visible spectra of Blue EXF dye solutions treated with *P. mirabilis* for different time intervals under static conditions

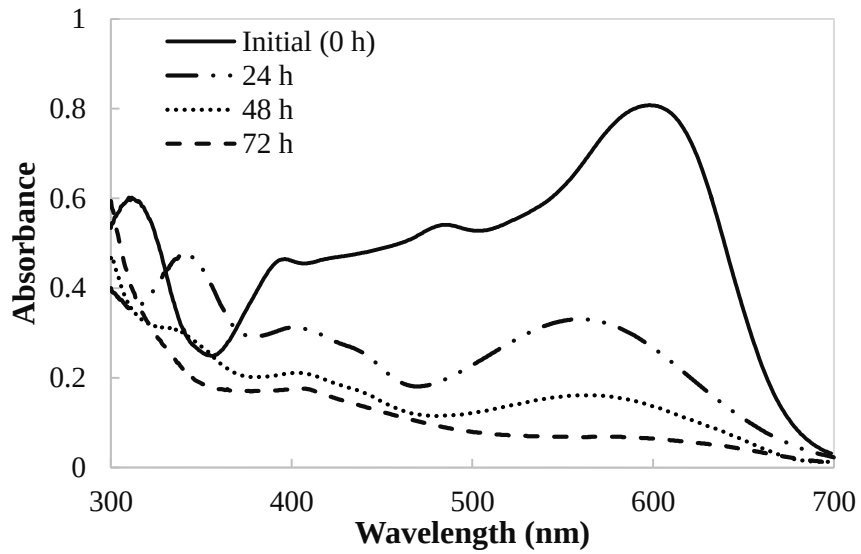


Figure 4.28: Variation of UV-visible spectra of Black WNN dye solutions treated with *P. mirabilis* for different time intervals under static conditions

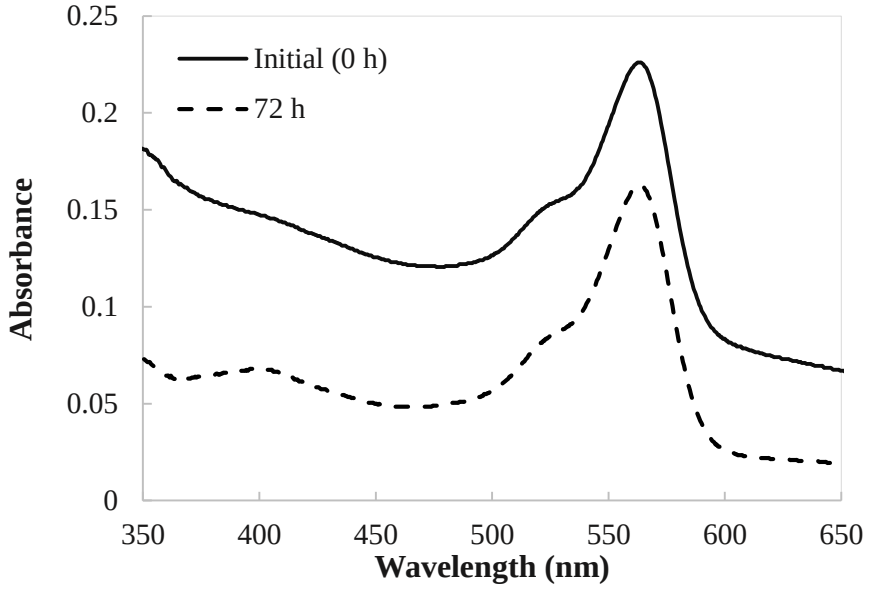


Figure 4.29: Variation of UV-visible spectra of Rhodamine dye solutions treated with *P. mirabilis* for different time intervals under static conditions

In this study, a peak was observed at 225 nm wavelength for the glucose containing medium prior to biological treatment of Yellow EXF however (Figure 4.30), this peak

was not visible in yeast extract containing medium due to noises in UV range of the spectrum (Figure 4.25).

This peak at 225 nm was not observed after biological treatments but a new peak was visible at 252 nm wavelength indicating dye degradation and formation of degraded compounds (Figure 4.30) [140]. Formation of aromatic amines during the degradation of azo dyes in oxygen limited conditions has been reported in literature [62, 64]. Peaks relevant to such aromatic amines can mostly be observed in the range of 190-380 nm wavelength in the UV-visible spectrum [141]. Hence, it can be explained that the peak at 252 nm as an indication of the formation of an aromatic amine.

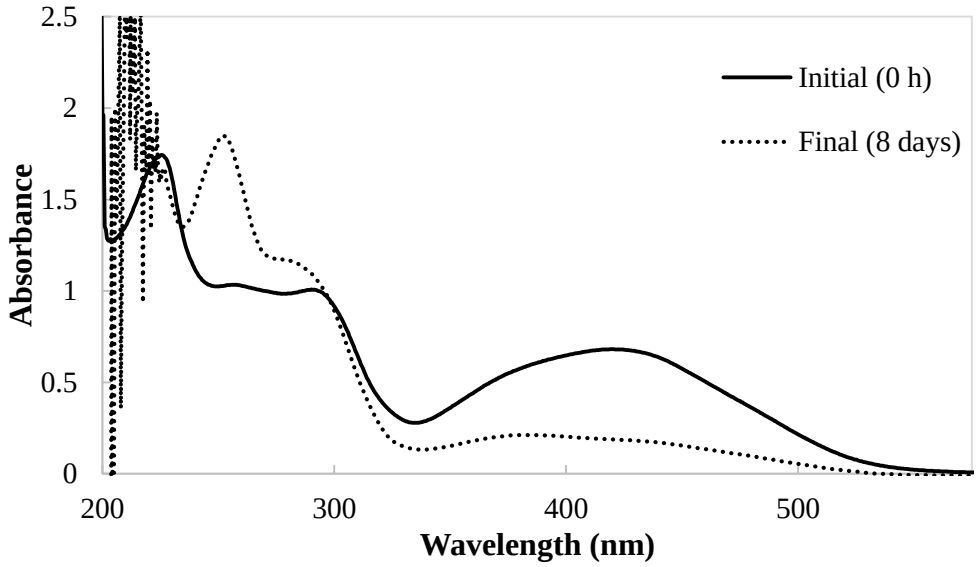


Figure 4.30: UV-visible spectra of Yellow EXF dye solutions decolourized with *P. mirabilis* in glucose containing medium

4.6 Determination of Dye Category

Studies on decolourization of Yellow EXF, Red EXF and Blue EXF dyes are not available in literature. According to material safety data sheets (MSDS), these dyes are mixtures of reactive dyes and further information is not revealed due to commercial secrets. Mansoor Iqbal [142] reported these as bi-functional dyes which carry two reactive groups.

Majority of the dyes used (more than 70% on a weight basis [6]) in textile industries are azo dyes. Peaks due to azo bond in dye structures mostly appear in the range 1500-1650 cm^{-1} in FTIR spectra [70, 138, 143]. Hence, the FTIR spectra of these three dyes were studied and the generated peaks were compared with the available literature. When peaks generated in the above wavelength range by these three dyes are investigated, it can be seen that peaks at 1569.92, 1548.15 and 1571.05 cm^{-1} for azo bond in Yellow EXF, Red EXF and Blue EXF, respectively (Figures 4.31). Further, the peaks formed around 3445-3451 cm^{-1} are due to the stretching of secondary amino (–NH–) group in the dyes [14, 70]. Absorbance bands at 1045 and 1046 cm^{-1} are indicative of S=O stretching vibrations [14, 144].

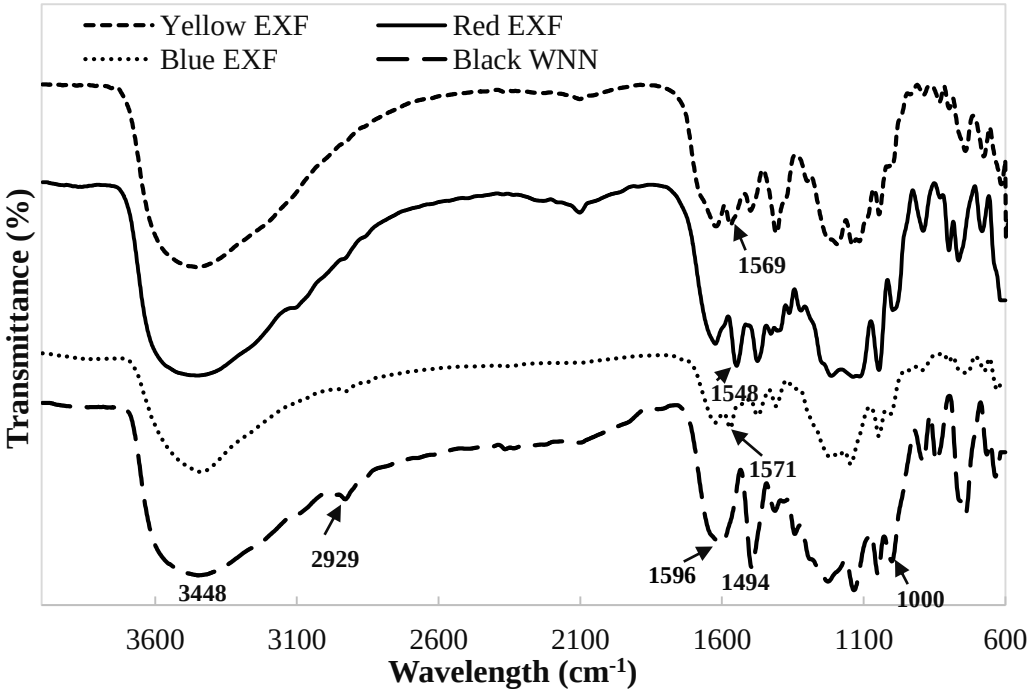


Figure 4.31: FTIR spectra of Yellow EXF, Red EXF, Blue EXF and Black WNN

According to MSDS, Black WNN is a mixture of azo dyes. Anamika and Sarabjeet [138] reported the ability of *Paenibacillus alvei* strain to decolourize di-azo Black WNN dye. FTIR spectrum of Black WNN indicated several peaks at 3448, 2929, 1596, 1494, 1413, 1000, 890-842, 738-636 cm^{-1} (Figure 4.31) which could be formed due to –NH (–NH2), C-H, -N=N-, -C=N, -C-H, C-N (aromatic ring), C-N (aliphatic) and -C=O bonds, respectively [138].

4.7 Dye decolourization by Bacterial Consortium

4.7.1 Decolourization of individual dyes

Dye decolourization by isolated bacteria was discussed in section 4.5.1. It was observed that some dyes can be decolourized by many of the isolates and hence it is worth investigating colour reduction when they are used as a consortium. According to Karunya et al. [145] , synergistic metabolic activities of microbial community in a consortium results in high dye decolourization and mineralization ability than individual bacteria.

Hence, to achieve a further enhanced dye decolourization, a bacterial consortium was developed using the most effective dye decolourizing isolates namely; *P. mirabilis*, *M. morganii* and *E. cloacae* identified during this study. As *E. fergusonii* was only able to decolourize Black WNN, it was omitted when developing the bacterial consortium. Further, as the growth and existence of *A. baumannii* was not possible under oxygen-limited conditions, this strain was also omitted from the bacterial consortium.

Figure 4.32 shows the percentage decolourization of the four dyes with time when microbial consortium was used as the inoculum. Out of the selected bacteria, I_1 was able to effectively decolourize all four dyes (section 4.5.1). Decolourization of Yellow EXF and Red EXF dyes were more rapid by I_1 than the bacterial consortium. However, the decolourization of Blue EXF and Black WNN dyes were more effective with bacterial consortium than with the individual bacterial strains.

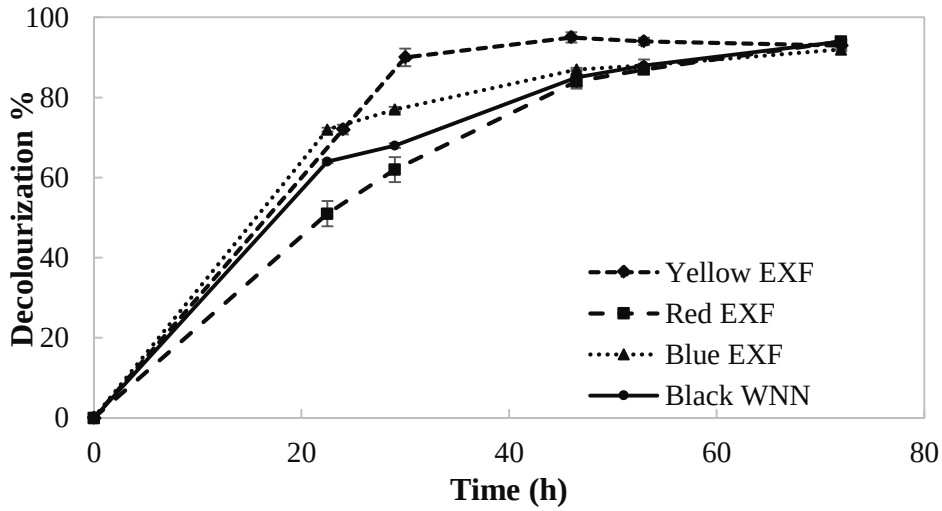


Figure 4.32: Effect of bacterial consortium on dye decolourization over time.

The developed bacterial consortium was more effective in colour reduction of Rhodamine than I_1 as shown in Figure 4.33. After 48 h of incubation, I_1 yielded 30% colour reduction whereas it was 36% with microbial consortium.

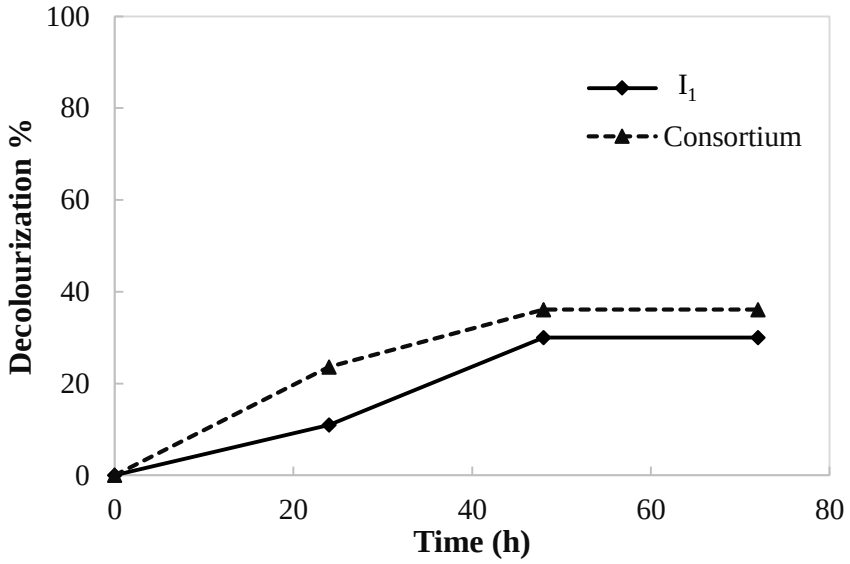


Figure 4.33: Effect of I_1 and the bacterial consortium on Rhodamine dye decolourization over time.

4.7.2 Decolourization of dye mixture

Investigations on the effect of decolourization of dye mixture with the bacterial consortium resulted in 95% colour reduction after 120 h incubation (Figure 4.34). With the increase of the number of microbial cells in the media with time, an enhanced colour removal was observed (Figure 4.34). Only a 72% colour removal was detected when I_1 was used with the dye mixture while the developed bacterial consortium decolourized 83%. These results indicate the effectiveness of the bacterial consortium over individual isolates for decolouration of dye mixture.

Figure 4.35 shows the UV-visible spectra at different incubation times of the dye mixture inoculated with the developed consortium. Prior to biological treatment, a clearly visible absorbance peak at 554 nm wavelength is present and this peak has gradually reduced with the treatment, indicating the destruction of the chromophore groups in dye mixture.

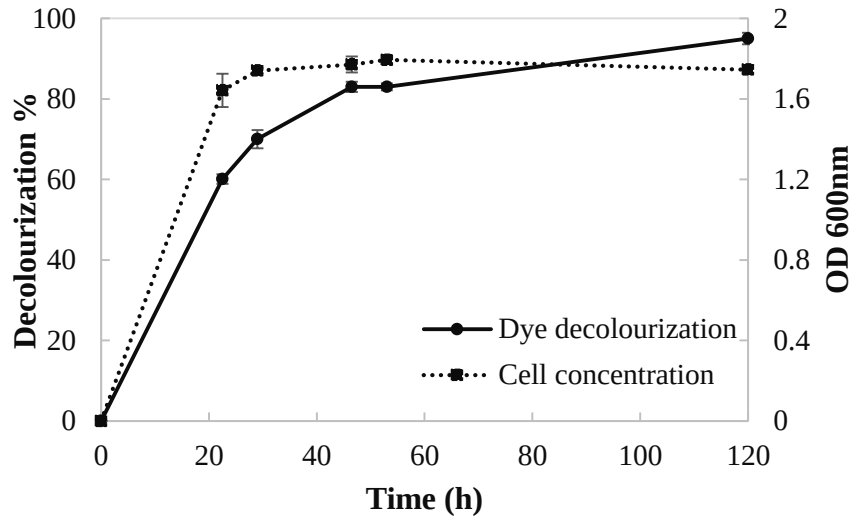


Figure 4.34: Time dependent cell growth and dye mixture decolourization by bacterial consortium

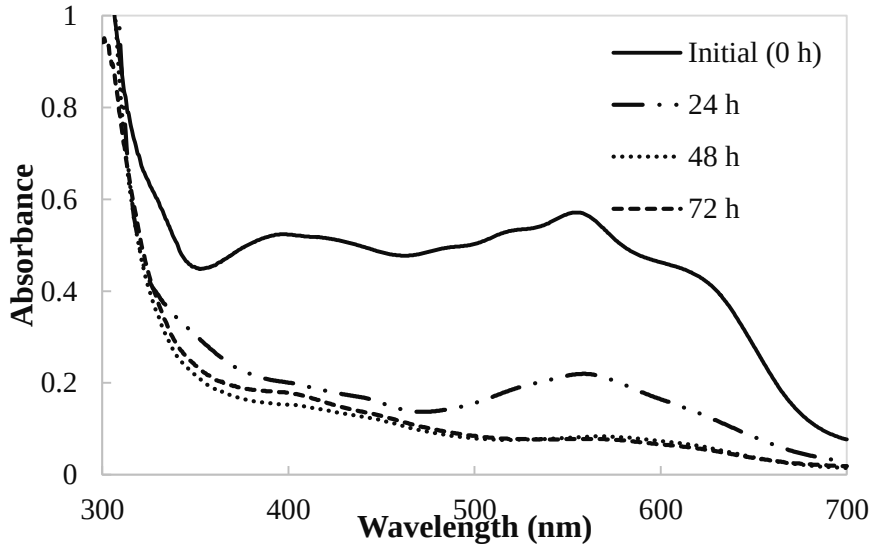


Figure 4.35: UV–visible spectra of the dye mixture decolourization with the bacterial consortium

Figures 4.36 and 4.37 show five dye samples prior to and after incubation with the developed bacterial consortium. Images were taken immediately after the decolourization without the removal of bacterial cells in the medium.

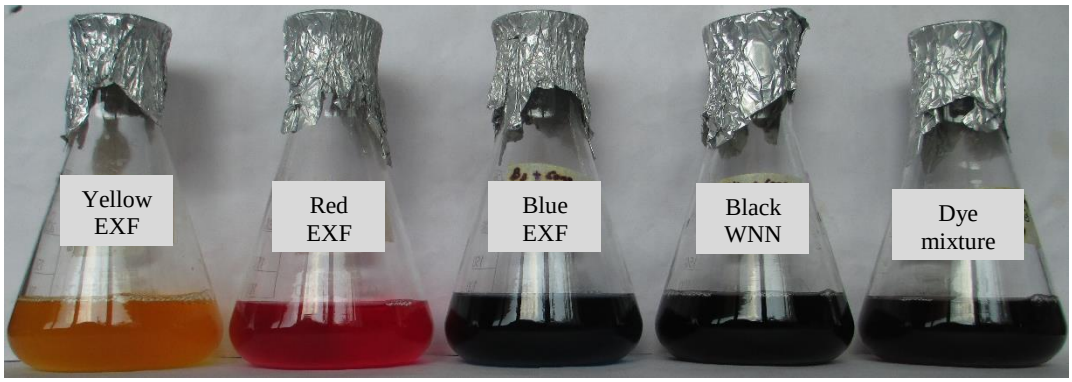


Figure 4.36: Dye containing nutrient media in flasks before biological treatments

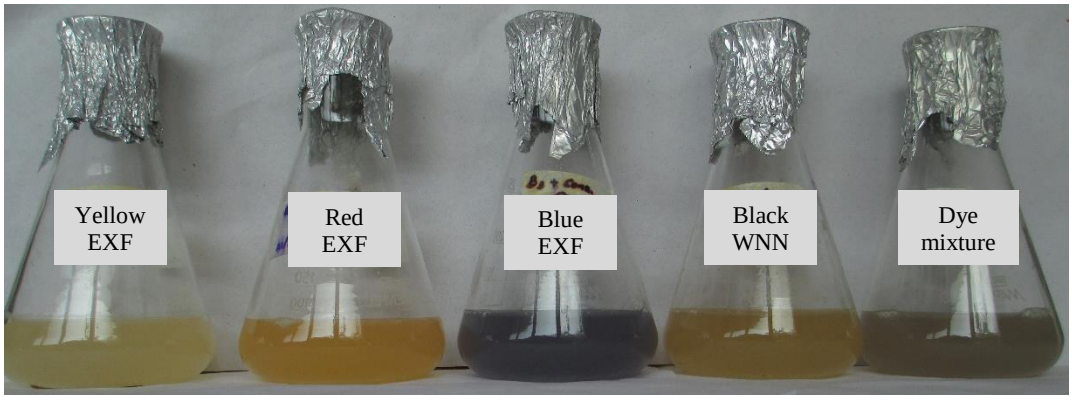


Figure 4.37: Dye containing nutrient media in flasks after 48 h biological treatments

Bacterial cells and supernatants of the decolourized dye samples separated by centrifugation are shown in Figure 4.38. As can be seen, the microbial cells from the flask containing blue dye were coloured (blue) due to absorption of the dye. However, the supernatant was clear indicating decolourization (Figure 4.38). Similarly, the cells separated from the samples that contained synthetic dye mixture after 48 h biological treatments were slightly black due to adsorption of dyes. However, clear supernatants obtained after decolourization showed the ability of the developed bacterial consortium to decolourize all these dyes effectively.

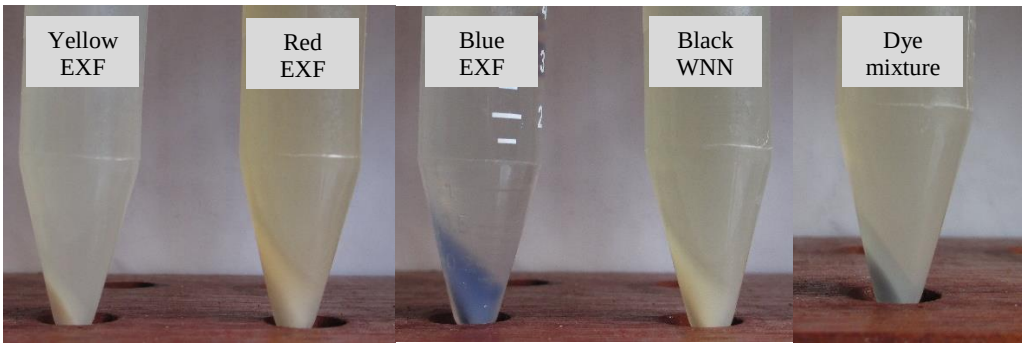


Figure 4.38: Bacterial cells and the supernatants of decolourized dye samples separated by centrifugation

4.8 Parameter Optimization

Out of the 5 bacterial strains isolated in this study, *P. mirabilis* showed the highest percentage colour removal in all dyes investigated. Hence, parameter optimization was conducted using this bacterial strain.

4.8.1 Dye decolourization under shaking conditions

As discussed in sections 4.5 and 4.7, all dye decolourization studies were conducted under static conditions if not specifically mentioned. Compared to static conditions, shaking conditions enhance oxygen transfer between the medium and cells, creating a favourable environment for the growth of aerobic and facultative bacteria. Yellow EXF dye decolourization by *P. mirabilis* under static conditions showed significant differences to that under shaking (Figure 4.39). In static conditions, more than 80% colour removal was observed within 24 h whereas, under shaking conditions colour removal was only 2%. However, biomass concentration was high under shaking conditions. Even though, high biomass concentrations should enhance biological decolourization, converse results obtained show that agitation has a negative effect on colour removal.

Bacterial degradation of azo dyes are prominently reported due to azo reductase enzymes under oxygen limited conditions whereas, the fugal dye degradation is prominent under aerobic conditions mainly due to exoenzymes such as oxidases and peroxidases [56]. Different mechanisms suggested for azo reduction under various

azoreductase enzyme activities are available in literature [146]. According to several hypotheses, azoreduction is facilitated by a flavoprotein in the microbial electron transport chain. Generation of reduced flavins (FMN or FAD) is catalyzed by flavoproteins via re-oxidation of reduced NADH or NADPH [146, 147]. These reduced flavins will provide electrons for the break of azo bond during the biological dye degradation.

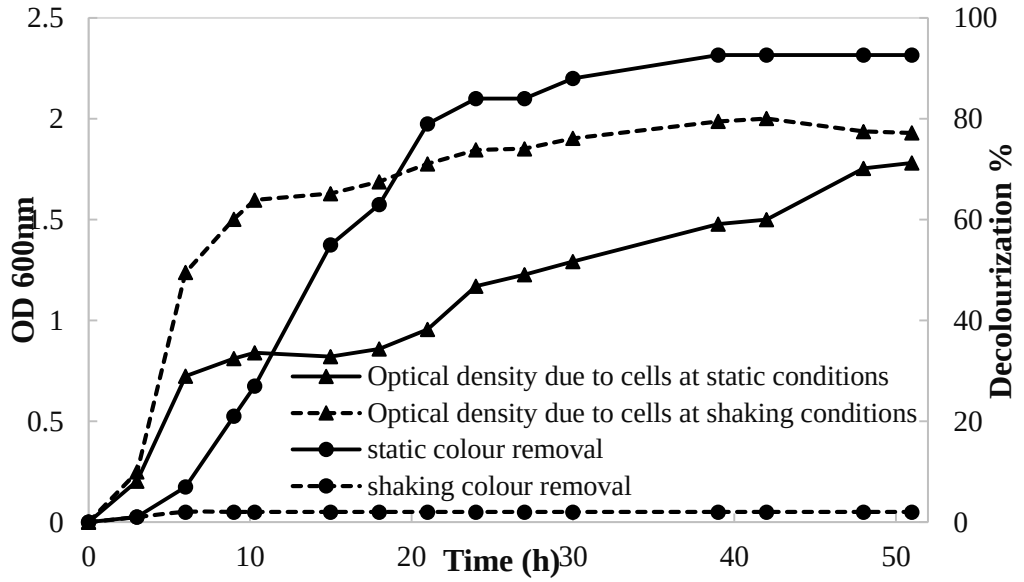


Figure 4.39: Effect of aeration on time-dependent cell growth and decolourization of Yellow EXF dye by *P. mirabilis*

Most of the azo dye decolourization studies conducted with bacteria showed lower colour reductions under shaking conditions [9, 148]. Azo dye decolouration occurs due to the cleavage of azo bond (N=N) of the dye by a transfer of four-electrons (reducing equivalents). This bond breakage proceeds in two stages, where in each stage two electrons are transferred to the azo dye, which acts as a final electron acceptor [56]. Electrons required for azo bond cleavage are not readily available under shaking conditions since, oxygen takes up the electrons available in the medium as it is a better electron acceptor than the azo dye [27, 149] (reduction potential of oxygen (O_2/H_2O) is 815 mV while for azo dyes in the range of -180 to -430 mV [7]).

Decolourization of Red EXF, Blue EXF and Black WNN dyes with I_1 gave similar results at shaking conditions. As shown in Figure 4.40, decolourization of Red EXF and Blue EXF dye was less than 15% whereas, Black WNN was decolourized up to 46% in 72 h under shaking conditions.

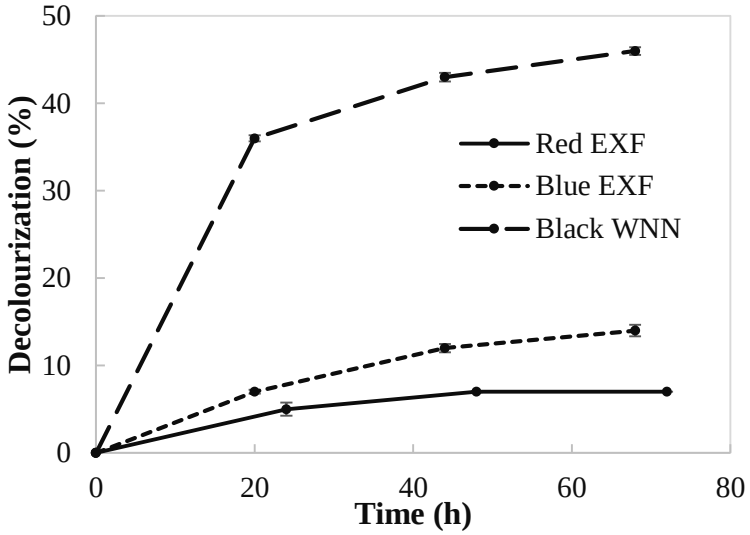


Figure 4.40: Effect of aeration on time-dependent decolourization of Red EXF, Blue EXF and Black WNN dyes by *P. mirabilis*

Further, low colour removal was observed when the synthetic dye mixture was incubated with I_1 under shaking conditions compared to static conditions. As shown in Figure 4.41, after 68 h incubation, 79% decolourization was achieved under static conditions where it was only 13% under shaking conditions. These findings are in agreement with the reported work on azo dye decolourization with different bacterial strains under shaking conditions [13, 68, 131].

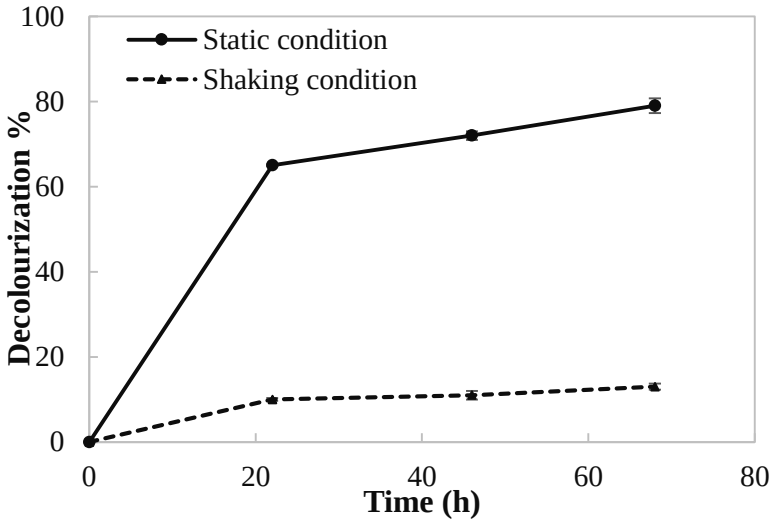


Figure 4.41: Effect of aeration on time-dependent decolourization of synthetic dye mixture by *P. mirabilis.*

4.8.2 Effect of pH on dye decolourization

As can be seen from Figure 4.42, pH value of the decolourization medium has a considerable effect on biological decolourization of Yellow EXF dye. After 18 h of incubation, the highest dye decolourization was observed in the culture of initial pH 7.5. However, final highest decolourization was exhibited by the culture with initial pH 8. When compared to other pH values tested, negligible dye decolourization was observed at pH 10.

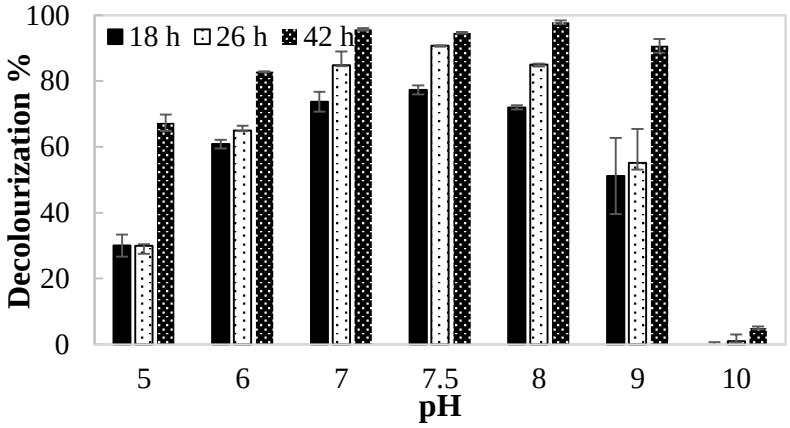


Figure 4.42: Effect of pH on time dependent decolourization of Yellow EXF dye by *P. mirabilis*.

Significance of pH value of the media on Red EXF dye decolourization was studied under a range of pH conditions as shown in Figure 4.43 and the optimum dye decolourization was achieved at pH 7 and 7.5. Similar to Yellow EXF, effectiveness of Red EXF decolourization by this bacterial species was highly reduced at pH 5 and 10.

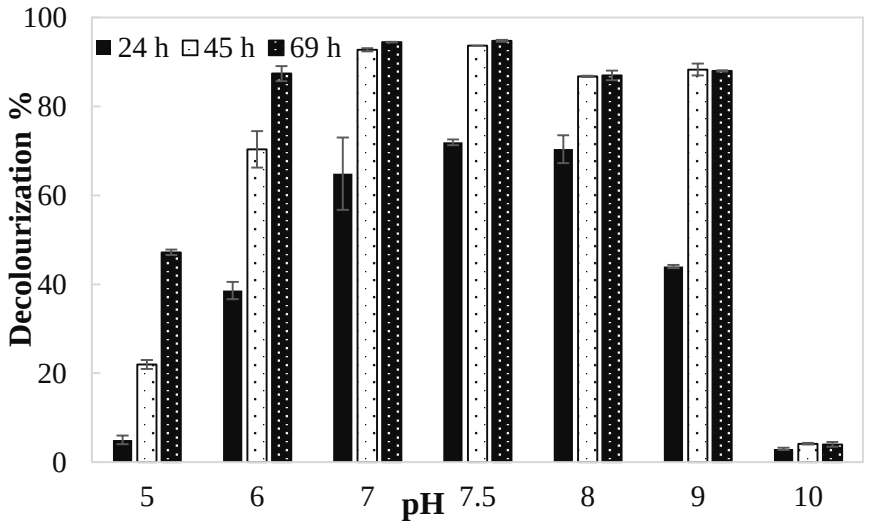


Figure 4.43: Effect of pH on time dependent decolourization of Red EXF dye by *P. mirabilis*.

As shown in Figure 4.44, synthetic dye mixtures of initial pH 7, 7.5 and 8 indicated the highest colour removal after 26 h incubation with the bacterial consortium. However, after 50 h, the highest percentage of decolourization was observed in the culture with initial pH 7. Samples in which the pH was adjusted to 10 indicated the lowest color reductions.

Growth of bacteria is highly influenced by the pH of culture media and the optimum growth of cells can be observed at optimum pH conditions. Further, optimum pH results high enzymatic activities of bacteria and therefore, enhanced dye degradation by respective enzymes (azo reductase) can be achieved at this pH condition. According to results obtained, it is clearly observed that pH range 7-8 is more effective for dye decolourization. Olukanni et al. [14] reported pH 7 as the optimum pH for decolourization of Reactive Blue 13 dye by *P. mirabilis.*

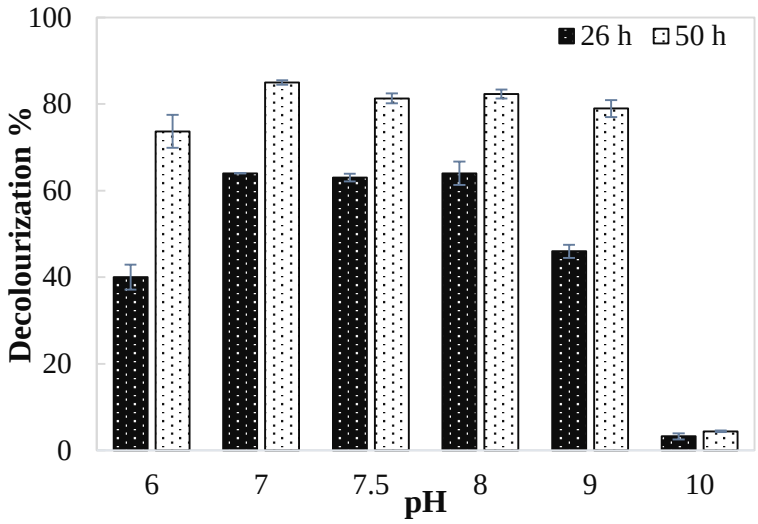


Figure 4.44: Effect of pH on time dependent decolourization of synthetic dye mixture by bacterial consortium.

According to literature, *Proteus* species have the ability to grow in 5-10 pH range and optimum growth has been observed at pH 7-8 [150]. Favourable cell growth of *P. mirabilis* around neutral pH conditions enhances the dye decolourization indicating that the dye degradation is associated with cell growth. Prasad et al. [134] reported the optimum decolourization of Congo red dye at pH 7 and progressive decrease of decolourization rates above pH 9 by *Enterobacter cloacae*. Further, it is reported that pH has an effect on transporting dye molecules through the cell membrane, which is a rate limiting step for decolorization [56]. Effectiveness of dye decolourization by *P. mirabilis* and consortium was reduced at pH 5 and 10. Similar observations of significant reductions in dye removal under strongly acidic or alkaline pH conditions are reported in literature [151]. Reason for this behavior could be the denaturation of enzymes in bacterial cells due to the disruption of protein structure (active site of the enzyme may be destroyed) with the change of H^+ ion concentration in the medium under extreme pH conditions [152].

4.8.3 Effect of temperature on dye decolourization

When the flasks containing Yellow EXF dye were inoculated with *P. mirabilis* and incubated at various temperatures, the maximum percentage dye decolourization was observed in the flask maintained at 40 ^{o}C (Figure 4.45). At this optimum temperature, 85% of colour has been removed within 18 h incubation. Even though samples incubated at temperatures 25, 30 and 45 ^{o}C indicated low percentage decolourization at 18 h, colour removal at all the considered temperatures were more than 85% after 42 h.

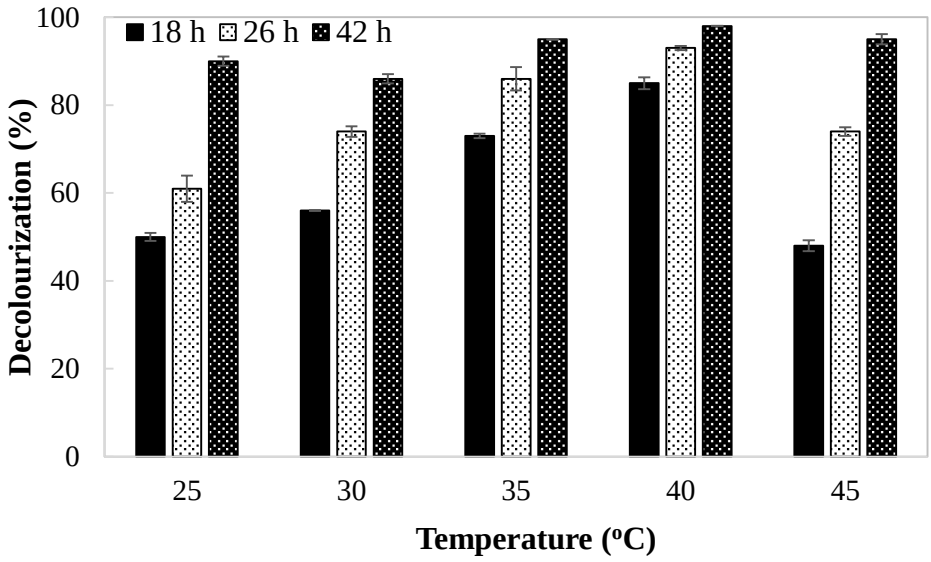


Figure 4.45: Effect of temperature on time dependent decolourization of Yellow EXF dye by *P. mirabilis*.

Influence of temperature on Red EXF dye decolourization is shown in Figure 4.46. As can be seen, all samples were decolourized to a level above 90% of the initial dye concentration after 72 h of incubation and the sample maintained at 30 ^{o}C showed the highest percentage of decolourization. Samples incubated at 25 and 45 ^{o}C indicated low colour reductions while samples incubated at 35 and 40 ^{o}C showed high levels of colour reduction during initial 31 h.

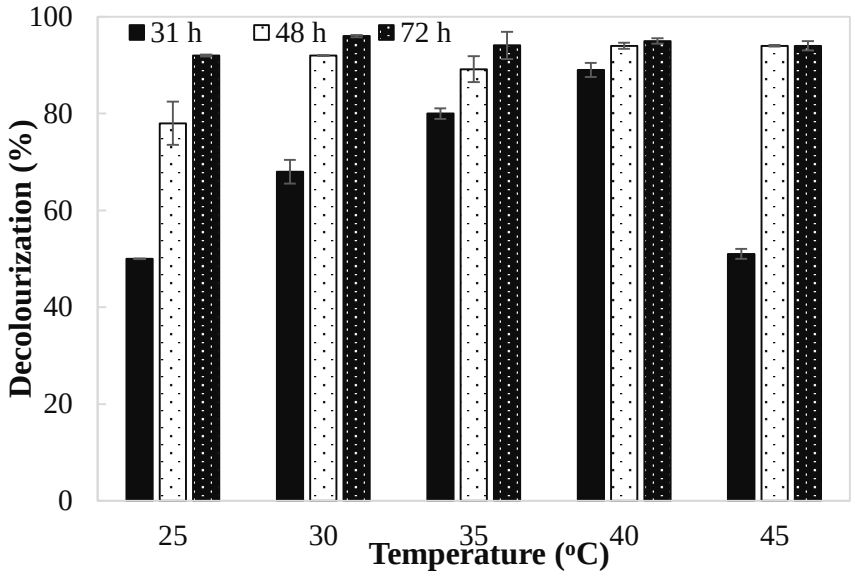


Figure 4.46: Effect of temperature on time dependent decolourization of Red EXF dye by *P. mirabilis*.

Within the initial 28 h, the highest color removal of the synthetic dye mixture by the bacterial consortium was observed in samples incubated at 35 ºC (Figure 4.47). However, after 45 h incubation, the highest dye decolourization was detected in samples incubated at 40 ºC. All samples showed more than 70% decolourization after 45 h incubation.

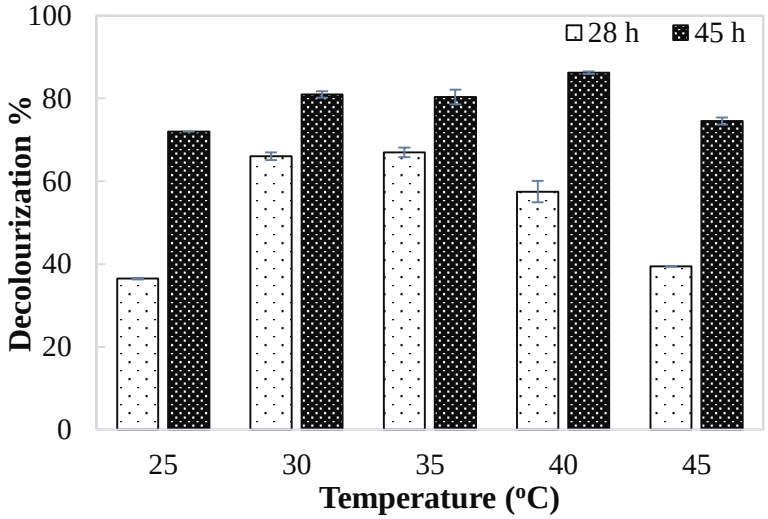


Figure 4.47: Effect of temperature on time dependent decolourization of synthetic dye mixture by bacterial consortium.

Similar to media pH, incubation temperature also has a great influence on bacterial growth. All the temperature optimization conducted in this study have resulted the highest decolourization at 40 ^{o}C. Anjaneya et al. [64] has observed the highest decolorization of Metanil Yellow with individual bacterial strains, *Bacillus* sp. and *Lysinibacillus* sp. at 40 ^{o}C. According to literature, optimal growth temperature for *P. mirabilis* [153] and *E. cloacae* [154] is 37 ^{o}C. Therefore, the high cell growth and enzyme activity around optimum growth conditions could be the reason for observed high decolourization rates at 40 ^{o}C [155].

Generally, the discharge temperature of textile dyeing industrial effluent is above ambient, since it gets mixed with hot water released from dyeing baths. The increased temperature could result in reductions in colour removal due to the loss of cell viability [156] and denaturation of enzymes [157] which are associated with dye degradation. However, in the current study effective decolourization was observed throughout 25-45 ^{o}C temperature range that will be beneficial when this biological treatment method is implemented in industry.

4.8.4 Effect of dye concentration

Industrial wastewater treatment facilities may frequently receive effluents with varying dye concentrations. Sulphonic-acid groups, which are generally present in aromatic rings of azo dyes act as detergents on microorganisms inhibiting cell growth [158] and further reported that textile dyes inhibit nucleic acid synthesis of microbial cells [159]. Therefore, investigations on biological decolourization were carried out with varying concentrations of Yellow EXF dye and I_1 as the microorganism.

With the increase of initial dye concentration, biodegradable organic load also increases and the percentage decolourization is expected to drop when the same size of inoculum is used (mainly due to improper cell to dye ratio), as observed in Figure 4.48. Similar results are reported for the decolourization of Navy Blue HE2R by *Exiguobacterium* sp. [160] and for decolourization of Red RBN dye by *P. mirabilis* [129].

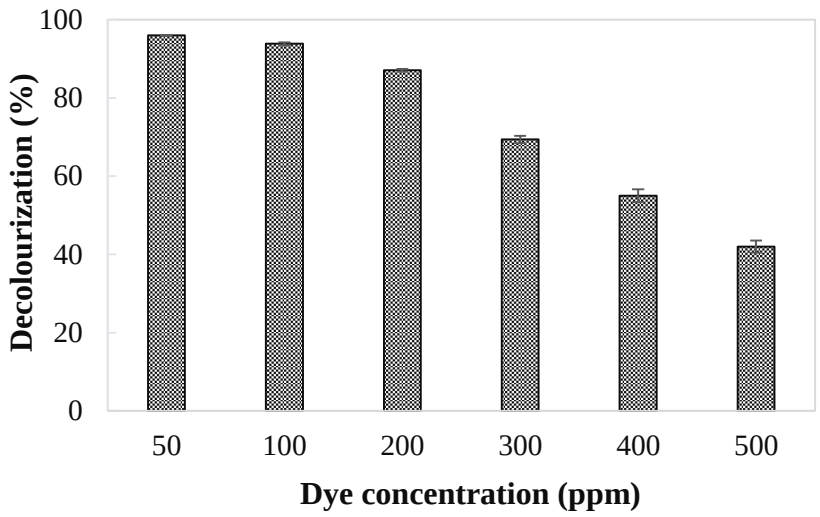


Figure 4.48: Effect of dye concentration on decolourization of Yellow EXF by *P. mirabilis* after 72 h of incubation

Even though it is reported that the toxic nature of highly concentrated textile dyes may inhibit the growth of most microorganisms in dye containing environments [111], Joshi et al. [131] effectively decolourized Acid Orange 7 dye by a bacterial consortium composed with *Proteus mirabilis* up to 200 ppm. However, in this work, *P. mirabilis* was able to grow in flasks containing up to 500 ppm of Yellow EXF and resulted in more than 40% decolourization. Ability of this bacterial strain to tolerate and decolourize highly concentrated dye solutions could be beneficial when implementing biological decolourization techniques in textile industries with high loads of residual dyes.

4.8.5 Effect of carbon sources on dye decolourization

Yellow EXF dye solution inoculated with *P. mirabilis* did not show considerable cell growth or colour removal without an external carbon source in the decolourization medium (data not shown). This is because carbon is essential for the microbial growth which provide energy and function as building blocks for microorganisms [161] and hence externally added carbon is required for decolourization of azo dyes [111]. Azo reduction has been identified as a form of microbial anaerobic respiration [162] which is the energy conservation process in anaerobic bacteria in the absence of the final

electron acceptor, oxygen in electron transport chain [163]. Carbon in the culture medium has the ability to behave as an electron donor; during electron transport phosphorylation (ETP), provide electrons required for the reductive cleavage of azo bond (final electron acceptor) under anaerobic conditions [9]. Hence, the quantity and the type of carbon source are major parameters that determine the effectiveness of bacterial dye degradation.

Out of the three tested carbon sources, yeast extract containing samples yielded the highest colour removal after 24 h and 48 h of incubation (Figure 4.49). However, when the concentration of yeast extract was reduced from 5 g/l to 2 g/l, a reduction in decolourization was observed. Even though, low colour reductions were observed in starch and glucose containing samples at 24 and 48 h incubation, after 8 days of incubation decolourization percentages were increased to 53 and 71% for starch and glucose containing samples, respectively (data not shown).

Starch is a commonly used additive in textile finishing processes, as well as a frequently used carbon source for microbial growth in biological studies. Hence, addition of external carbon sources may not be required if starch has the potential to enhance colour removal. However, a significant enhancement of dye decolourization was not observed with starch in this study. Similarly, Joshi et al. [131] reported yeast extract as a more effective co-substrate than glucose or starch for decolourization of azo dyes by a bacterial consortium containing *P. mirabilis*. Chen et al [159], have observed reduction of dye decolourization and cell growth of *A. hydrophila* when glucose was used as the source of carbon which were due to the reduction of media pH caused by consumed glucose or converted organic acids. According to literature, carbohydrates such as glucose and starch can effectively be utilized as co-substrates for dye decolourization under methanogenic conditions, although yeast extract is preferred under static (microaerophilc) conditions [131, 164].

Utilization of different carbon sources such as glucose, starch, acetate, yeast extract, ethanol, whey and tapioca in various dye degradation experiments with different microbial strains are reported in literature [13, 63, 165]. Type and quantity of the carbon source required for optimum decolourization of a particular dye will vary depending on the microbial species utilized for the experiment.

Yeast extract provides nitrogen and vitamins such as riboflavin for the growth of bacteria in addition to carbon [166]. During dye degradation, yeast extract serves the function of both co-substrate which provides reducing equivalents (NADH or NADPH) for the reductive cleavage of azo bonds [63] and redox mediator that shuttle electrons to extracellular dyes, accelerating azo reductase activity. It has found that riboflavin component of yeast extract as the reason for its ability to behave as a redox mediator which is not present in other co-substrates such as starch and glucose [166]. However, colour removal is significantly low when 2 g/l of yeast extract was used in the medium.

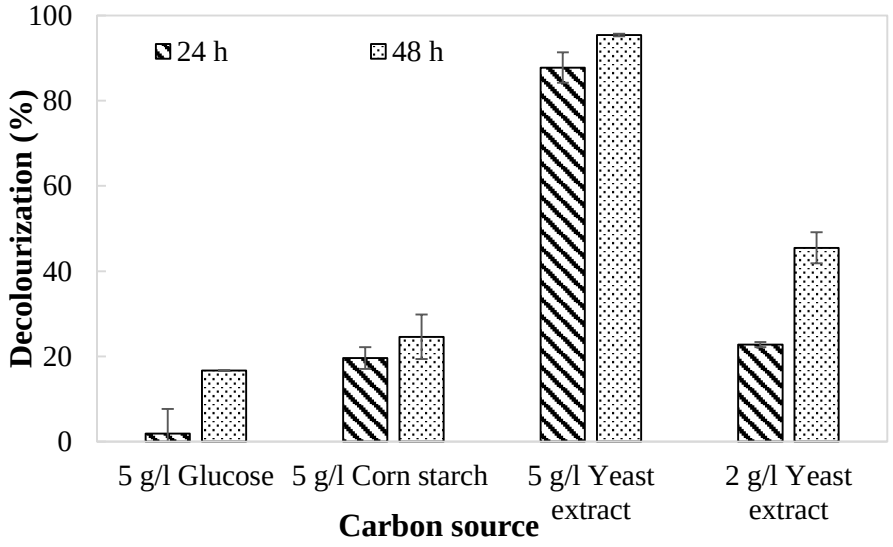


Figure 4.49: Effect of carbon sources on Yellow EXF decolourization by *P. mirabilis*

Results obtained from the experiments conducted to decolourize 50 mg/l of synthetic dye mixture with the bacterial consortium are shown in Figure 4.50. Nutrient media that contained 2, 1 and 0.25 g/l yeast extract concentrations indicated 41, 22 and 14% colour removals, respectively after 48 h incubation (Figure 4.50). OD values due to bacterial cells at 600 nm wavelength were 0.846, 0.66 and 0.31 in the above media at 48 h. Observed low colour reductions could be a result of slow growth of bacterial cells in the media at reduced yeast extract concentrations. These results show that the decolourization of synthetic dye mixture is not effective in the media that contained less than 2 g/l of yeast extract.

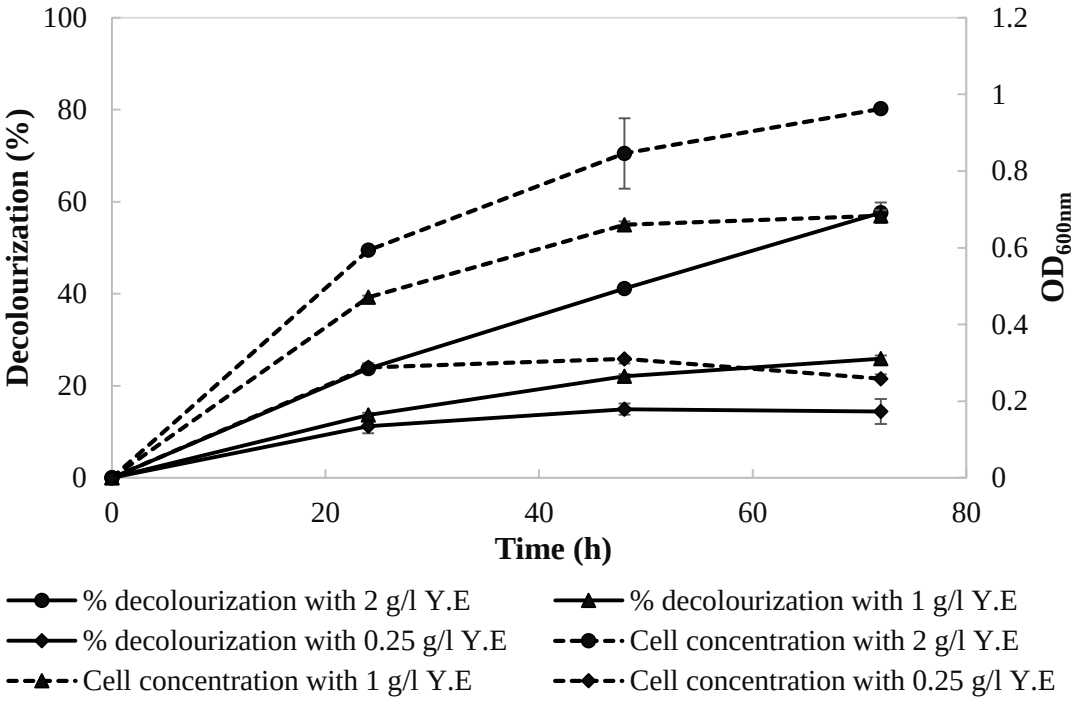


Figure 4.50: Effect of yeast extract concentration in decolourization of the synthetic mixture of dyes and cell growth by bacterial consortium

Among the dyes considered, Yellow EXF was effectively decolourized by several bacterial isolates compared to other four dyes. Hence, parameter optimization was conducted only for Yellow EXF. Additionally, temperature, pH and shaking condition optimization were conducted for Red EXF dye and results obtained were similar to that of Yellow EXF. Optimized conditions were similar in both of these dyes and hence condition optimisation was not repeated for other individual dyes. However, condition optimization was carried out for the dye mixture using bacterial consortium and the resulted optimum conditions are similar to that of Yellow EXF and Red EXF dyes. Hence it can be concluded that the optimum conditions for dye decolourization are the same for the selected dyes by the isolates.

4.8.6 Decolourization of Malachite green

All four reactive dyes used in this study were proven to contain azo structures and the Rhodamine dye was composed of a Xanthene structure. Therefore, the decolourization studies were conducted with Malachite green (triarylmethane structure) to evaluate the ability of the developed bacterial consortium to decolourize structurally different dyes. Malachite green is a basic dye which is commonly used as a biological stain and extensively used in the textile industries [167]. Developed bacterial consortium was able to remove 64% and 97% colour of Malachite green dye after 24 and 48 h incubation in a medium containing 5 g/l yeast extract. However, only a partial colour removal was observed (55% after 48 h incubation) when the decolourization was conducted with a medium containing 2 g/l yeast extract (Figure 4.51).

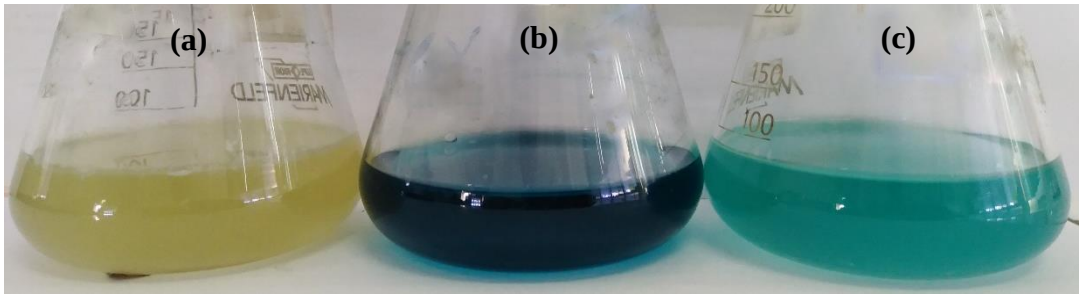


Figure 4.51: Malachite green containing samples after 48 h incubation with bacterial consortium in (a) 5 g/l (b) 5 g/l control and (c) 2 g/l yeast extract containing media

As can be seen from Figure 4.52, Malachite green resulted a maximum absorbance peak at 617 nm wavelength before biological treatment. However, this peak has gradually reduced with the treatment and not visible at 48 h incubation. Formations of new peaks were not observed at the wavelength range considered in this study. However, Daneshvar et al. [168] reported the formation of new peaks at UV range of the UV-visible spectrum due to formation of new compounds by biological degradation of Malachite green.

These results prove the ability of the developed bacterial consortium to decolourize structurally different Malachite green dye.

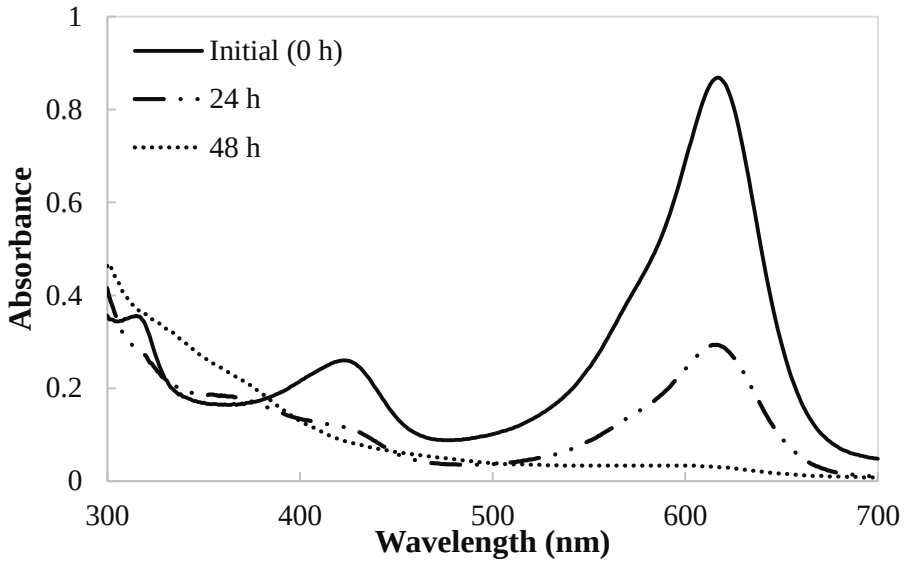


Figure 4.52: UV-visible spectra for Malachite green decolourization by bacterial consortium

4.9 Textile Effluent Decolourization by Bacterial Consortium

Decolourization of textile dye by bacteria in synthetic dye media are widely reported in literature [64, 62, 68, 111], however, studies with textile industry effluent are not commonly reported. Industrial wastewater contain various quantities of different chemical substances such as salts, detergents, and organic acids [4], therefore, resulting in a different composition than synthetic dye media use in laboratories. High salt concentrations and toxicity of these chemical substances may negatively affect the microbial growth. Further, textile wastewater contains a number of dyes in various concentrations and hence, use of biological decolourization techniques for laboratory investigations is considered to be difficult.

However, in this study, effluents collected from three different textile-dyeing facilities were used to evaluate the ability of the developed bacterial consortium to decolourize textile effluents.

UV-visible spectra of effluent obtained from companies A and Z did not show any peaks in the visible range of the spectrum (Figure 4.53 and 4.54). In textile dyeing industries, a number of different dyes are simultaneously used (for different fabrics) and all of these dyes end up in wastewater. The UV visible spectrum for a dye mixture

is the resultant absorbance of all absorbing functional groups of the dyes present in the effluent. Therefore, it is not possible to identify a maximum absorbance wavelength for most textile dyeing effluents and these absorbance values can vary with the change of effluent composition.

Hence, for the calculation of percentage decolourization of wastewater from company A and Z, three wavelengths; 436, 525 and 620 nm were selected as representative points to cover the visible range as per ISO 7887:2011 standard [169]. 34%, 46% and 61% color reductions were observed in effluent from company A at 436, 525 and 620 nm wavelengths respectively after 24 h incubation (Table 4.11). However, further considerable color reductions were not observed in this effluent even with 138 h incubation (data not shown). Effluent from company Z showed 31%, 41% and 55% colour removals at 436, 525 and 620 nm wavelengths after 24 h incubation and further colour reductions were observed with incubation.

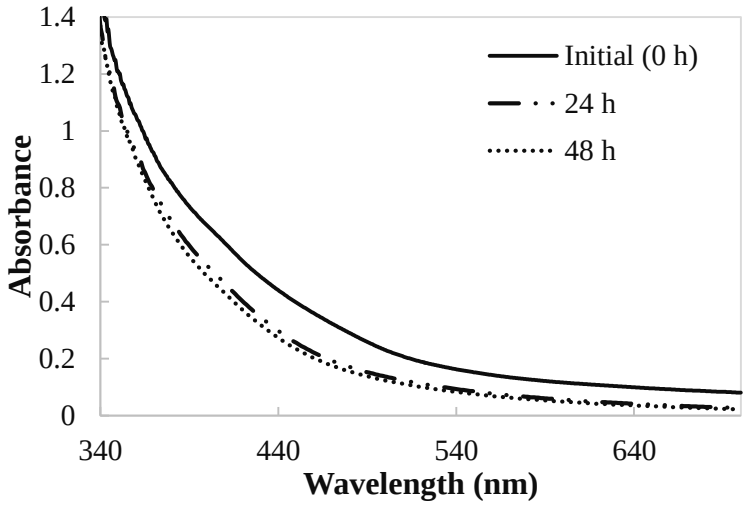


Figure 4.53: UV-visible spectra of company A effluent before and after biological treatments

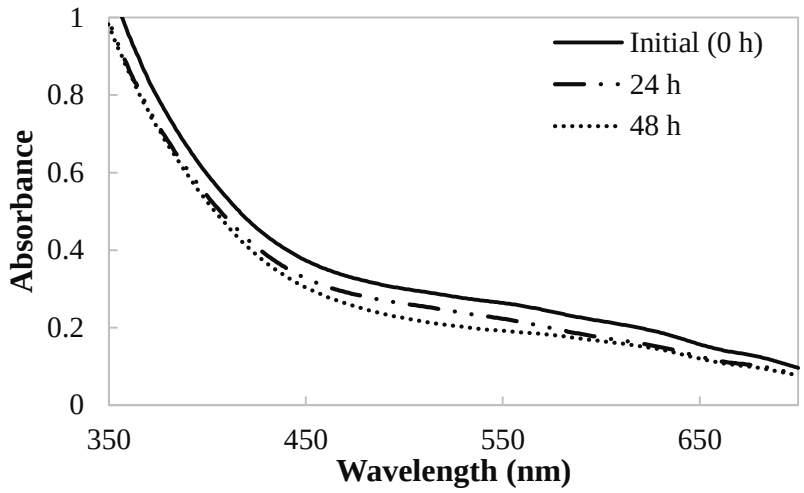


Figure 4.54: UV-visible spectra of company Z effluent before and after biological treatments

Table 4.11: Percentage decolourization of textile effluents at different wavelengths

Wavelength (nm)	**% decolourization of company A effluent after**			**% decolourization of company Z effluent after**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
436	33.6	35.4	37.1	30.9	35.4	37.1
525	45.7	46.1	48.5	40.7	46.1	48.5
620	60.7	59.9	62.1	54.6	59.9	62.1

Colour of textile effluent is influenced by the type and composition of dyes used in textile dyeing processes. During this study, effluent samples collected from company D at two occasions indicated maximum absorbance peak at dissimilar wavelengths (study I- at 521 nm and study II- at 401 nm). After biological treatments, clear shifts of UV-visible spectra of each effluent sample were observed due to reductions in absorbance values, indicating the dye decolourization (Figures 4.55 and 4.56). Time-dependent decolourization conducted with the bacterial consortium for effluents obtained from company D indicated 60% (study I) and 56% (study II) decolourization within 138 h (Table 4.12).

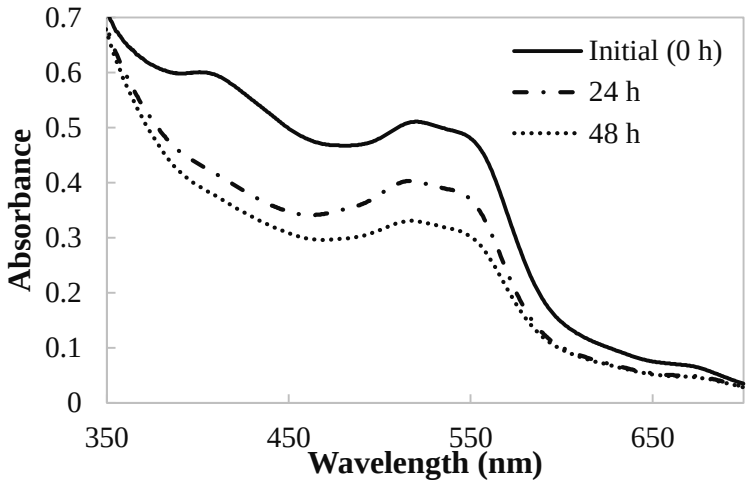


Figure 4.55: UV-visible spectra of company D effluent before and after biological treatments (study I)

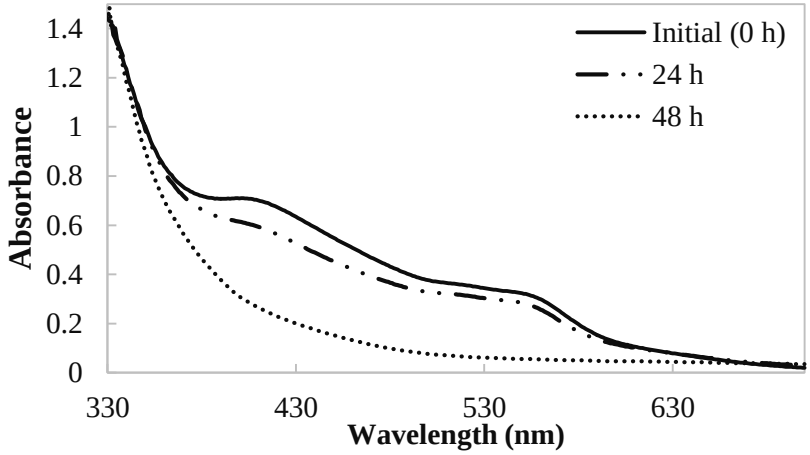


Figure 4.56: UV-visible spectra of company D effluent before and after biological treatments (study II)

Table 4.12: Percentage decolourization of effluent obtained from company D by bacterial consortium after 48 h of incubation

Time (h)	% decolourization during study I at 521 nm	% decolourization during study II at 401 nm
24	22.1	10.5
48	36.5	52.6
72	45.3	55.1
138	60.3	56.4

As seen in Figure 4.57, visible colour reductions were observed in all the samples owing to biological treatment. Company A is a sewing thread manufacturing company and prominently uses disperse and vat dyes for the dyeing process. Company D is a weft-knitted fabric manufacturing company, which widely uses reactive textile dyes in their processing. Therefore, based on the decolourization results obtained, it can be considered that the developed bacterial consortium has the ability to decolourize textile effluents consisting of different types of dyes.

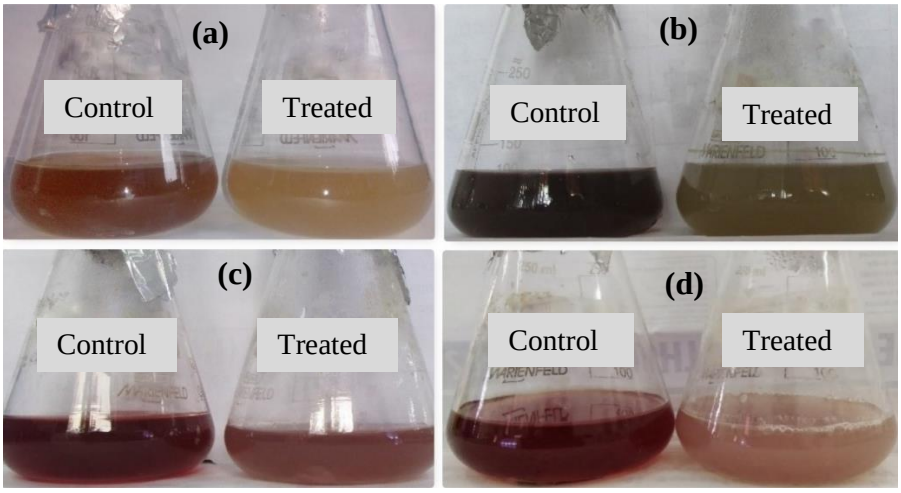


Figure 4.57: Decolourization of textile effluents obtained from company (a) A (b) Z and (c) D (study I) (d) D (study II)

4.10 Yellow EXF Degraded Compounds Analysis

4.10.1 HPLC analysis

High performance liquid chromatogram of Yellow EXF dye and dye-degraded compounds (metabolites) are shown in Figures 4.58 and 4.59 respectively. Major peaks appeared in original dye chromatogram at retention times 1.44, 1.547, 4.916, 5.441 and 10.772 min with peak areas of 557, 666, 1158, 1283 and 830 mAU.s respectively were not observed in the chromatogram of degraded compounds. Few minor peaks were observed at retention times 11.730, 12.423 and 26.753 min with peak areas of 81, 60 and 148 mAU.s respectively in the HPLC chromatogram of degraded compounds. Absence of prominent peaks observed in the HPLC chromatogram of the dye after the biological treatments can be attributed to

degradation of original dye structure and minor peaks observed at new retention times demonstrated the formation of dye metabolites due to biological treatments.

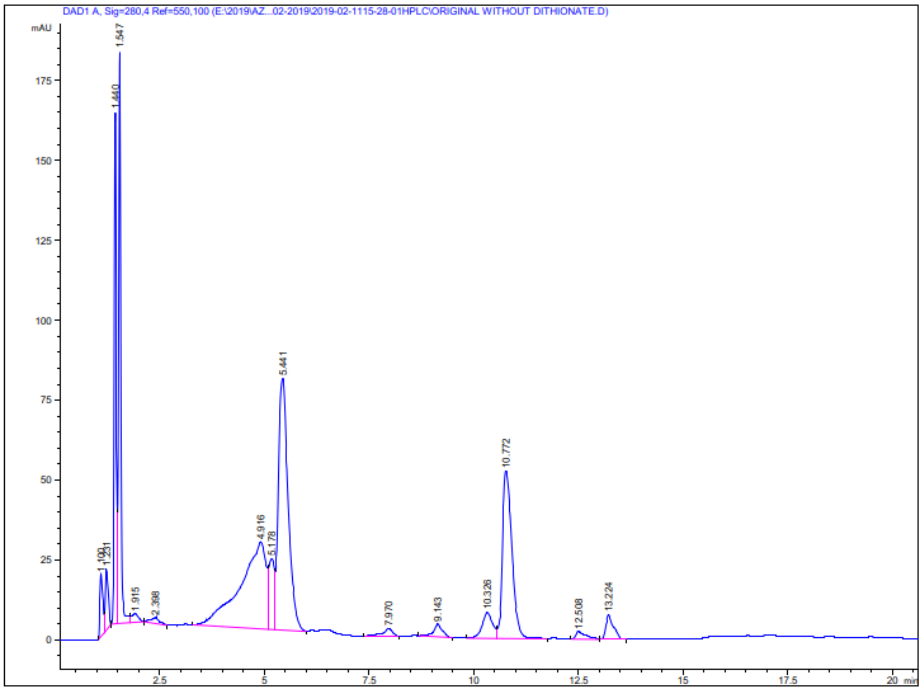


Figure 4.58: HPLC chromatogram of Yellow EXF dye

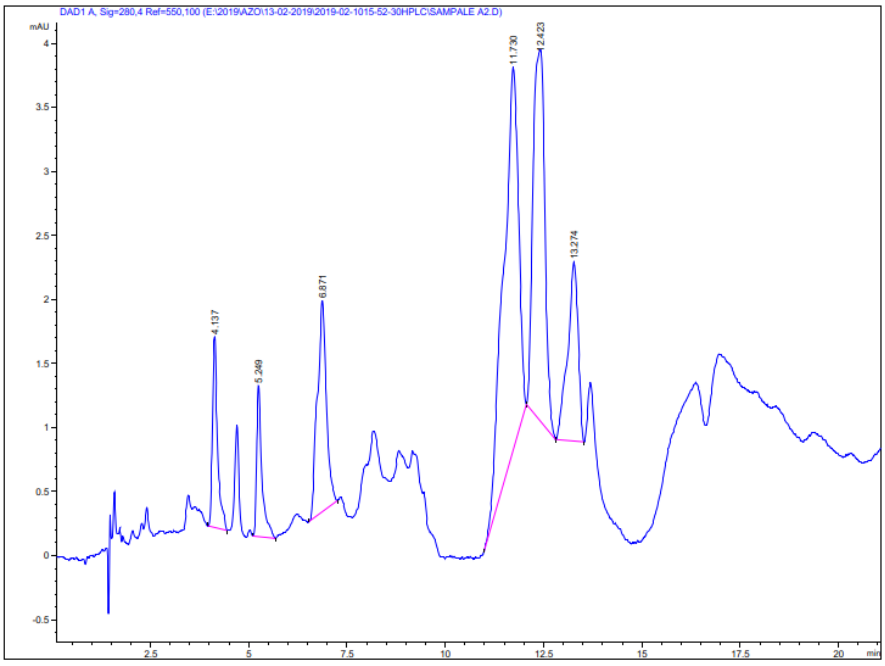


Figure 4.59: HPLC chromatogram of dye degraded compounds

4.10.2 GCMS analysis

GC chromatograms of the Yellow EXF dye after static treatment and after both static and aerobic treatments (shaking) are shown in Figures 4.60 and 4.61 respectively. As shown in Figure 4.60, GC chromatogram of the sample treated under static condition indicated prominent peaks at 4.825, 10.604, 11.521, 12.208, 14.875 and 15.108 (min) retention times. However, the compounds that indicated more than 89% NIST similarity score were only considered in the analysis of dye metabolites. Based on the library search, peaks observed at retention times 4.825, 11.521,12.208, 10.604, 3.954 min were identified as Phenylethyl alcohol, 1H-Indole-3-ethanol, 1H-Indole-3-carboxaldehyde, Benzenemethanamine N-(phenylmethyl)- and Benzyl alcohol and mass chromatograms of these compounds are shown in Figure 4.62.
According to gas chromatogram (Figure 4.60), Phenylethyl alcohol is the major compound present in dye metabolites and contributed to 41.98% peak area while Benzenemethanamine N-(phenylmethyl)- contributed to 1.53 %.

Cleavage of azo bond in dye structure is the main mechanism of biological decolorization and further degradation and mineralization of degraded compounds can be achieved under aerobic conditions [7]. In the presence of oxygen, aromatic amines can be further degraded in to simpler compounds by the insertion of oxygen in to those molecules. Some aromatic amines are reported to undergo oxidative deamination producing different intermediate products under aerobic conditions [67] whereas some microbial species have the ability to completely mineralize aromatic amines in to CO_2, H_2O and NH_3 [5].

Peaks observed in GC chromatogram of anaerobically treated sample (Figure 4.60) have almost disappeared after aerobic treatments, however, few major peaks have been formed at 15.114 and 16.516 min retention times (Figure 4.61) showing formation of metabolites. Furthermore, out of the previously detected compounds, only Phenylethyl alcohol was present after aerobic treatments (Figure 4.61) but a significant (87%) reduction of GC chromatogram peak area was observed, compared to the sample treated under static conditions.

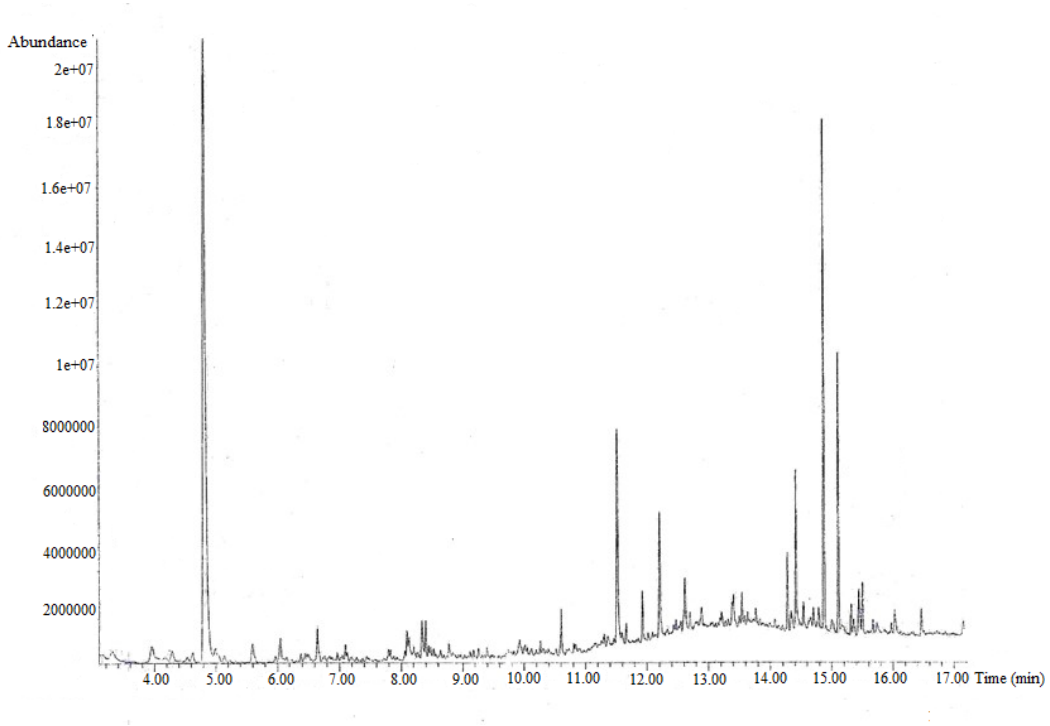


Figure 4.60: Gas chromatogram of the dye degraded compounds formed after static treatment

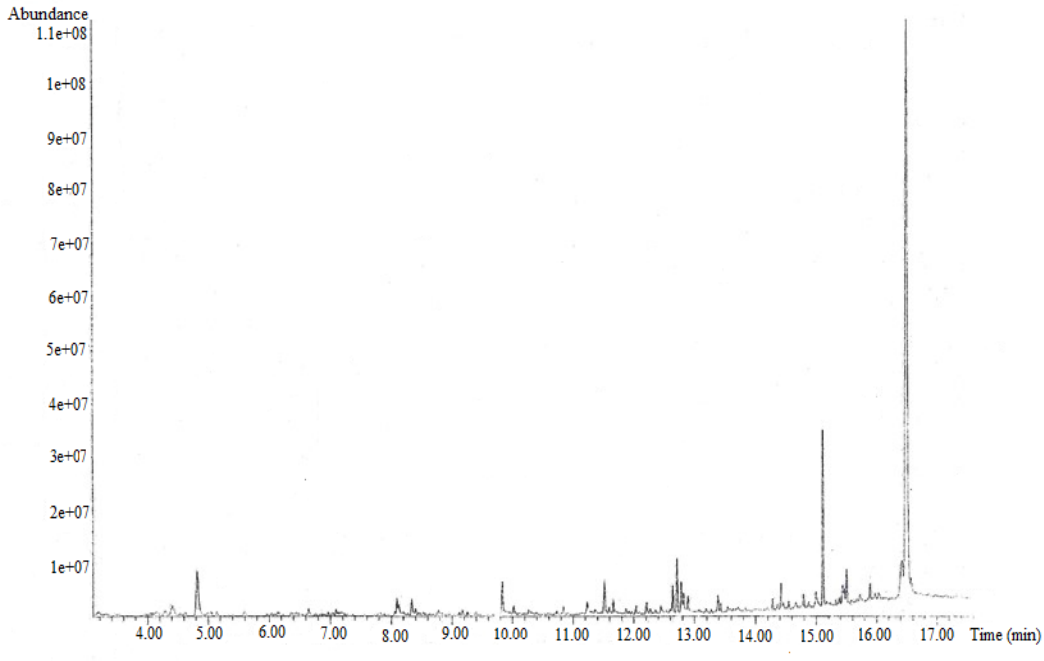
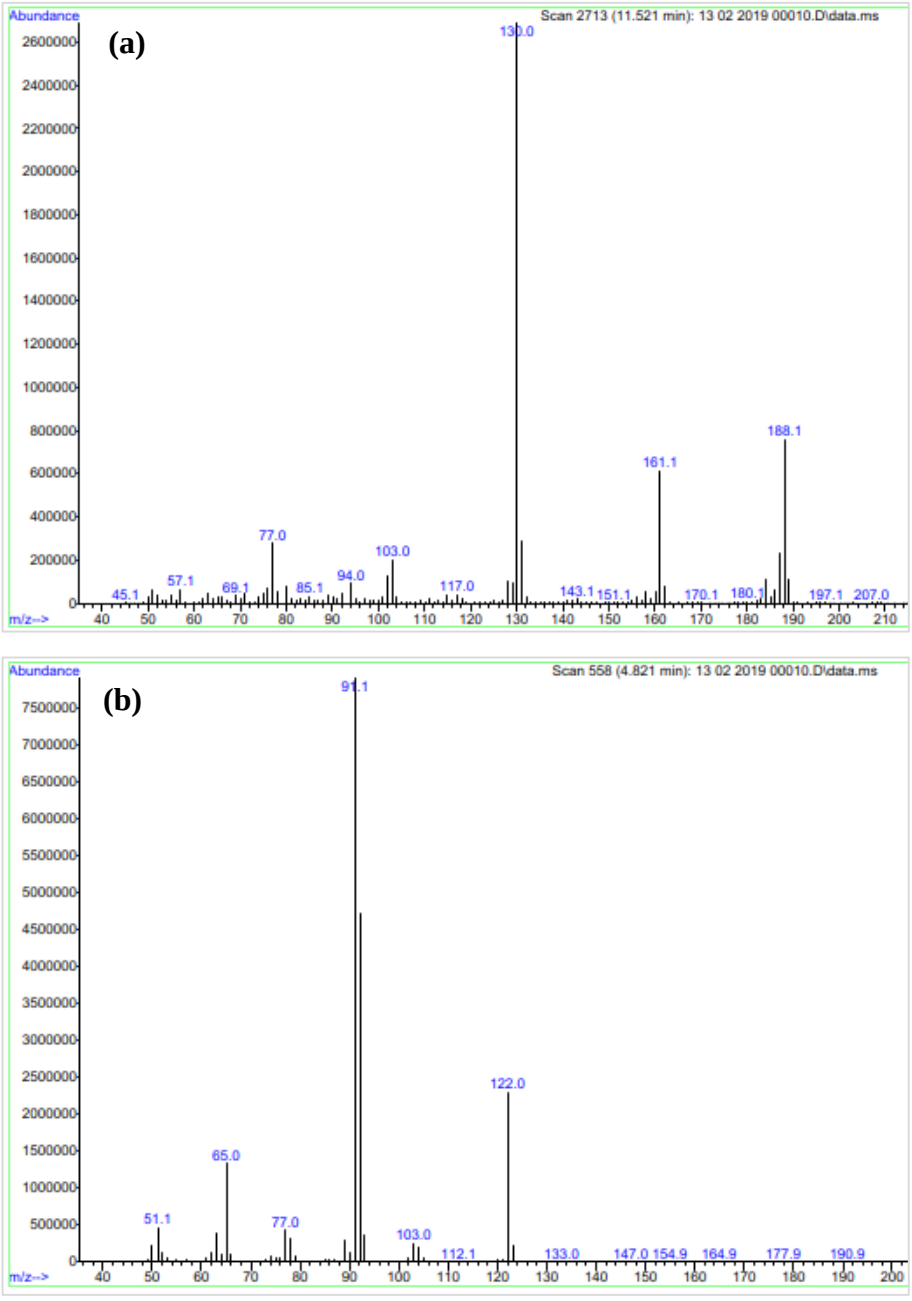


Figure 4.61: Gas chromatogram of the mineralized degraded compounds formed after both static and shaking treatments

Hence, it can be assumed that this bacterial strain could decolourize yellow EXF dye under static conditions and further breakdown degraded compounds under shaking conditions. Even though, a large number of studies reported on the dye decolourization ability of different bacteria, only few reported mineralization of dye degraded compounds [62, 67, 170]. Hence, this bacterium, *P. mirabilis* can be considered as a favorable and eco-friendly solution for textile dye decolourization applications.



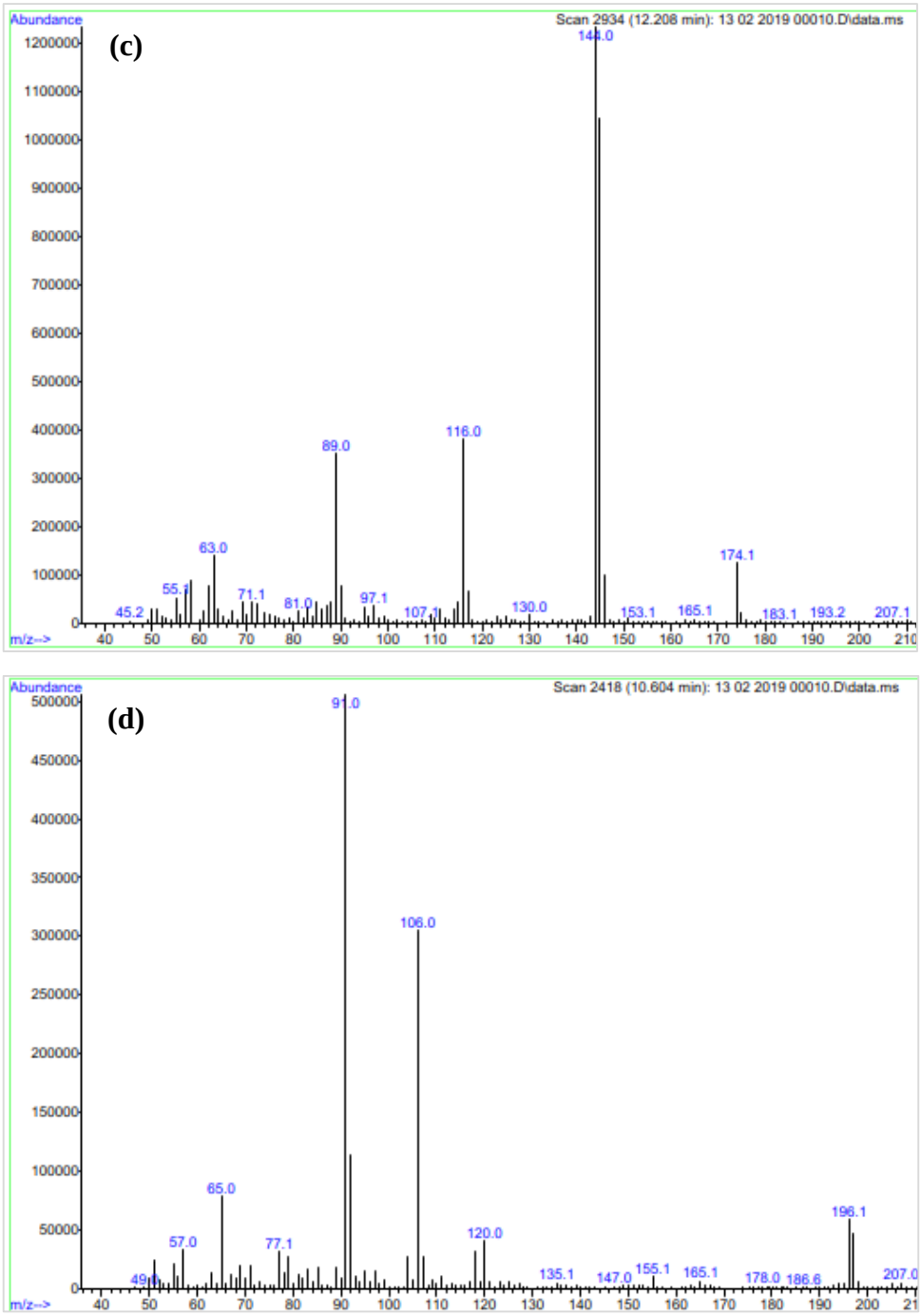


Figure 4.62: GC–mass spectra of degraded compounds of Yellow EXF by *P. mirabilis* for the peaks corresponding to RT value of (a) 4.825 (b) 11.521 (c) 12.208 and (d) 10.604

4.11 Decolourization of Textile Dyes and Effluent in FBBR

Even though the developed bacterial consortium was able to decolourize textile dyes and effluents effectively, all corresponding batch studies were conducted in flasks with limited sample volumes (100 ml). However, industrial effluent treatment units are fed continuously and need to treat massive volumes of wastewater. Hence, investigation of the ability of bacterial consortium to decolourize dyes and effluents in both batch and continuous modes is important.

At the beginning of each decolourization study conducted in flasks (sections 3.11 and 3.14-3.17), fresh cultures of free (unimmobilized) bacteria were used as the inoculum. However, immobilized cells were used as the reactor seeding material in the FBBR as it could be more beneficial over the use of free bacterial cells; immobilized cells may eliminate the non-productive growth phase, enhance the resistance of bacterial cells over environmental stresses such as temperature, pH, salts and toxic chemicals and avoid cell washout during continuous operation of the FBBR [171].

Hence, three cell immobilization techniques were investigated for the preparation of seeding materials for the reactor.

4.11.1 Reactor seeding material preparation

Three methods; cell immobilization with calcium alginate, incorporation of cells into activated sludge and attached growth of cells were tested for cell immobilization.

When alginate immobilized cells were used as the seeding material for dye decolourization, 64 % colour removal was resulted after 72 h incubation. However, these alginate beads (cell immobilized) were not stable and solubilized in the decolourization media after few days of operations. Nevertheless, when decolourization media was prepared without Na_2HPO_4 .$7H_2O$ reagent, beads were more stable and did not dissolve in the medium. As reported [172], calcium alginate matrix is sensitive towards chelating compounds, such as citrate, phosphate and lactate or non-gelling cations such as magnesium or sodium. To stabilize calcium alginate beads, various methods are available and the simplest method is to maintain the sodium: calcium ratios at specific values [173]. Ramsay et al. [174] also reported the breakage of calcium alginate beads during the storage in NaCl due to exchange of

calcium ions in the alginate matrix with sodium ions in the storage medium. Dye decolourization conducted with different microbial species immobilized in calcium alginate beads in synthetic dye media are reported in literature [175, 176, 177]. However, decolourization of textile effluent using alginate immobilized cells is rarely reported [114]. Textile effluents may contain sodium and calcium ions in varying ratios [2]. Therefore, the maintenance of the stability of alginate beads in textile effluent decolourization will be problematic. Hence, seeding material prepared by immobilizing cells in calcium alginate was considered as inappropriate for the colour removal in textile effluent and therefore was not used for the further studies.

When the mixture of dye was decolourized using cells incorporated in sludge, 77% colour removal was achieved. Microscopic observations of prepared sludge samples showed that there was no proper incorporation of bacterial cells with the sludge. Activated sludge consists of organic fibres, inorganic particles, extracellular polymeric substances, filamentous bacteria, and ions (calcium, magnesium etc.). Activated sludge in wastewater treatment units may form when the bacteria develop a sticky layer of slime around the cell wall that enables them to clump together and separated from the liquid phase. As reported in literature, bacteria form this slime layer when sufficient food is unavailable for their energy and cell production. At this condition, bacteria lose its flagella to conserve energy thereby reduce motility and form flocs [178]. As culture broth contained nutrients, majority of bacteria were observed in the liquid broth without being incorporated with the sludge. Further, sludge samples were autoclaved in this work to remove any living microorganisms, which may change the structure and the composition of sludge. Therefore, the sludge sample did not settle to the bottom of the flask but remained as a suspension in the solution.

Further, when the sludge containing media were centrifuged, a separate layer of bacterial cells was observed. Hence, the observed colour removal could be due to the free cells in the media but not due to the cells incorporated in sludge. Due to these reasons, seeding material prepared by incorporation of bacterial cells with the sludge was also considered as inappropriate to be used for investigation of dye decolourization in the bioreactor.

All three bacterial species used in the consortium namely *P. mirabilis, M. morganii* and *E. cloacae* have cell structures such as pili or flagella [179, 180, 181] which

enhance the biofilm formation. Further, the ability of each of these bacterial isolate to form biofilms on different surfaces were reported in literature.

4. 11. 1. 1 Microtiter plate (MTP) assay

Biofilm formation ability of these bacterial isolates were confirmed by MTP assay. As shown in Figure 4.63, all three strains showed biofilm formation on the wells of the microtiter plate. Ability of these bacterial strains to form biofilms is in agreement with the previously reported work [182, 183, 184].

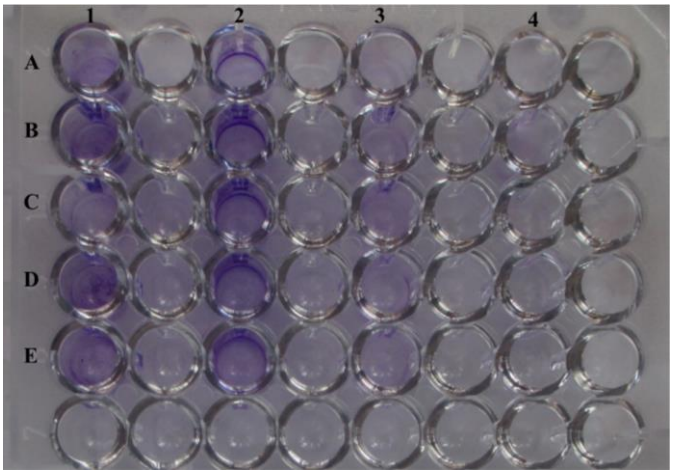


Figure 4.63: Detection of biofilm formation by each microorganism by microtiter plate method (wells 1A to 1E: *P. mirabilis*, 2A to 2E: *M. morganii*, 3A to 3E: *E. cloacae* and 4A to 4E: control)

Compared to the control, higher biofilm formation was observed in the wells containing *P. mirabilis* and *M. morganii* cells and comparably lower biofilm formation was indicated by *E. cloacae* (Table 4.13). Colin et al. [185] also reported *E. cloacae* as a weak biofilm producer. However, direct comparison of results obtained from MTP assay with work reported in literature is not possible due to minor differences in assay protocols and bacterial strains used.

Table 4.13: Optical density values measured from ELISA reader at 595 nm wavelength

	Bacterial strain	**OD at 595 nm**
I_1	*P. mirabilis*	0.1486
I_3	*M. morganii*	0.2278
I_4	*E. cloacae*	0.0946

4. 11. 1. 2 Investigation of biofilm formation on plastic carrier materials

As reported in section 3.19.1, selected three species were introduced individually to three plastic boxes containing the selected carrier material. Substrate (nutrient media) was transferred through the carrier materials to allow the initial attachment of the bacterial cells to the carrier surfaces and thereafter for the development of mature biofilms as described in section 2.5.2. When carrier materials in section 3.19.1 were observed after 55 days of inoculation, biofilm was visible. As most bacteria are hydrophilic [186, 187], adhesion is favored to the hydrophobic PP materials [188]. SEM images of the plastic carrier material (control) and biofilms formed by each bacterial strain on carrier surfaces are shown in Figures 4.64, 4.65, 4.66 and 4.67 respectively. However clearly visible biofilms were observed only on carrier materials, located close to the nutrient feeding point of the carrier-holding box. Nevertheless, results obtained by this study assure the biofilm formation ability of all three bacterial strains on the selected carrier material.

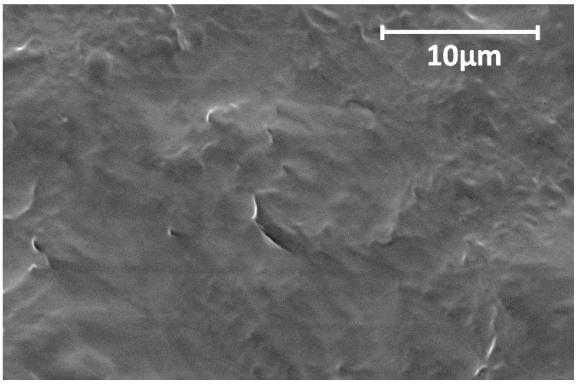


Figure 4.64: SEM image of biofilm support material (x7500) (control)

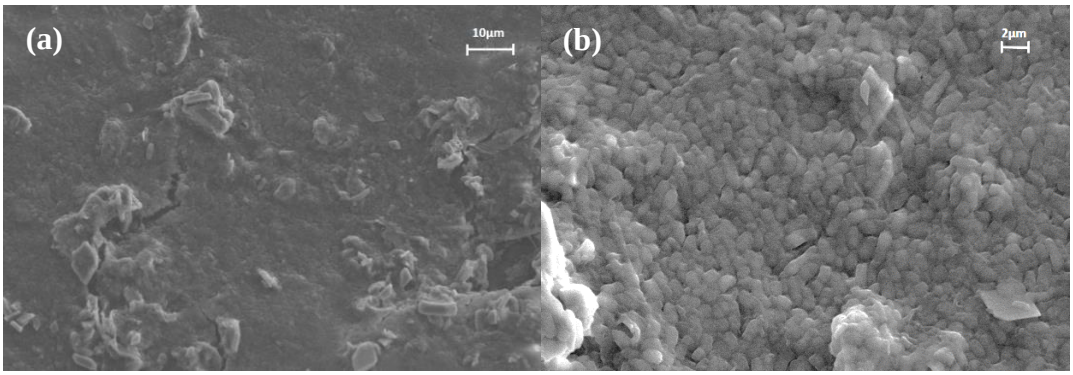


Figure 4.65: SEM images of *P. mirabilis* biofilms at (a) 2500x and (b) 7500x magnifications

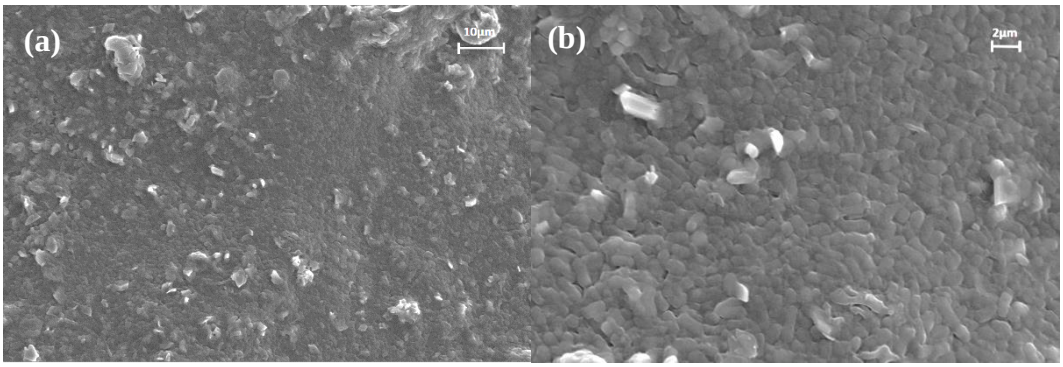


Figure 4.66: SEM images of *M. morganii* biofilms at (a) 2500x and (b) 7500x magnifications

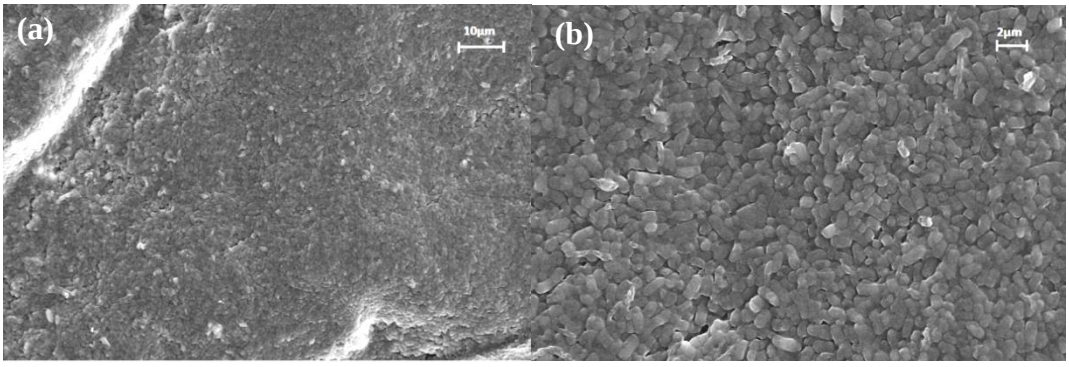


Figure 4.67: SEM images of *E. cloacae* biofilms at (a) 2500x and (b) 7500x magnifications

SEM images showed the ability of each bacterial species to form biofilms on the plastic carrier material used. Hence, this biofilm technique was selected as the most appropriate method for reactor seeding material preparation.

Figure 4.68 shows the fixed bed biofilm reactor used for dye decolourization in this study.

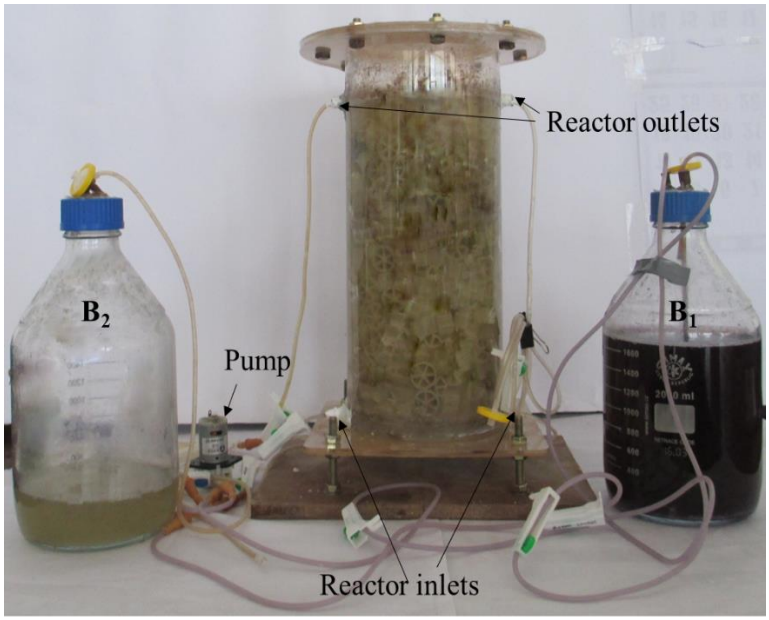


Figure 4.68: Fixed bed biofilm reactor

4.11.2 Decolourization of synthetic dye mixture in batch FBBR

Figure 4.69 shows the percentage decolourization of the synthetic dye mixture (50 mg/l) in media containing different concentrations of yeast extract due to the treatment in FBBR in batch mode.

As discussed in section 4.8.5, concentration of yeast extract has a direct influence on colour removal when decolourization was conducted in flasks using free cells. When decolourization was conducted with free cells (Figure 4.49), firstly the medium was inoculated with respective cultures of bacteria and allowed to grow in flasks. Therefore, yeast extract is utilized for both bacterial growth and colour removal. When low concentrations of yeast extract are used (2 and 1 g/L), colour removal was low (41 and 22%) which could be due to utilization of yeast extract mostly for cell growth or

could be due to low cell concentration resulted by low concentration of yeast extract. However, when the synthetic dye mixture was treated in the FBBR operated in batch mode, where already formed biofilm is present, decolourization was not highly influenced by the amount of yeast exact (Figure 4.69). 90% decolourization was observed in media having 2 and 1 g/l yeast extract after 44 h operation of FBBR in batch mode. Moreover, 77% decolourization of synthetic dye mixture was observed at 44 h when the concentration of yeast extract was further reduced to 0.25 g/l.

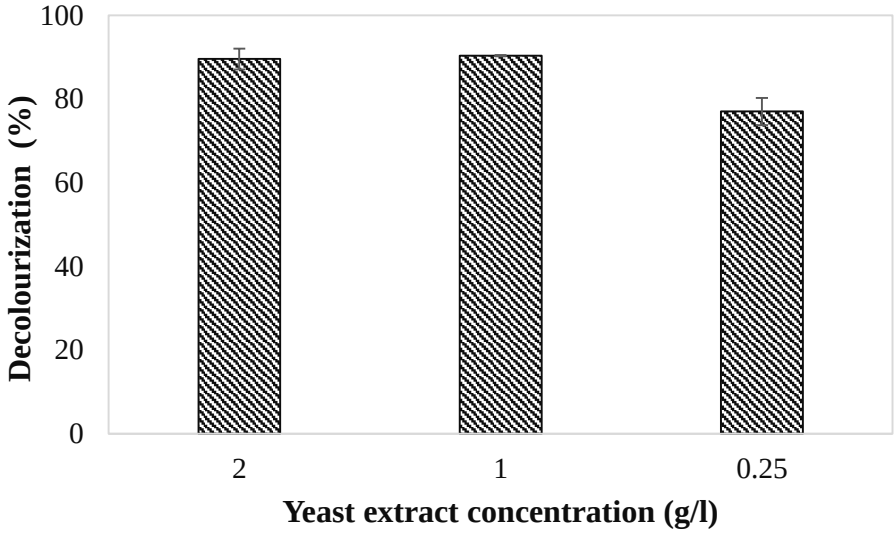


Figure 4.69: Effect of the concentration of yeast extract on decolourization of the synthetic dye mixture after 44h in batch FBBR

Optical densities of free microbial cells in reactor fluid (measured as in section 3.12) were 0.3 and 0.065 (average) when the influent has 1 and 0.25 g/l yeast extract respectively. These free cell concentrations observed in batch operation of the FBBR are significantly lower than the free cell concentrations observed in dye decolourization in flasks (section 4.8.5). Hence, high decolourization percentages achieved in batch operation of FBBR could be due to the attached cells (biofilm). Anjaneya et al.[99] reported that the colour removal of Amaranth dye by a bacterial biofilm grown on surface of bone chips was more effective than freely suspended cells. Formation of biofilms on a surface depends on several factors such as surface properties, microbial cell properties and environmental conditions [91]. For the

multiplication of microorganisms that are attached to a surface, and for the formation of biofilms, nutrients are required. However, the microorganisms within biofilms can withstand nutrient deprivation better than free cells and also have a reduced rate of growth relative to free bacterial cells growing in broth culture [88, 189]. Moreover, dense microbial communities in packed bed biofilm have stable operating performances and excellent dye decolourization characteristics [99, 104]. In the fixed bed biofilm reactor operated in batch mode there was a ~88% colour removal in the synthetic dye mixture with 20-fold decrease of yeast extract concentration compared to batch operated flasks with free cells. Barragán et al [103], observed ~80% removal of Reactive Black 5 dye in a packed bed reactor with 6 fold decrease of nutrient concentration compared to cells grown in flasks.

Yeast extract in the decolourization medium may serve as an electron donor for the cleavage of azo bonds in dyes other than being a source of carbon (providing building blocks) for the growth of bacteria [7]. The addition of external carbon sources influences the reductive conditions in the reactor by providing electrons required for the breakage of azo bonds and therefore increase colour removals. Reductive conditions in a reactor can be measured using values of oxidation reduction potential (ORP). It is reported that, with the addition of a nutrient solution that contribute to initial COD of 2500 mg/l, ORP value of a "granular activated carbon-biofilm configured sequencing batch reactor" dropped to -480 mV improving colour removals [101]. In this work, more than 88% colour removal in synthetic dye mixture containing media was observed after 68 h of operation when the batch FBBR was fed with the lowest tested concentration of 0.25 g/l yeast extract in the medium. Even though the reactor media has low amount of yeast extract, electrons provided by yeast extract appears to be sufficient for the reductive cleavage of azo bonds of the dyes by bacteria in FBBR. The ability of this bacteria in FBBR to decolourize the synthetic dye mixture with significantly low quantity of carbon source is economically favourable when applied in industrial biological dye decolourization.

4.11.3 Decolourization of synthetic dye mixture in continuous FBBR

When decolourization was conducted in continuous mode in the FBBR for 73 days (Figure 4.70), average of 75% colour removal of the dye mixture (25 mg/l concentration) was observed during phase I (Table 3.3) with 40 ml/h influent flow rate. In the second phase, where the concentration of dye mixture was 50 mg/l and the influent flow rate was increased to 83 ml/h (1.9 d HRT) with a dilution rate of 0.0105 h^{-1}, gradual increase of dye decolourization from 76 to 83% was observed. Even though the concentration of yeast extract in the feed was reduced to 1 g/l in the third phase, considerable reduction of dye decolourization was not observed (similar to section 4.11.2). Mean decolourization of FBBR treated water during phase III was 85%.

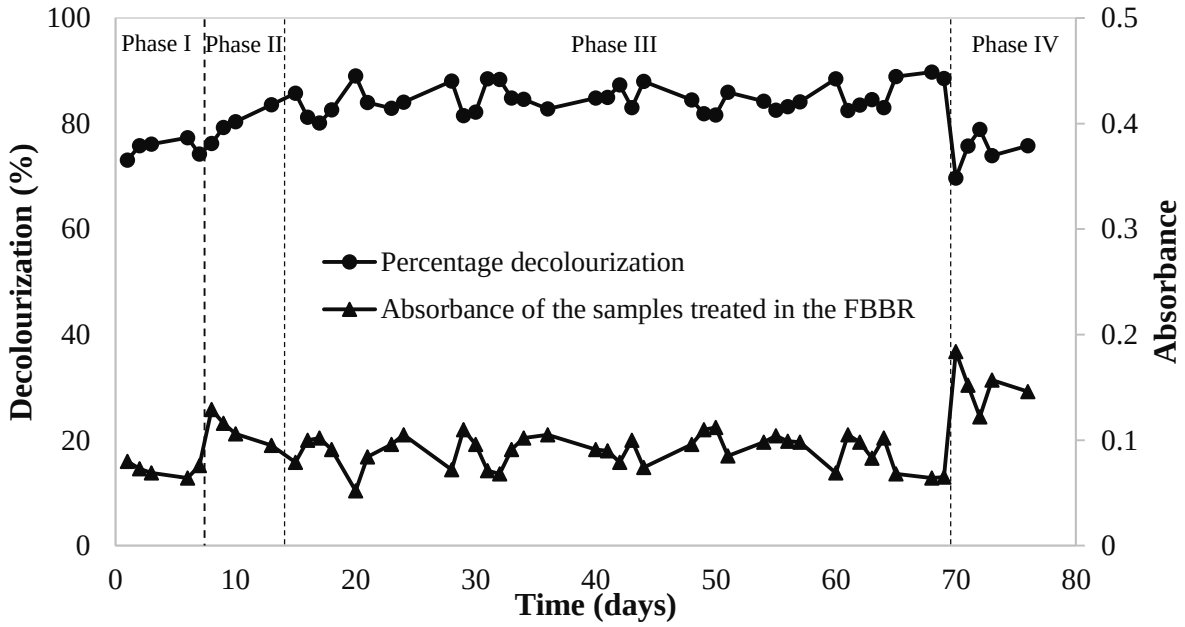


Figure 4.70: Decolourization of synthetic dye mixture in FBBR in continuous mode

Concentration of yeast extract in the feed was further reduced to 0.25 g/l after phase III and the colour removal was investigated. As shown in Figure 4.70, considerable reduction of colour was observed and the average colour removal during this phase was calculated as 75% even with the limited yeast extract concentration.

As observed, both batch and continuous FBBR operations resulted in more than 75% reduction of colour of the synthetic dye mixture containing media even at low concentration of yeast extract (0.25 g/l). Ong et al. [101] has observed high oxidation-reduction potential and almost oxygen free condition at the bottom of a granular activated carbon compartment of the sequencing batch biofilm reactor which was used to decolourize Acid Orange 7. Considering the observed high colour removals, existence of highly reductive environment with low concentrations of dissolved oxygen can be expected in the FBBR used in this study.

Dry cell weights of biofilm grown on carrier material of the continuous FBBR on day 1, 32, 52 and 69 were 5.83, 9.83, 10.12 and 10.33 mg/piece respectively. Biofilm formations were high at the initial 32 days of FBBR operation and gradually reduced thereafter. This could be due to the reduction of the concentration of yeast extract in the reactor influent after phase II.

As shown in Figure 4.71, dark colour of the reactor feed (generated due to 50 mg/l dye mixture) was not observed after the biological treatment in FBBR.

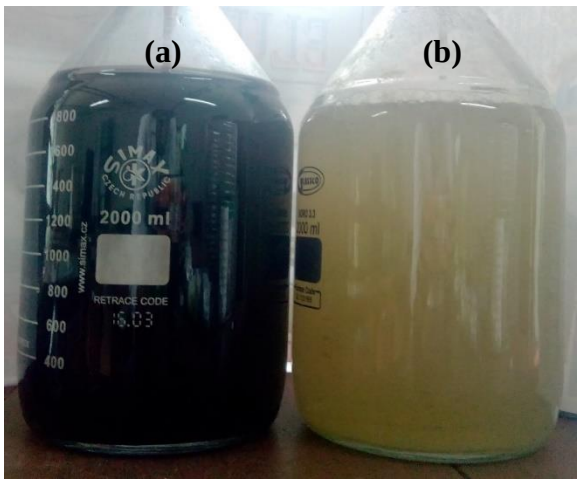


Figure 4.71: a) Influent (50 mg/l synthetic dye mixture containing medium) and b) treated water obtained during phase III operation of FBBR

4.11.4 Decolourization of textile effluent in batch and continuous FBBR modes

Decolourization conducted with 50% diluted wastewater in FBBR operated in batch mode showed average colour removals of 32% and 35% after 24 and 48 h of reactor operation. When the FBBR was operated in continuous mode with 50% diluted wastewater (1.9 days HRT), there was a gradual increase in percentage decolourization from 33 to 45% within 26 days (Figure 4.72). Observed increase in percentage decolourization indicated that the toxic environment [1, 190] in the FBBR (due to textile effluent) has not affected biological activities of bacteria.

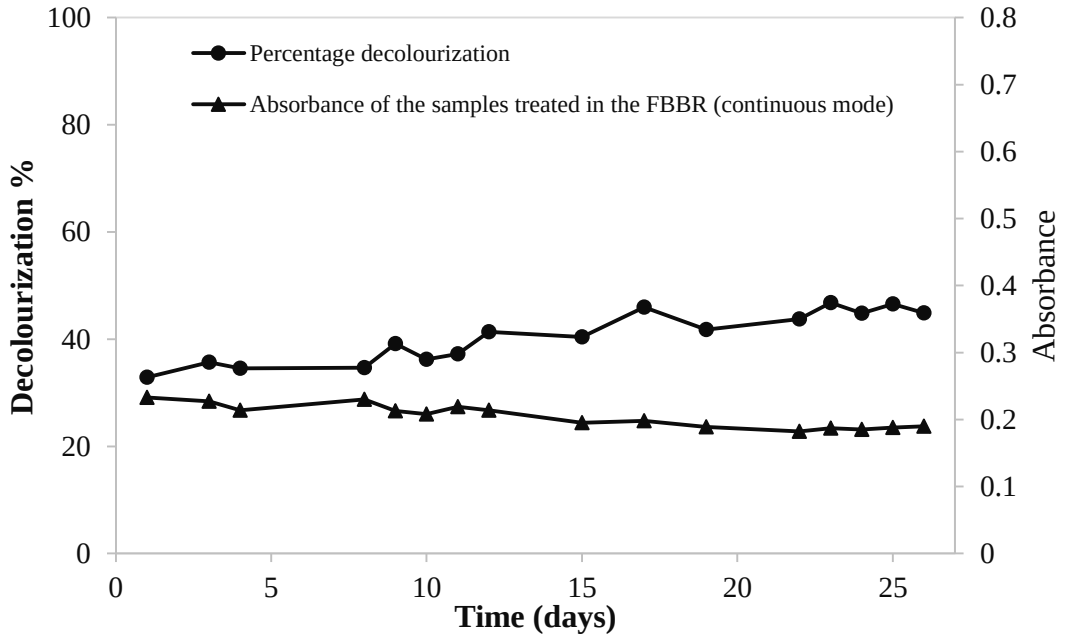


Figure 4.72: Decolourization of 50% diluted textile effluent in continuous FBBR

When undiluted textile wastewater was decolourized in batch mode, after 48 h, 70% colour removal was achieved. Figure 4.73 shows the colour change (Maroon to pale yellow) of textile effluent due to the batch treatment in the FBBR.

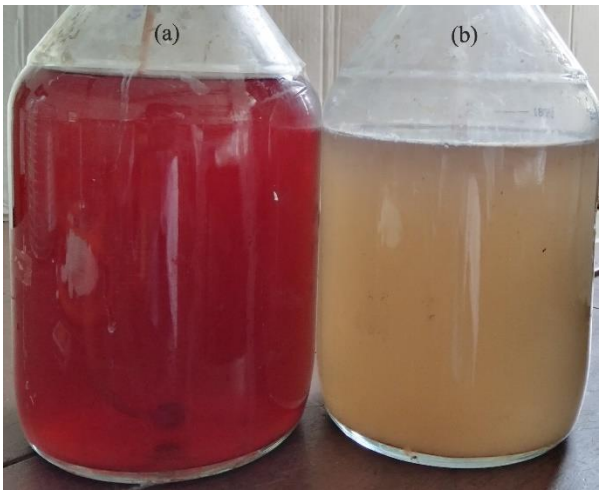


Figure 4.73: a) Feed (Undiluted textile effluent containing medium) and b) 48 h treated water obtained from the batch FBBR

Ultra violet-visible spectra of undiluted textile effluent before and after the biological treatment in FBBR are shown in Figure 4.74. Degradation of colour compounds in effluent after the biological treatment is indicated by the decrease of absorbance values in the visible range of the spectrum of undiluted effluent.

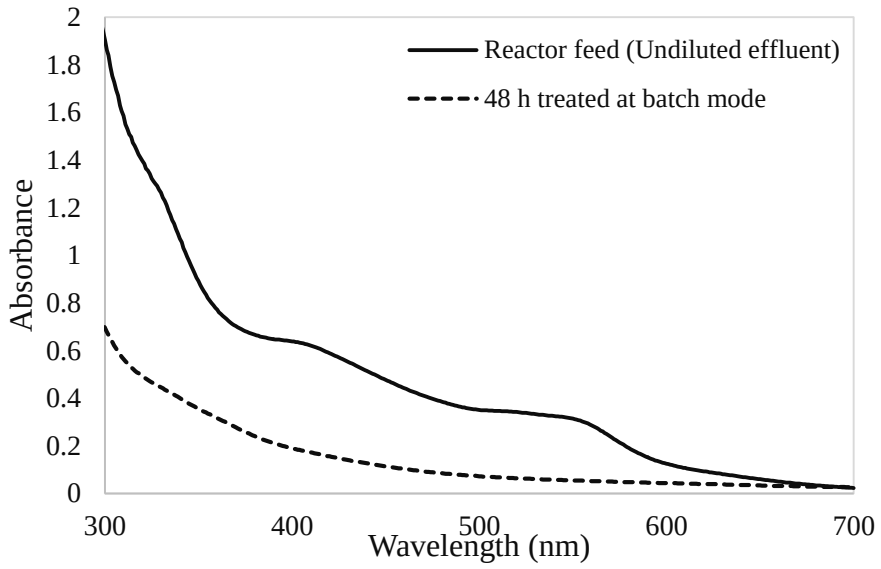


Figure 4.74: UV-visible spectra of undiluted textile effluent before and after treat in FBBR

Table 4.14 shows the maximum spectral absorption coefficient values of the reactor influent and effluent samples at three wavelengths. The last column of the table indicates the permissible textile effluent colour discharge limit (standard value) in Sri Lanka. As shown in columns three and four, maximum spectral absorption coefficients of dye mixture (continuous reactor – 69th day) and undiluted textile effluent (batch reactor) have reduced significantly after treatment in bioreactor. After the biological treatment, colour of the dye mixture has reached permissible discharge limits at 436 and 525 nm wavelengths however; further decolourization is required at 620 nm.

When textile industry effluent was treated in the reactor, considerable colour removals were observed in treated samples (effluent) compared to wastewater (influent) samples. However, the final absorption values of the treated effluent were not sufficient to meet the effluent discharge standards. Hence, minor improvements (such as increase of HRT, increasing reductive conditions in the reactor etc.) in the treatment are required to enhance the quality of the treated effluent to permissible discharge limits. Long HRT may enhance the interaction between biofilms and wastewater therefore high colour removals can be achieved [191, 192].

Further, biological treatments can be combined with advanced chemical treatment methods such as ozonation [193, 194] and Fenton's process [195] to obtain efficient and cost effective colour removal in textile effluent.

Table 4.14: Maximum spectral absorption coefficient of reactor feed (influent), treated water samples (effluents) and the permissible discharge limits

Sample	**Wavelength**	**Maximum spectral absorption coefficient (m^{-1})**		
		Influent	**Effluent**	**Standard value**
Dye mixture	436 nm	47.1	4.7	7
	525 nm	57.2	4.5	5
	620 nm	49	6.7	3
Undiluted textile effluent (batch)	436 nm	53	13.2	7
	525 nm	33.8	6.2	5
	620 nm	9.3	4	3

Most of the reported reactor studies have been conducted directly using sludge samples obtained from industrial effluent treatment facilities without specific analysis (identification) of the microorganisms present in the sludge. Dye decolourization in FBBRs of more than 1 l volume with isolated bacterial strains (consortium) is not reported in literature. Absence of such work may be due to difficulty in handling and operating bioreactors of large volumes under sterile conditions.

However, during this study, both synthetic dye mixture and textile industry effluent decolourization was effectively conducted in the FBBR (working volume 3.8 l) operated at HRT of 1.9 days.

Further, the majority of dye decolourization studies conducted in the fixed/packed bed biofilm reactors is limited to synthetic decolourization media that contain a single dye or a mixture of dyes. Reactor studies conducted with actual textile industry effluent are rarely reported [191, 196]. Decolourization of textile effluent in a bench-scale experimental system, comprising of an anaerobic biofilter (2.7 l total volume), an anoxic reactor and an aerobic membrane bioreactor is reported in literature [196]. There, the anaerobic biofilter that was seeded with activated sludge indicated 70% removal of effluent colour.

Khelifi et al.[191] studied Indigo dye-containing textile effluent decolourization in a fixed film bioreactor packed with rippled cylindrical polyethylene support. The reactor has been seeded with an acclimated microbial consortium obtained from a textile wastewater treatment plant and 80% removal of colour has been obtained.

Eventhogh biofilm growth on PP support materials have been studied and reported [197, 188], dye decolourization conducted with biofilms grown on PP support materials were not found in literature. In this study, biofilm formation ability of *P. mirabilis, M. morganii* and *E. cloacae* on PP carrier materials was investigated. Biofilm composed with these three bacterial species showed considerable colour reduction of synthetic dye mixture and textile industry effluents after the treatments in the FBBR.

4.12 Analysis of the Biofilm Formed on the Carrier Materials in FBBR

4.12.1 Biofilm formation on the carrier materials in the fixed bed biofilm reactor (FBBR)

A visible growth of biofilm was observed on carrier surface after one month of nutrient supply. Biofilms formed on plastic carrier materials during reactor operation are shown in Figure 4.75. Approximately 1 mm thick wet biofilm was observed on the surfaces of the carrier.

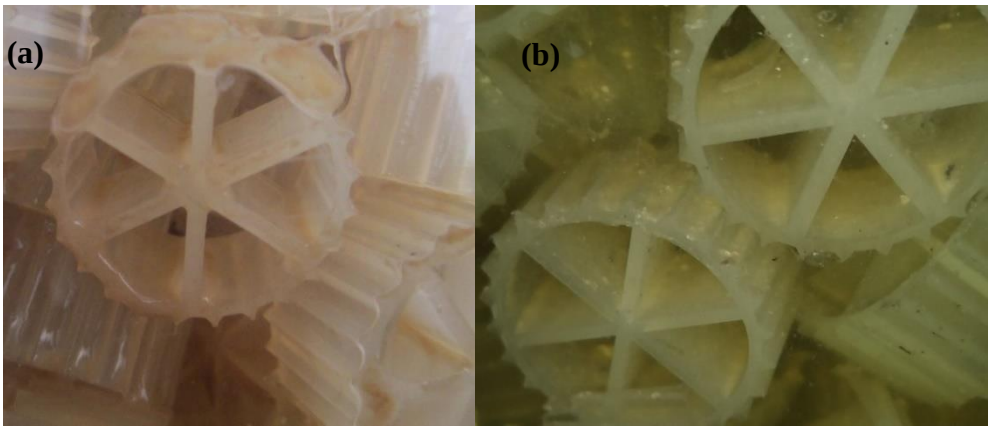


Figure 4.75: Biofilms formed on the plastic carrier material after (a) one month and (b) two months of reactor operation

The presence of biofilm on plastic carrier material in the reactor was confirmed by SEM analysis. SEM images of the plastic carrier material (control) and biofilms grown on carrier surfaces are shown in Figure 4.76.

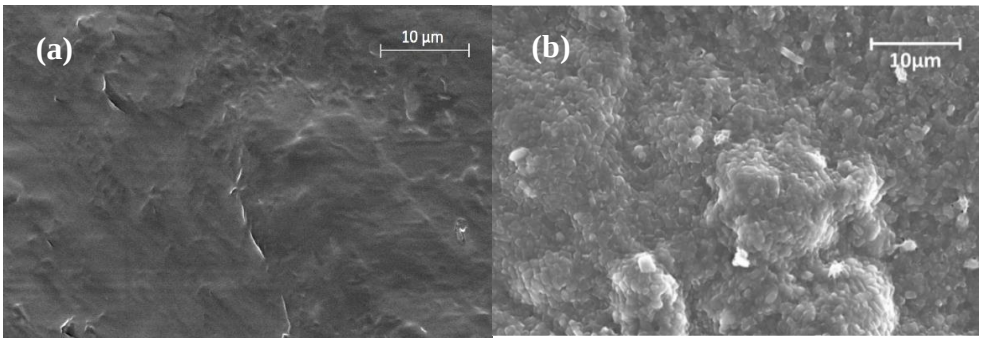


Figure 4.76: SEM images of a) carrier material (x5000) and b) biofilms grown on the carrier material (x5000)

Figure 4.77 shows plastic carriers with attached biofilms obtained from the final stages of operation of FBBR (after 26 days of continuous operation of the FBBR with textile effluent)

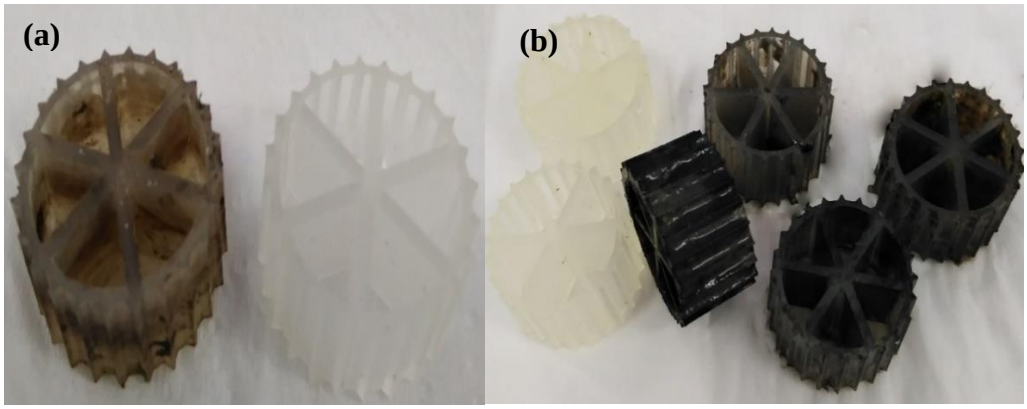


Figure 4.77: a) biofilm attached on carrier particle (dried) and control b) biofilms attached on carriers (wet) and controls

As discussed in section 2.5.2, extracellular polymeric substances (EPS) are one of the major chemical compounds present in biofilms. EPS are mainly composed of polysaccharides, nucleic acids, lipids and proteins. Figure 4.78 shows an SEM image of a biofilm grown on the surface of the carrier material obtained during this study. Similar to Anjaneya et al. [64], bacterial cells embedded in EPS were detected in this SEM image (Figure 4.78).

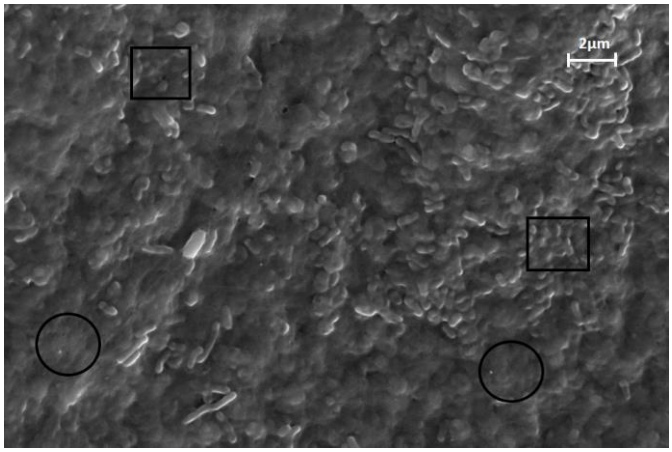


Figure 4.78: SEM image showing the adherence of bacterial cells on to the surface of carrier material. Cells covered with EPS (□); EPS (o).

4.12.2 FTIR analysis of biofilm

FTIR spectrum revealed the presence of typical EPS macromolecules in the developed biofilms. As shown in Figure 4.79, FTIR spectrum of the biofilm has three major peaks at 3384, 1656 and 1089 cm^{-1} wavenumbers. The peak at 1656 cm^{-1} is attributed to amides I which is a protein secondary structure [120, 198]. The prominent peak at 1089 cm^{-1} indicates the presence of carbohydrates in EPS [120]. The broad peak observed at 3384 cm^{-1} could be attributed to the N–H stretching vibrations of amides and amines and stretching vibrations in hydroxyl functional groups of carbohydrates [198, 199]. Even though any significant peak related to lipids was not observed in the FTIR spectrum of the biofilm, a slightly visible peak at 2900-2960 cm^{-1} region might be due to lipids [120]. Based on spectral data it can be concluded that the biofilm contains high quantity of carbohydrates, which is an active group of polysaccharides in EPS compared to proteins and lipids. Similarly, Hu et al. [120] reported, carbohydrates as the main component and lipids and proteins as minor components of EPS in the biofilms obtained from MBBR.

Extracellular polymeric substances (EPS) are of crucial importance to the biofilms and control the living conditions of microorganisms in biofilm by affecting mechanical stability, porosity, charge, water content, sorption properties, hydrophobicity, and density [200, 201]. Even though, EPS does not contribute to the colour removal by degradation of dyes, it has been reported as a biosorbent in removing certain types of cationic dyes such as basic blue 54 and methylene blue [202, 203]. Further, Janaki et al. [204] reported decolourization of reactive dye containing effluent using polyaniline/extracellular polymeric substance composite. Adsorbing groups such as carboxyl, hydroxyl and amino groups in the EPS are assumed to be responsible for dye decolourization [203, 204]. Colour change in the biofilm was not observed when the synthetic dye mixture was treated in the biofilm reactor and hence it can be considered that there was no significant colour removal due to biosorption. However, in undiluted textile effluent decolourization, there was a visible colour change in the biofilm. This could be due to adsorption of some dyes into the biofilm matrix.

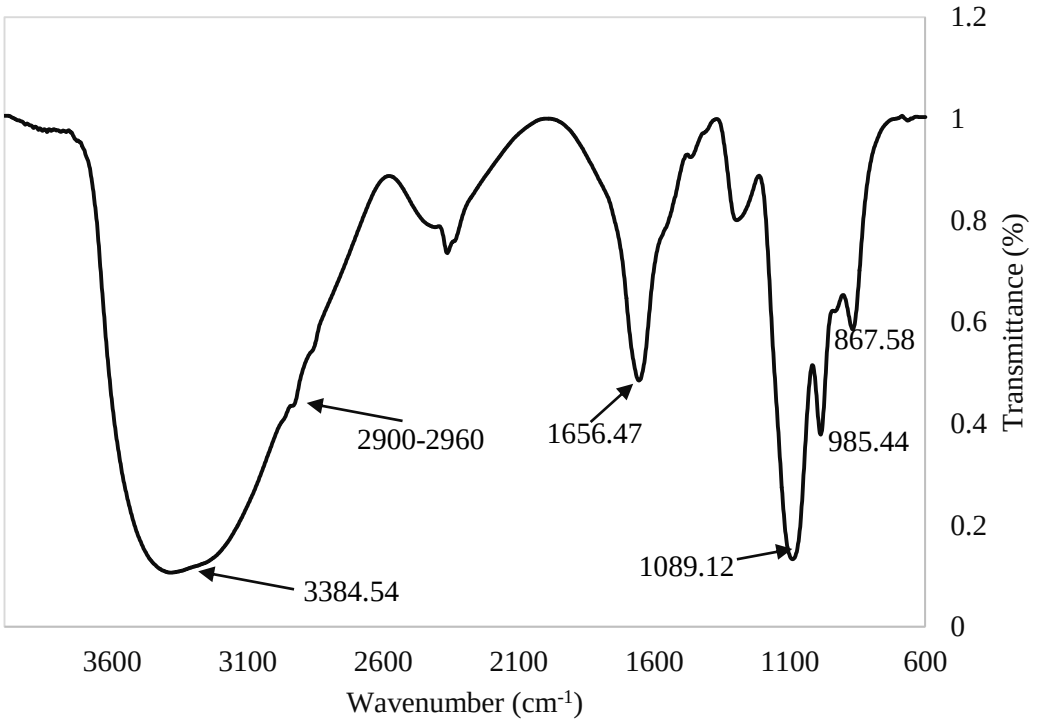


Figure 4.79: FTIR spectrum of the biofilms attached to the carrier

4.13 Determination of Quality of Treated Water in FBBR

4.13.1 COD analysis

High values of COD indicate the presence of large quantities of organic matter in the water. Textile dyes which are organic compounds also contribute to a portion of COD in dye containing liquid samples. Hence a reduction in COD can be expected as dyes degrade.

Chemical oxygen demand of water samples collected from FBBR at different phases are given in Table 4.15. 45% and 50% reduction of influent COD due to the treatment in FBBR was observed respectively during phase III and phase IV of synthetic dye mixture decolourization studies. As influent of FBBR contained synthetic dye mixture and nutrients (3.19.4), measured COD could be due to both these components. COD of 50 ppm synthetic dye mixture (without nutrients) was 225 mg/l. As more than 90% colour removal was observed, it can be assumed that COD reduction detected in these phases of FBBR operation could be mainly due to the degradation and mineralization of textile dyes in the FBBR. Hence, the residual COD in the treated reactor broth could mainly be due to the remaining nutrients (yeast extract).

Table 4.15: COD of the water samples collected from FBBR

Analyzed sample	**COD before treatments in FBBR (mg/l)**	**COD after treatments in FBBR (mg/l)**
Synthetic dye mixture containing media collected at phase III of FBBR operation	1074	585
Synthetic dye mixture containing media collected at phase IV of FBBR operation	675	338
50% diluted effluent containing media collected at continuous operation of FBBR	1350	1020
Undiluted effluent containing media collected at batch operation of FBBR	1830	1450

Compared to synthetic dye mixture, textile effluent decolourization studies conducted in FBBR indicated low COD removals (Table 4.15). During these studies high COD values were observed in FBBR feed as it contained textile effluent with different organic compounds including dyes and added nutrients. Observed COD removals could be attributed to the degradation of dyes in textile effluent. As reported by Grilli et al. [196] and Ong et al. [101] further reduction of COD could be obtained by combining this FBBR with an aerobic treatment system.

4.13.2 Phytotoxicity analysis

Cowpea is a common agricultural crop in Sri Lanka, which can grow under local climate conditions. Cowpea seeds germinate easily, and rapid growth of the plant can be observed at early stages of growth. Therefore, cowpea was selected for this study.

Seed germination and plant growth bioassays were done to evaluate the phytotoxicity of the treated water (effluent of FBBR) samples. Percentage germination of seeds watered with biologically treated wastewater and of control sample were similar while less seed germination was observed when watered with untreated wastewater (influent

of FBBR) (Tables 4.16 and 4.17). Similar observation has been reported by Vallinachier et al. [39] for seed germination assay conducted with textile dye containing wastewater decolourized by *C. arietinum.*

Table 4.16: Effect of effluent on cowpea seed germination.

Sample	Time (days)	Control	Untreated effluent	Treated effluent
Germination %	2	90	60	90
Average length of plumule (cm)	4	1.8±0.23	0.9±0.16	1.5±0.17
Average root length (cm)	4	2.4±0.26	1.50±0.25	2.1±0.21

The seeds watered with distilled water resulted the highest average plumule, shoot and root lengths. However, compared to the untreated wastewater, seeds watered with biologically treated wastewater indicated higher shoot and root lengths (Tables 4.16 and 4.17). Seedling vigour indices of control, untreated effluent and treated effluent samples calculated in this study were 378,144 and 324 respectively. This index can be used to study the inhibitory activity of phytotoxic substances on germination process [205]. Higher SVI values obtained for treated effluent compared to untreated effluent shows the toxicity reduction of textile effluent due to this biological treatment method. Images of cowpea seeds taken during seed germination and plant growth are shown in Figures 4.80 and 4.81 respectively. Results obtained from these two assays imply the toxicity reduction of textile effluent due to the treatments in FBBR.

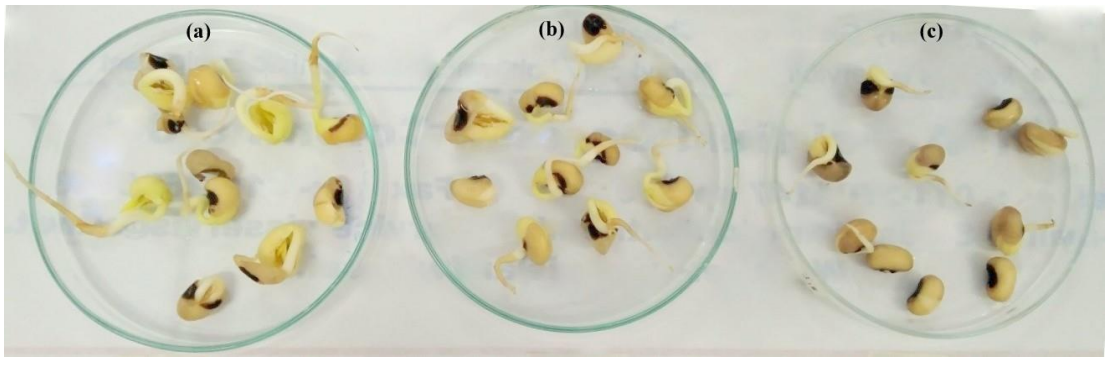


Figure 4.80: Germination of cowpea seeds watered with (a) control water (b) treated wastewater and (c) untreated wastewater

Table 4.17: Results of the plant growth assay

Sample	Time (days)	Control	Untreated effluent	Treated effluent
Germination %	3	71	43	71
Average shoot length (cm)	5	10.6±3.5	8±4.4	9.3±4.6
Maximum shoot length (cm)	5	15	11	13
Average shoot length (cm)	6	15.3±2.6	10±4.4	12.7±4.0
Maximum shoot length (cm)	6	19	13.5	16

Figure 4.81: Growth of cowpea seeds watered with (a) control water (b) treated wastewater and (c) untreated wastewater

4.13.3 Analysis of the metabolites formed by biological degradation of synthetic dye mixture in FBBR

4. 13. 3. 1 UV-visible spectrophotometric analysis

Different groups and units in dye structures form peaks in the UV-visible spectrum based on the size of their conjugated double bond system. Due to the large conjugated structure around the chromophore group, it can absorb light at low frequencies indicating maximum absorbance peak in the visible range of the spectrum. Presence of other conjugated systems such as benzene and naphthalene are possible in dye molecules which indicate absorbance peaks in UV range of the spectrum [206]. As a dye gets degraded, dye structure is broken down in to simpler compounds and therefore, the strength of the conjugated system is reduced. Hence, the degraded compounds generally indicate peaks at the UV-range of the UV-visible spectrum.

Figures 4.82, 4.83 and 4.84 show UV-visible spectra of the reactor feed (50 mg/l dye synthetic mixture containing media) and treated water samples obtained at different stages of FBBR operated in batch and continuous modes. Maximum absorbance peak of the synthetic dye mixture containing feed observed at 554 nm wavelength, has reduced after biological treatment indicating the destruction of chromophore groups of dye molecules (Figures 4.82, 4.83 and 4.84). The peak at 286 nm (in the UV-visible spectra of synthetic dye mixture containing feed) indicated the presence of additional conjugated structure (which is unknown) in the synthetic dye mixture other than the chromophore group.

Ultra violet-visible spectra of the treated water sample collected from the FBBR after 68 h batch operation (when feed consisted of 0.25 g/l yeast extract containing media) showed a peak at 291 nm (Figure 4.84). Absorbance value of this peak was less than 0.5 and could be generated due to the degradation of the compound which previously indicated a peak at 286 nm. Hence, it can be assumed that the bacteria in biofilm can degrade the chromophore group of dyes as well as the other conjugated structures which could be present in dye media. However, no peak was observed closer to 286 nm wavelength in the spectra of water treated in FBBR when feed consisted of 2 and 1 g/l yeast extract (Figure 4.82 and Figure 4.83). This could be due to the overlapping of the peak at 286 nm with the peak at 249 nm.

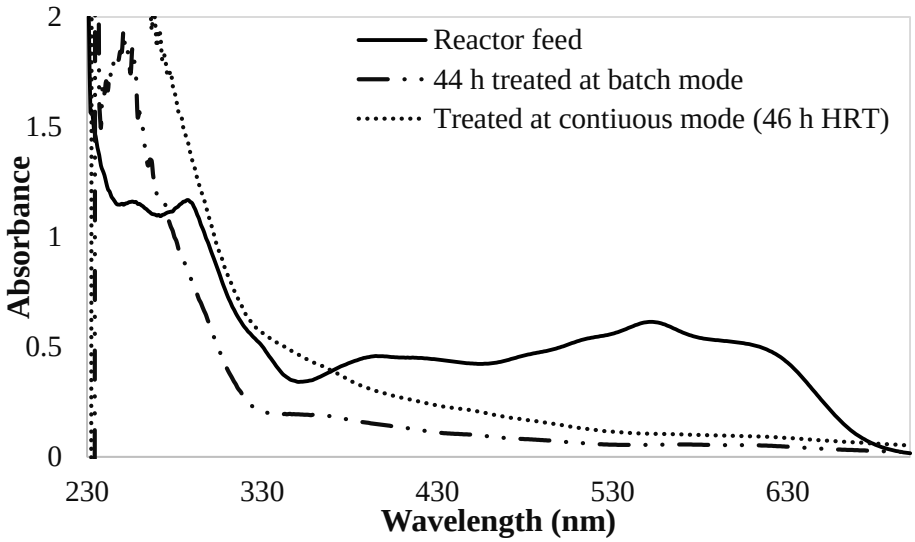


Figure 4.82: UV-visible spectra of reactor feed (2 g/l yeast extract containing media with 50 mg/l synthetic dye mixture) and treated water samples obtained from FBBR operated in batch and continuous mode

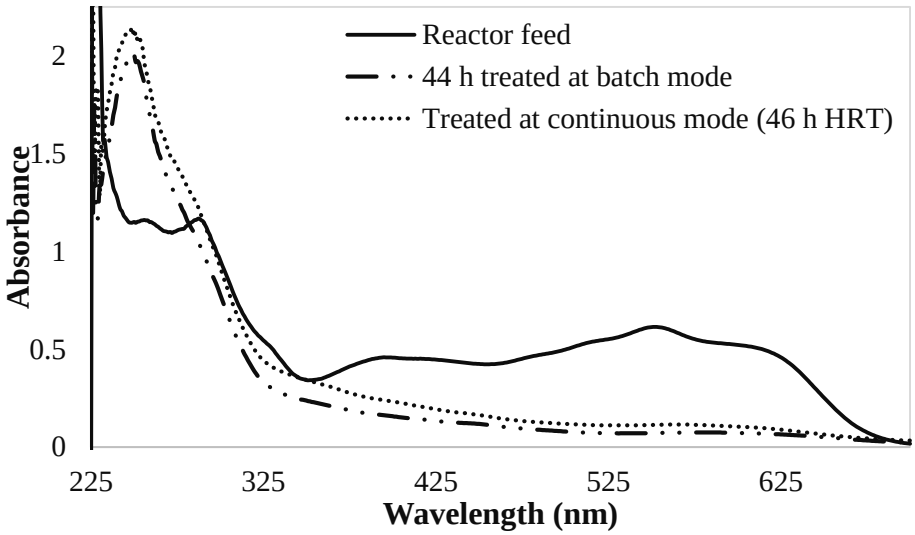


Figure 4.83: UV-visible spectra of reactor feed (1 g/l yeast extract containing media with 50 mg/l synthetic dye mixture) and treated water samples obtained from FBBR operated in batch and continuous mode

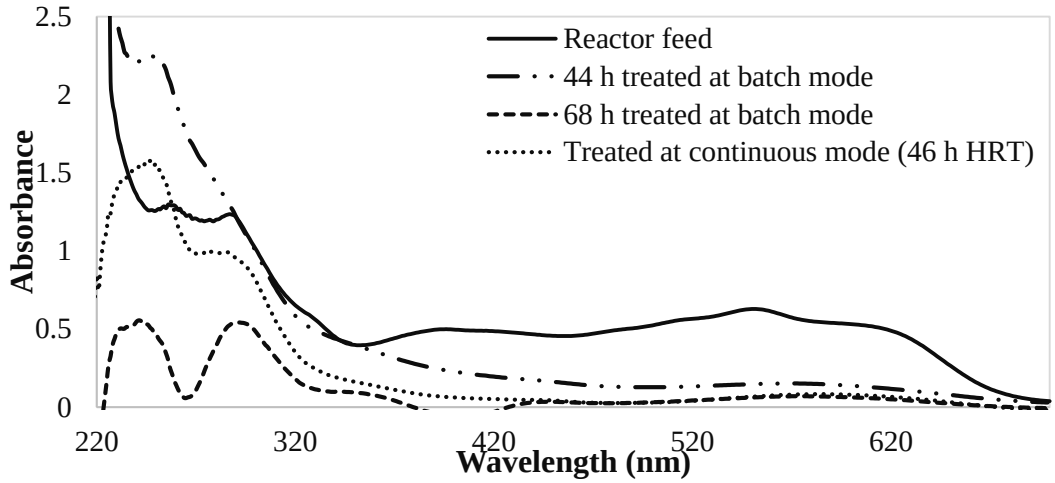


Figure 4.84: UV-visible spectra of reactor feed (0.25 g/l yeast extract containing media with 50 mg/l synthetic dye mixture) and treated water samples obtained from FBBR operated in batch and continuous mode

Formation of a new peak within 241-251 nm wavelength range of the spectra was observed in all treated water samples collected. This new peak detected in the UV range of the spectra could be due to a compound formed by degrading a dye. UV-visible spectra of synthetic dye mixture containing water samples treated for 68 h in FBBR in batch mode (when feed contained 0.25 g/l yeast extract containing media) has shown a clear reduction of this peak (Figure 4.84). Hence, it is assumed that this bacterial consortium can decolourize the synthetic dye mixture by biodegradation and further breakdown the compounds formed into simpler under the operating conditions of the reactor.

4. 13. 3. 2 HPLC analysis

High performance liquid chromatograms of synthetic dye mixture and the treated water from the batch operation of the FBBR during decolourization of dye mixture are shown in Figures 4.85 and 4.86.

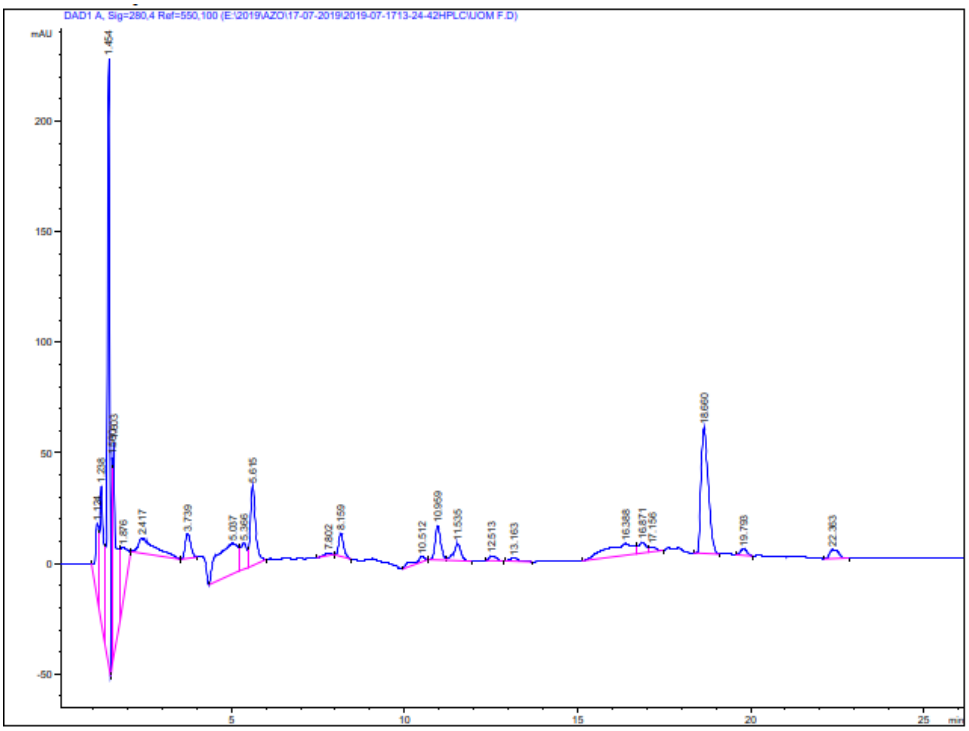


Figure 4.85: HPLC chromatogram of the reactor feed that containing 50 mg/l of synthetic dye mixture

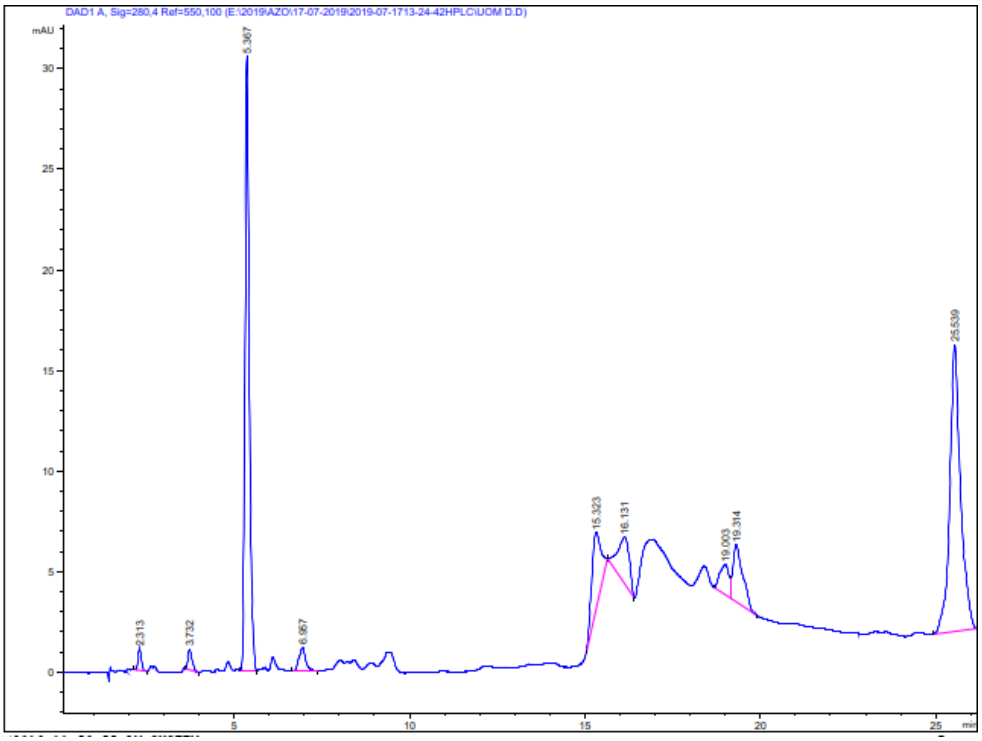


Figure 4.86: HPLC chromatogram of the feed treated in FBBR in batch mode

HPLC chromatogram of the synthetic dye mixture has a number of prominent peaks at 1.238, 1.454, 1.603, 1.876, 5.037, 5.615, 18.66, 28.858 and 30.072 min retention times with peak areas of 499, 1218, 631, 327, 615, 402, 837, 332 and 781 mAU.s respectively. On the other hand, chromatogram of the biologically treated samples has major peaks at 5.367, 25.5, 28.861, 29.92 and 30.264 min with peak areas of 261, 302, 299, 460 and 983 mAU.s. Absence of the previously observed peaks and the formation of new peaks indicate the structural changes occurred in dye molecules due to biological treatment.

4. 13. 3. 3 GCMS analysis

Two compounds present in the biologically treated synthetic dye mixture (4.11.2) were identified as phenol, 4-methyl- and 1H-Indole, 3-methyl from GCMS analysis. Peaks relevant to these two compounds were detected at 4.134 and 7.930 min retention times in gas chromatogram (not shown) and their mass spectra are shown in Figure 4.90. Even though aromatic amines were the major expected metabolite of azo dye degradation under oxygen-limited conditions, aromatic amines were not detected in treated water samples. Although most bacteria are not capable of degrading aromatic amines under anaerobic/oxygen limited conditions, few studies have reported mineralization of aromatic amines (formed as dye degraded compounds) in biological reactors under anaerobic conditions [101, 207, 208]. Bacterial species such as *Moraxella osloensis* [67] and *Lysinibacillus* sp. [148] are reported to have the ability to mineralize textile dyes. Olukanni et al. [14] have reported mineralization of reactive Blue 13 dye by *Proteus mirabilis* under static conditions. Hence, the reason for not detecting aromatic amines in the GCMS analysis of biologically treated synthetic dye mixture could be due to the mineralization of these molecules by bacteria in biofilm (including *P. mirabilis*).

Any carcinogenic or toxic metabolites were not detected by the degradation of synthetic dye mixture in FBBR. Hence, this biological treatment method can be considered as an effective and environmental friendly method for the decolourization of textile dyes. Development of dye degradation mechanisms was not possible in this study as the original structures of the dyes are unknown.

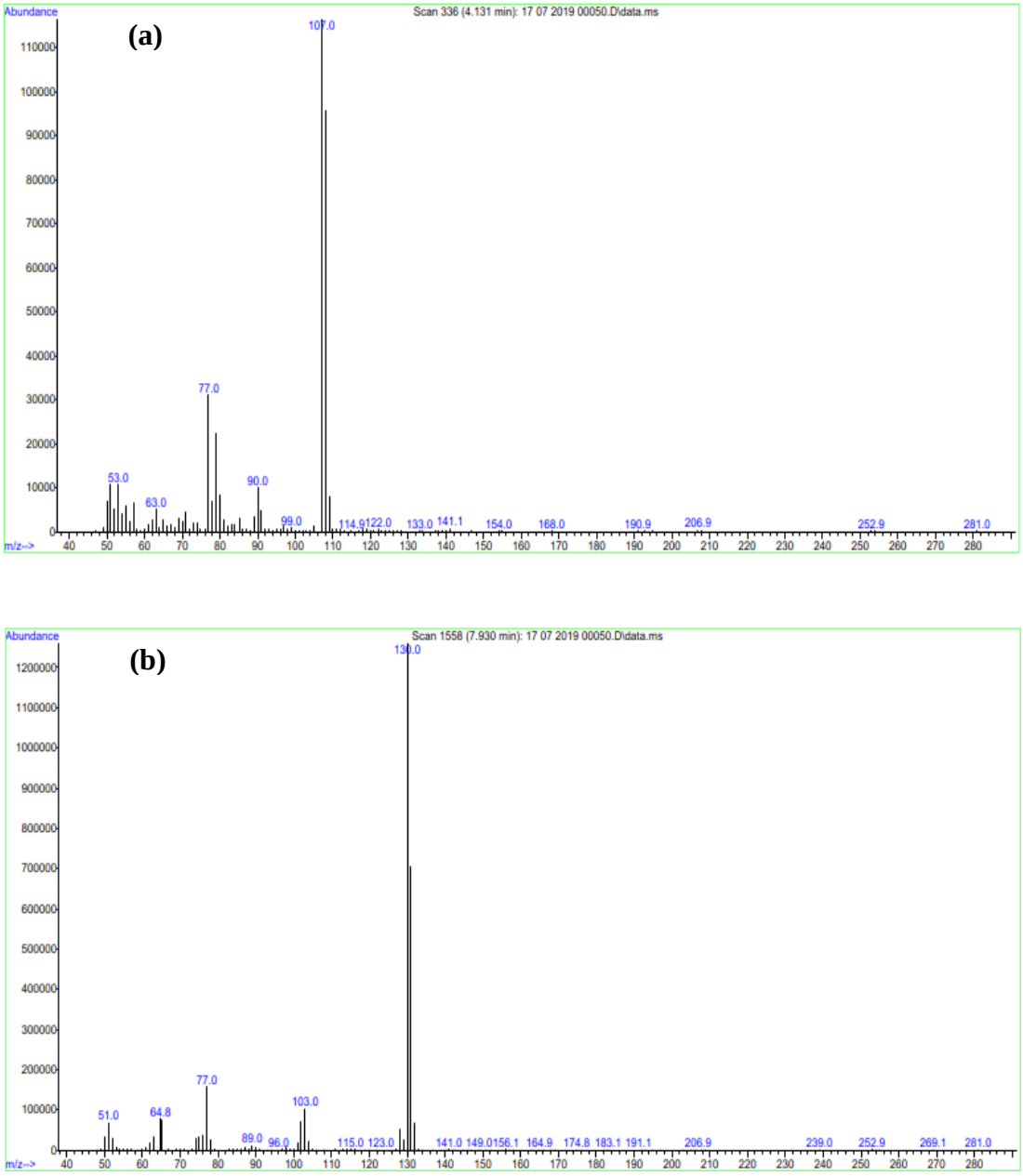


Figure 4.87: GC– mass spectra of compounds present in the biologically treated synthetic dye mixture (a) phenol, 4-methyl- (b) 1H-Indole, 3-methyl

CHAPTER 5

5 CONCLUSIONS

- Reactive dyes were identified as the most widely used dye category and chemical coagulation and flocculation as the most commonly used effluent decolourization technique in Sri Lanka.
- Five bacterial strains; *Proteus mirabilis, Morganella morganii, Enterobacter cloacae, Escherichia fergusonii* and *Acinetobacter baumannii* isolated from a local textile effluent treatment facility were able to decolourize textile dyes used.
- Out of these bacterial strains, *Proteus mirabilis* showed the highest capability to decolourize dyes and decolourize 50 ppm; Yellow EXF, Red EXF, Blue EXF, Black WNN and Rhodamine up to 96, 94, 83, 95 and 30% respectively after 72 h incubation.
- Bacterial consortium consisted with *Proteus mirabilis, Morganella morganii* and *Enterobacter cloacae* resulted, more than 90% color removals in all four reactive dyes and 36% decolourization in Rhodamine dye after 72 h of incubation.
- Colour removal of the synthetic dye mixture was more effective with the bacterial consortium due to synergistic metabolic activities of microbial community in the consortium than the individual bacterial strains.
- Optimum decolourizations of dyes were observed at pH 7-8, 40 ºC and oxygen-limited, static conditions.
- Out of the three methods tested namely; cells immobilized by alginate beads, activated sludge and biofilm, biofilm was identified as the most appropriate seeding material for the reactor to achieve the objectives of this research.
- A fixed bed biofilm reactor with 3.8 l working volume was fabricated and a biofilm consisted of *Proteus mirabilis*, *Morganella morganii* and *Enterobacter cloacae* strains were grown in it.
- Corrugated cylindrical tubes made of polypropylene were found to be a good support material for the development of bacterial biofilms.
- When FBBR was operated in batch mode with culture media having 2 and 1 g/l yeast extract and 50 ppm of synthetic dye mixture, 90% colour removal was observed after 44 h. Colour removal was only 85% when the reactor was operated in continuous mode

with 1.9 days of HRT. Even when the concentration of yeast extract in the feed was reduced to 0.25 g/l, more than 75% decolourization in 50 ppm dye mixture was achieved in both batch and continuous FBBR cultures.

- UV-visible spectra and HPLC analyses indicated structural changes in synthetic dye mixture due to biological treatment. Formation of toxic metabolites due to degradation of the dye mixture was not observed in this study.
- When the FBBR was operated continuously with 50% diluted textile effluent as the feed, there was a 45% colour removal while there was a 70% colour removal in undiluted textile effluent treated in batch mode for 48 h.
- Biological treatment methods used in this work are effective, economical and environmentally friendly compared to most widely used dye decolourization technique (coagulation and flocculation) in Sri Lanka. Therefore, the FBBR can be considered a highly promising alternative technique for the treatment of textile dye-containing wastewater in industry.

CHAPTER 6

6 RECOMMENDATIONS AND FUTURE WORK

- The ability of the isolated bacteria to decolourize textile dyeing industry effluent was investigated in flaks and in a laboratory-scale reactor. Before, the method is applied to industrial scale it is always good to conduct biological effluent decolourization in pilot-scale and hence the work should be extended to pilot scale with a reactor which can treat large volumes of wastewater.
- Utilization of pure cultures of bacteria is not practically possible when this biological dye decolourization is implemented in pilot or industrial-scale reactors. However, to maintain the effectiveness of the treatment process, it is recommended to frequently monitor biofilms in the FBBR and assure the survival of dye decolourizing microorganisms in the reactor.
- The effect of different sources of carbon in biological decolourization of Yellow EXF was investigated using glucose, corn starch and yeast extract in the medium. Out of the tested carbon sources, yeast extract containing medium resulted the highest colour removal with free bacterial cells. However, yeast extract cannot be recommended as an appropriate carbon source to be utilized in industrial wastewater treatment. Therefore, further studies should be conducted on cost-effective sources of carbon which can be used in industrial effluent decolourization processes.
- Even when the media in FBBR contained low concentrations of external carbon (0.25 g/l) to treat the dye mixture, there was an acceptable level of colour removal. This biological treatment method will be further cost-effective if dye decolourization could be achieved without the addition of external carbon sources. Hence, it is recommended to conduct textile effluent and synthetic dye decolourization without or with further low concentrations of external carbon to determine the minimum dosage of carbon required for the effective removal of colour.

- When FBBR was fed with synthetic dye mixture and textile industry effluent, colour reduction to a considerable level was observed in all operations at HRT of 1.9 days. However, further enhanced colour removals can be achieved by changing the operating conditions of FBBR (increasing HRT, reducing dissolved oxygen concentrations etc.). Hence, it is recommended to test different FBBR operating conditions to obtain treated wastewater with expected level of colour that fulfills the effluent discharge limits.
- In this study, the FBBR was operated under oxygen-limited conditions without aeration. Even though oxygen-limited conditions are more appropriate for the biological decolourization of textile dyes/effluents, mineralization of dye degraded compounds is commonly reported under aerobic conditions. Hence, it is recommended to combine FBBR with an aerobic reactor to further enhance the quality of the treated effluent.
- Even though the reactor was operated in both batch and continuous modes to investigate decolourization of 50% diluted textile effluent, decolourization of undiluted textile effluent was investigated only in batch mode due to practical issues faced during the study. Hence, it is recommended to conduct undiluted textile effluent decolourization continuously in the reactor to further investigate the appropriateness of this treatment method in industrial scale applications.

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APPENDIX A

INDUSTRIAL SURVEY QUESTIONNAIRE

Purpose of this industrial survey is to gather information related to the M. Phil. Degree titled "Development of a Novel Biological Decolourization System for Treatment of Textile Effluent"at the Department of Chemical and Process Engineering, University of Moratuwa. The information received from this questionnaire will be held in strict confidence and no information will be released or published.

Please provide answers for the following questions with regard to your company.

1. Company name:

……

…….....

2. Company products (eg: thread, cloth, finished garments):

……

……

……

3. Most commonly used dye types (please mention the names of the dyes) and their quantities:

Name of the dye	Quantity required (kg/month)

5. Main dye supplying companies:

   ……

   ……

   ……

6. Please fill the following table considering your company effluent.

Components of the textile effluent	Composition (as a %)
Organic matter	
Suspended solids	
Dye	
Salts	
Other	

7. Characteristics of the company effluent:

	Effluent Parameter	Value (prior to treatments)
1	pH	
2	BOD (mg/l)	
3	COD (mg/l)	
4	Temperature (^oC)	
5	Colour	

8. Types of effluent treatment methods use in company effluent treatment plant (if available):

……

……

9. Treatment methods use to remove colour from water:

……....

.....

10. Effectiveness of the currently using methods for decolourization of textile effluent: Assume that the effectiveness of effluent decolourization method is divided in to 10 portions. Right edge represents the complete decolourization and left edge represents zero decolourization (no change in effluent colour). Select the most appropriate treatment stage for your company effluent.

1	2	3	4	5	6	7	8	9	10

No change → Complete decolourization

11. Names of the dyes which are adding colour to industry effluent:

……

……

12. What are the most problematic dyes to treat with currently using decolourizing method?

……

……

13. Characteristics of the treated water (releasing from the company)

	Effluent Parameter	Value (After the treatments)
1	pH	
2	BOD (mg/l)	
3	COD (mg/l)	
4	Temperature (^{o}C)	
5	Colour	

----------Thank You----------

APPENDIX B

FABRICATION OF THE REACTOR COMPONENTS

i) Reactor lid

Reactor lid consisted of top plate (P_1) and top-guide plate (P_2). Eight holes were cut on both these plates for clamping purposes using laser technology. The lid was constructed by bonding P_1 and P_2 together. Plates were bonded to generate a groove in the lid (Figure 1) and a layer of silicon glue was applied around the edge of the groove which was used as a sealing material as the reactor fastened.

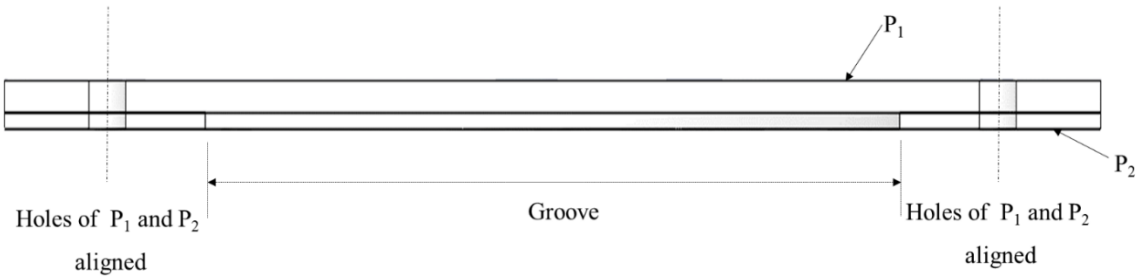

Figure 1: Sectional view of the reactor lid

ii) Reactor base

Reactor base was prepared by bonding bottom plate (P_8) and bottom-guide plate (P_7) together to generate a groove as shown in Figure 2. Both these plates contained four holes in the edges in order to fix the base to the support plate

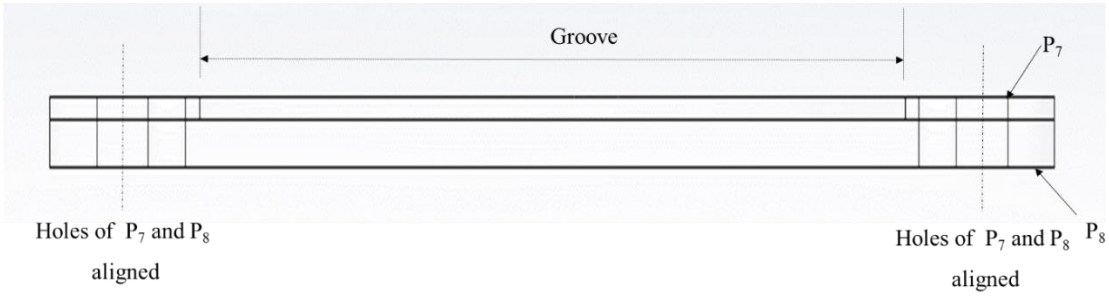

Figure 2: Sectional view of the reactor base

iii) Reactor column

Reactor column was composed with the tube (P_4), tube clamping plate (P_3), top grid plate (P_5) and bottom grid plate (P_6). Tube clamping plate was bonded to the external surface of the tube (0.5 cm below from the upper end of the tube) as shown in Figure

3. Holes were generated on the wall of the tube, to connect inlet and outlet connectors to the reactor (Figure 3).

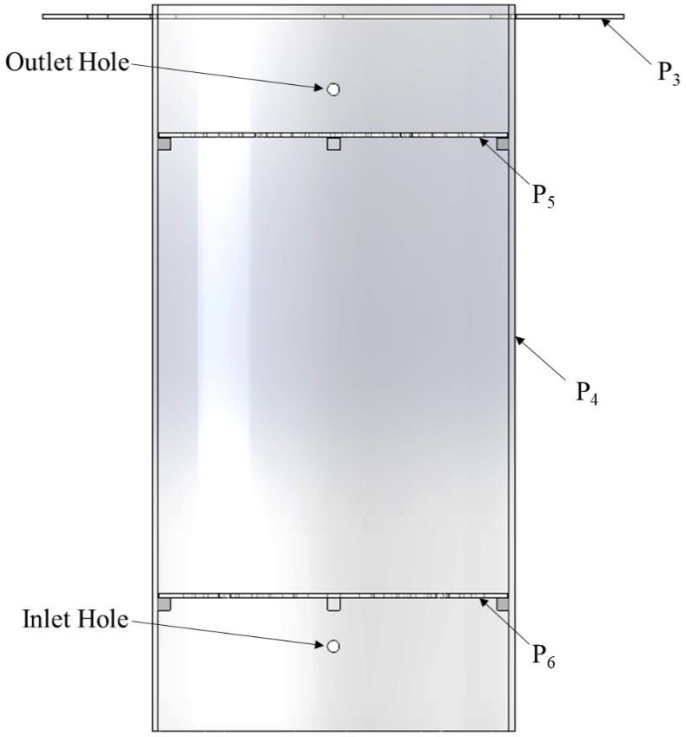

Figure 3: Sectional view of the reactor column

Both grid plates have symmetrically distributed holes which were used for proper distribution of influent throughout the reactor (P_6) and for collection of treated water over the bed of packing (carrier) material (P_5). P_6 was used to hold the carrier material and P_5 was used to pack the plastic carriers tightly and to avoid the free floating of carriers in the reactor.

iv) Reactor holding stand

Reactor holding stand was constructed using support plate (made out of wood), thread bars, nuts and bolts.

Initial assembling of the reactor

Initial assembling of the reactor components was done in laboratory environment (not inside the biosafety cabinet). Initially, the reactor column was bonded to the reactor base (Figure 4). Then the base was fixed with the support plate using thread bars, nuts and bolts as shown in Figure 5.

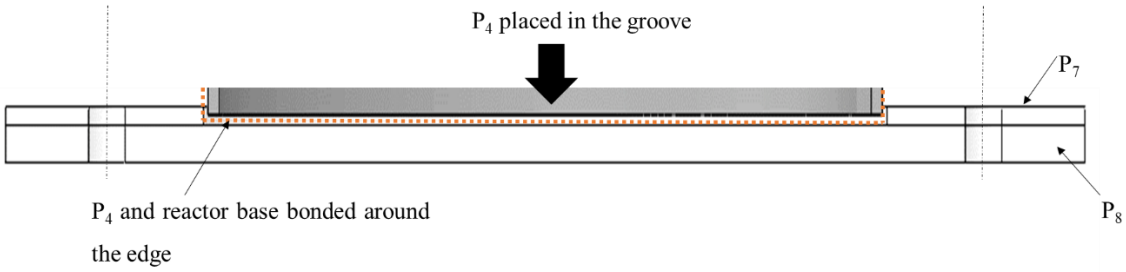

Figure 4: Bonding of the reactor column to the reactor base

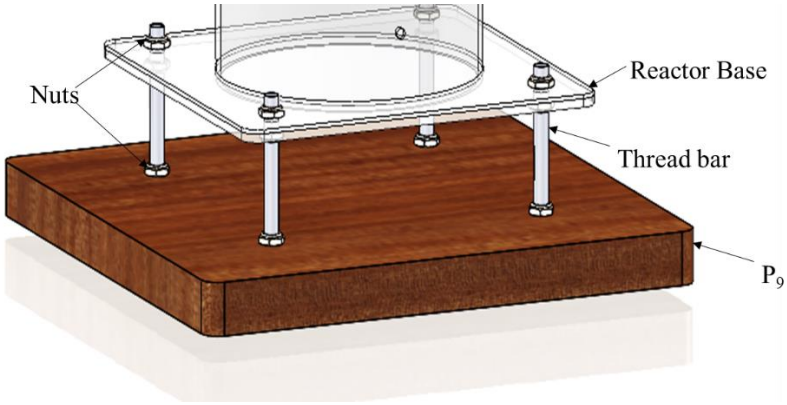

Figure 5: Fixing of the reactor base to the reactor holding stand

According to the design, reactor lid was to be placed on the reactor column as shown in Figure 6. However, lid was placed at the final assembling.

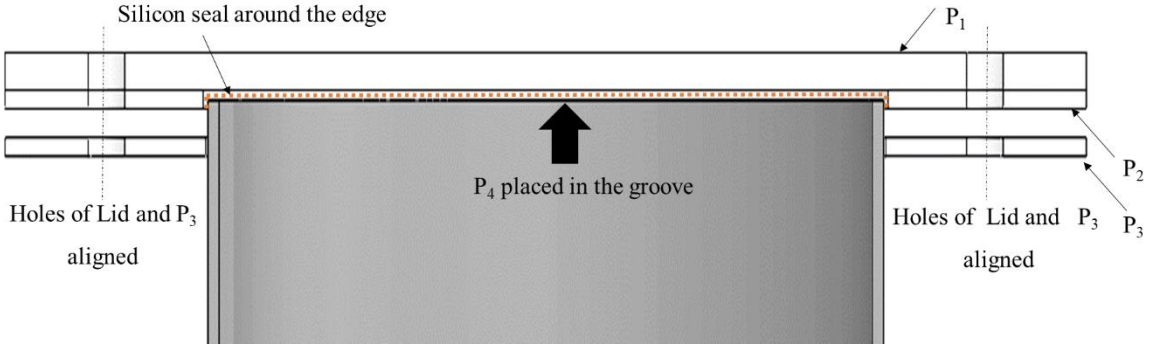

Figure 6: Fixing of the reactor lid to the reactor column

LIST OF PUBLICATIONS

No	Description	Category
1	Madhushika HG, Ariyadasa TU, Gunawardena SHP (2019) Biological decolourization of textile industry wastewater by a developed bacterial consortium. Water Sci Technol 80:1910–1918. https://doi.org/10.2166/wst.2020.010	Indexed Journal SCIE, Scopus
2	Madhushika HG, Ariyadasa TU, Gunawardena SHP (2019) Decolourization and Degradation of Reactive Textile Dyes by Isolated Strain Proteus mirabilis. Asian J Water Environ Pollut Vol. 16:1–6. https://doi.org/DOI 10.3233/AJW190042	Indexed Journal Scopus
3	Madhushika HG, Ariyadasa TU, Gunawardena SHP (2018) Decolourization of Reactive Red EXF Dye by Isolated Strain Proteus Mirabilis. In: 2018 Moratuwa Engineering Research Conference (MERCon). pp 231–234	Refereed Conference Scopus indexed
4	H. Gimhani Madhushika, T. Ariyadasa, and S. Gunawardena, 'Enhanced Decolourization and Biodegradation of Textile Azo and Xanthene Dyes by Using Bacterial Isolates,' In Proc. ICWWTP 2017 : 19th International Conference on Water and Wastewater Treatment Plants, Singapore, 11, 2017	Abstract - Conference Proceeding
5	H. G. Madhushika, T. U. Ariyadasa, and S. H. P. Gunawardena, "Biodegradation of reactive yellow EXF dye: optimization of physiochemical parameters and analysis of degradation products," *Int. J. Environ. Sci. Technol.*, Mar. 2021, doi: 10.1007/s13762-021-03220-6	Indexed Journal SCIE, Scopus
6	Manuscript titled 'Fixed bed biofilm reactor for decolourization of synthetic dye mixture and coloured effluent from textile industry' was submitted to Chemical Engineering Journal	Manuscript submitted