STUDIES ON DECOLOURISATION OF SYNTHETIC DYES USING SPENT MUSHROOM SUBSTRATES

A

Thesis

Submitted in fulfillment of the requirements for the award of degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

by

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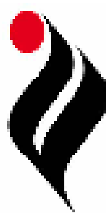

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2013

CERTIFICATE

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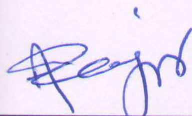

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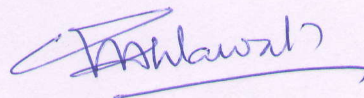

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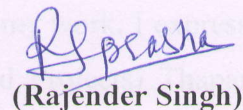
DECLARATION

I hereby declare that the work which is being presented in this thesis "**Studies on decolourisation of synthetic dyes using spent mushroom substrates**" submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. O.P. Ahlawat, Principal Scientist, Directorate of Mushroom Research, Solan, India and Dr. Anita Rajor, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.



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ACKNOWLEDGEMENT

I would here like to express my thanks to the people who have been very helpful to me during the time it took me to write this thesis. First I bow my head to "Almighty God" to give me strength to complete this work. I wish to express my profound gratitude to my thesis advisor **Dr. O.P. Ahlawat**, Principal Scientist, Directorate of Mushroom Research, Solan (HP) India and **Dr. Anita Rajor**, Assistant Professor, Department of Biotechnology and Environmental Science, Thapar University, Patiala, India for his dynamic spirit, pertinent guidance, patronage, prudent advises and dedication for research rendered to me during my work. I sincerely thank for his/her confidence and faith on me throughout my research. I am highly thankful to **Dr. Dinesh Goyal**, Head, Department of Biotechnology and **Dr. A.S. Reddy**, Head, School of Energy and Environmental Sciences for their comments and suggestions during the course of my work. I would like to express my deep appreciation to my committee members **Dr. Susheel Mittal** and **Dr. M.S. Reddy** for their valuable suggestions during the course of investigation. I am immensely grateful to **Dr. Manjit Singh**, Director and **Dr. R.P. Tewari**, Ex-Director, Directorate of Mushroom Research, Solan, for kind support in Directorate of Mushroom Research throughout my work. I express my gratitude to **Prof. P. K. Bajpayee**, Dean (Research and Sponsored Projects) Thapar University, Patiala, for encouragement and support during my research work in the University. I would also like to thank my friends Mamta Chauhan , Hemlata, Babita, Ashutosh Pathak, Kunal, Gurdeep, Rajkumar, Pankaj and Tina for their support when I was low with tough time and fighting back to get on the track.

Lastly, and most importantly, I would like to thank my family members. Their endless sacrifies, the numerous times when they remained awake so that I could sleep their unrelating support, encouragement and understanding have helped me to reach where I stand today. To them I dedicate this thesis. Thank you all!! I finish with a final silence of gratitude for my life.

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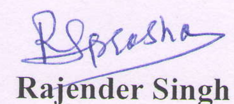

Rajender Singh

The following publications are the outcome of the research work carried out in the Thesis:

1. Singh, R., Ahlawat, O.P., Rajor, A., 2012. Identification of the potential of microbial combinations obtained from spent mushroom cultivation substrates for use in textile effluent decolourisation. Bioresource Technology **125**: 217-225.
2. Singh, R., Ahlawat, O.P., Rajor, A., 2013. Potential of spent substrate of *Pleurotus sajor-caju* for Methyl violet 2B decolourisation. Journal of Pure and Applied Microbiology **7**: 1099-1106.
3. Singh, R., Ahlawat, O.P., Rajor, A., 2013. Screening of mycelia and spent substrate of edible mushroom species for their dyes decolourisation potential. Mushroom Research **22**(2) 127-136
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5. Ahlawat, O.P**.,** Singh, R., 2009. Influence of pH, Temperature and Cultural medium on decolourisation of synthetic dyes through spent substrate of different mushrooms. Journal of Scientific and Industrial Research **68**: 1068-1074.
6. Singh, R., Ahlawat, O.P., Rajor, A. Decolosrisation of textile dyes by *Schizophyllum commune* and *Pezizomycotina* sp. isolated from spent substrate of *Pleurotus sajor-caju*. Applied Biochemistry and Biotechnology (Under Revision).

Conferences

1. Rajender Singh, O.P. Ahlawat and Anita Rajor. 2010. Effect of cultural conditions for the decolourisation of different dyes using spent mushroom substrate of *Pleurotus* spp. **Global Warming and Human Health**. Jamia Hamdard University, New Delhi, India **(Awarded with best poster)**.

2. Rajender Singh, O.P. Ahlawat and Anita Rajor. 2012. Potential utilization of spent substrate of *Pleurotus sajor- caju* for decolourisation of Methyl violet 2B. **International Conference on Industrial Biotechnology**. Punjabi University, Patiala, Punjab, India.

3. Rajender Singh, O.P. Ahlawat and Anita Rajor. 2013. Screening of mycelia and spent substrate of edible mushroom species for their dyes decolourisation potential. **Indian Mushroom Conference, 2013**. Punjab Agricultural University, Ludhiana, India.

// ABSTRACT

Mushroom cultivation generates two main end products, comprised of the mushroom fruit body and the substrate known as spent mushroom substrate (SMS). Substrate is considered as spent when one full crop of mushroom is harvested and further extension of the period become unremunerative. For each metric tons of fruit bodies of mushrooms produced, at least an equivalent amount of spent mushroom substrate is generated, and this needs to be disposed . This disposal can be costly because it is bulky in nature. During the previous year's efforts have been made to find out amicable ways to dispose of spent mushroom substrate after crop harvest because if it is left unattended it may cause environmental problems including nuisance. On the other side, wastewater from textile industries poses a threat to the environment in a large part of the world. The use of microorganisms or their enzyme systems are the two different biological approaches widely practiced for treatment of hazardous chemicals and effluents. The present study was planned after giving due considerations to the physico-chemical, microbiological and biochemical properties of spent mushroom substrate, as it may act as a good bioremediative agent. In the present study spent substrate of *Pleurotus sajor-caju* and *Agaricus bisporus* had been studied for extracellular ligninolytic enzymes and dyes decolourisation potential.

Among SMS of different mushrooms, the SMS from *P. sajor-caju* exhibited highest population along with diversity of both fungi and bacteria. Five fungi viz., *Aspergillus fumigatus*, *Paecilomyces variotii*, *Pichia guilliermondii*, *Schizophyllum commune* and *Pezizomycotina* sp. with potential dye decolourisation potential have been recorded to thrive on SMS of different mushrooms by using 5.8S rRNA gene sequencing and BLASTn techniques. Similarly, by using 16S rRNA gene sequencing and BLASTn tecnhniques, six potential bacteria *viz*., *Bacillus subtilis*, *B. pumilus*, *B. licheniformis*, *Pseudomonas fluorescens*, *Sphingobacterium multivorum* and *Rummelibacillus stabekissi* have also been identified, out of which *B. licheniformis* isolated from *P. sajor-caju* SMS, exhibited highest decolourisation potential of 66.10% against Orange II sodium salt, followed by *Bacillus pumilus* (57.7%). Out of isolated fungi, *Schizophyllum commune* from *P. sajor-caju* SMS has been recorded to exhibit highest decolourisation potential (95%) within 10 days against Chicago sky blue. This fungus also exhibited dye decolourisation potential of 100, 92.50, 81.60, 73.40 and 67.80% against Starch azure, Methyl violet 2B, Rhodamine B, Orange II sodium salt and Methylene blue, respectively.

Moreover, the study also evaluated the ligninolytic enzymes activities, dyes decolourisation by spent substrate filtrate and biomass production during dyes decolourisation. In optimization of cultural parameters role of pH, temperature, growing medium, additional carbon sources, heavy metals, initial concentration of dyes, addition of veratryl alcohol and manganese ions, maintaining of agitated/stationary conditions and inoculation with pellet and intact forms of mushroom mycelia were also studied for achieving highest dyes decolourisation with spent substrate of *P. sajor-caju*. Lower concentration of Methyl violet 2B, Rhodamine B and Azure B showed higher decolourisation efficiency (100%) with spent substrate of *P. sajor-caju* compared with higher initial concentrations of dyes. Decolourisation of Methyl violet 2B showed that lower concentration of Manganese ions, veratryl alcohol and addition of carbon and starch enhances decolourisation of Methyl violet 2B. Presence of lead, cadmium, cobalt and nickel ions supported higher decolourisation with spent substrate of *P.sajor-caju*.

The study also presents variation in microbial population of *Agaricus bisporus*, *Pleurotus sajor-caju* and *Volvariella volvacea* spent substrates along with ligninolytic enzymes activity and textile effluent decolourisation potential of microorganisms isolated from these SMSs. The effect of temperature, pH, carbon sources and immobilizing agents on effluent decolourisation using different combinations of these microorganisms has also been studied. *Schizophyllum commune* and *Pezizomycotina* sp. from *P. sajor-caju* SMS, exhibited highest activities of laccase (11.8 and 8.32 UmL^{-1}) and lignin peroxidase (339 and 318 UL^{-1}), while *Pseudomonas fluorescens* of Manganese peroxidase. Highest decolourisation was in presence of glucose and sucrose at 30 $^{\circ}$C, and microbial consortium comprised of the immobilized forms of *S. commune* and *Pezizomycotina* sp. on wheat straw and broth cultures of *P. fluorescens*, *Bacillus licheniformis* and *Bacillus pumilus*. First order kinetics was recorded for the dye decolourisation process by using SMS of *P. sajor-caju*, *A. bisporus* and potential fungi isolated from SMS. Bioremediation of synthetic dyes by SMS and their ligninolytic enzymes may be one alternative and effective way for textile effluent treatment and solid waste management of mushroom industry.

Keywords: Spent mushroom substrate; Fungi; Bacteria; Ligninolytic enzymes; Dye Decolourisation; *Pleurotus sajor-caju*

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Abbreviations

Abbreviation	Word (s)
%	Percentage
°C	Degree centigrade
ANOVA	Analysis of Variance
BLAST	Basic local alignment search tool
bp	Base pair
BS	Bootstrap value
C	Carbon
C.D.	Critical difference
cfu	Colony forming unit
cm	Centimeter
DNA	Deoxyribonucleic acid
dNTP	2’-deoxynucleoside-5’-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
g	Centrifugal force
g	Gram
hr	Hour(s)
ITS	Internal transcribed spacer
kb	Kilo base
KOH	Potassium hydroxide
L	Litre
M	Molar
MEA	Malt extract agar
mg	Milligram
Min	Minutes
ml	Millilitre(s)
mm	Millimetre(s)

mM	Millimolar(s)
MQ	Milli Q
N	Nitrogen
NA	Nutrient agar
NaCl	Sodium chloride
NCBI	National centre for biotechnology and information
NaOH	Sodium hydroxide
Ng	Nanogram(s)
nm	Nano meter
O.D.	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
ppm	Parts per million
rpm	rotation per minute
SD	Standard deviation
SE	Standard error
SMS	Spent mushroom substrate
Tris	Tris-(hydroxymethyl-)amino methane
U.V	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume
µg	Microgram
µl	Microlitre
µM	Micromolar

Chapter-1

Introduction

1.1 General introduction

The increasing industrialization has specially put pressure on natural resources like water bodies, agricultural land and the physical area by adding unwanted chemicals in the environment. Textile industry is one of the largest water consuming industries in the world. The manufacturing and use of dyes and pigments is a multibillion-dollar industry. The use of these chemicals is an integral part of almost all manufacturing processes. Dyes are synthetic chemical compounds having complex aromatic structures, which are extensively used in the textile, cosmetics, plastic, food, paper printing, colour photography and pharmaceuticals industries (Forgacs *et al.,* 2004). Approximately 10,000 different dyes and pigments are used at industrial scale and over 0.7 million tonnes of synthetic dyes are produced annually, worldwide (Knapp *et al.*, 1997).

United States Department of Commerce has predicted a 3.5 fold increase in textile manufacturing by 2020 (Ganesh *et al.,* 1994; Walsh *et al.,* 1980). In India, an average textile mill producing 60 x 10^4 m of fabric per day is likely to discharge approximately 1.5 Megalitres per day (MLD) of effluents (COINDS, Comprehensive Industry Document Series, 1999-2000). During industrial dyeing process a substantial amount of dye is lost in the waste water. It is estimated that nearly 40% of the total dyes used in the dyeing process may find their way in wastewater (Vaidya *et al.,* 1982). Even in modern days of industrialization, an industrial waste effluent is being often discharged into water bodies without any adequate pre treatment. When such wastewater effluent is discharged onto the land adjoining the agriculture crops, it causes the gradual deterioration of land quality and ultimate reduction in crop yields. This has led to an enhanced concern and awareness, forcing Government of India

to strict legislations for controlling the discharge of untreated effluents into the environment, may it be land, water or air.

Major classes of synthetic dyes are azo, anthraquinone and triaryl methane, and majority of them are toxic and even carcinogenic with long turnover times. With the increased use of a wide variety of dyes, pollution due to dye wastewater is becoming increasingly alarming. Colour is the first recognised contaminant in textile wastewater and has to be removed before discharging it into receiving water body (Padamavathy *et al.,* 2003). Among various industrial effluents involved in an increase of chemical load (COD) of water systems, effluents from textile dyeing industries are the major source, resulting discharge of coloured water causing toxicities to aquatic life. The presence of a variety of dyes in the wastewater emanating from textile dyeing industries is a serious concern because of their resistance to environmental conditions like light, temperature and pH, resulting in low biodegradability. The presence of high level of heavy metals viz., copper, zinc, cadmium, chromium in wastewater further aggravates the problem (Pagga and Taeger, 1994). Dye house effluents typically contain 0.6 to 0.8 g dye/l of effluent. Most of dyes used in the textile dyeing industries are derived from hydrocarbon-benzene, toluene, naphthalene, xylene, anthracene, phenol, cresol, carbazols, etc. and among the oldest and commonly used are azo dyes. Textile industry is also one of the water intensive industries, which consumes large quantity of water for various processes and discharges equally large volume of wastewater containing a variety of pollutants. It is estimated that 10-70 liters of water may be required for processing one meter of cloth, which produces about 20 to 30 g of BOD (Pandey and Carney, 1989). According to an estimate, a mill having production capacity of 35,000 m cloth per day consumes 1,000 kl water and produces effluents of 800 kl day^{-1}. Textile industry in India is one of the oldest industries. Large textile mills are (Fig. 1) mainly situated in Ahmadabad, Surat, Mumbai, Coimbatore, Kanpur and Delhi.

Fig 1. Location of major textile industries in different parts of India

The exact data on the number of processing houses operating in the cottage and household sector is not available and it has remained by and large unorganized. The textile units are scattered all across the country and out of 21,076 units, Tamilnadu tops with 5,285, whereas Maharashtra has the next highest number of units (ISPCH, 1995).

The problem of treating textile industry effluents is very complex, as the process involved in raw material utilization and intermediate products generation is very vast and it generates heterogenous effluents. Chemical processes like equalization, segregation, sedimentation, aeration, etc., have been studied in details for treating the textile effluents. The physical processes are capable of removing only the suspended solids with negligible removal of organic load. Physical processes if followed by chemical or biological process have shown good results. However, wide variation in BOD and other parameters necessitates

the requirement of equalization of various physio-chemical processes viz., chemical coagulation / flocculation, adsorption, ion exchange, etc. Chemical coagulation employing various chemical coagulants followed by setting, have proven to be highly efficient in removing solids, BOD and COD from textile effluents and attained its reliability as physical and chemical unit (Mohan *et al.,* 1997; Robinson *et al.,* 2001 a). Activated carbon adsorption has also demonstrated its potential in treating textile effluents (Mckay *et al.,* 1979; Mohan *et al.,* 1997). However, adsorption process is employed, where recycling of water is in need as a tertiary unit. Removal of colour from the effluents is difficult by using conventional physico-chemical treatment system. However, biological treatment may be the best alternative, as the operational costs involved in it are relatively low compared to conventional technologies (Arutchelvan *et al.,* 2003).

The use of microorganisms to detoxify environmental pollutants is generally referred as bioremediation (Boopathy *et al.,* 2000). Environmental issues are now of increasing concern and biological methods are being more directed towards technologies to minimize pollution or to remediate it if it occurs. Mushroom species like, *Pleurotus*, *Agaricus*, *Volvariella* along with white rot fungi and their ligninolytic enzymes appear to have wide industrial applications. The biotechnology based processes involving dead or live microbial cells or their components, as biota agents are more user friendly under such conditions, as these restrict the introduction of new forms of chemicals in the environment. The important criteria for treatment of effluents containing hazardous chemicals are the reduction in toxicity, odour and dissolved materials up to acceptable levels. Decolourisation of effluent is the primary target in steps involved in water purification process.

Mushroom cultivation is an eco-friendly method of converting a wide range of agro-residues and agro-industrial wastes in to a protein rich food in the form of mushrooms. After several cycles of mushroom cultivation, the mushroom productivity decreases and the

residual by-product, known as "spent mushroom substrate" (SMS) required to be properly disposed off to maintain hygiene and avoid environmental problems. If not properly disposed off, it leads to environmental problems, such as soil and water contamination, due to high load of organic carbon, nitrogen and salt in SMSs of different mushrooms. SMSs are mainly comprised of lignocellulosic materials, decomposed and permeated by the fungal mycelia. These are also source of phenol oxidases and harbour a great diversity of microorganisms.

The use of SMS for biodegradation of pollutants is well documented in the literature. Studies on polycyclic aromatic hydrocarbons (PAH) (Eggen, 1999; Lau *et al.,* 2003) and phenolic compounds (Trejo *et al.,* 2001) such as pentachloro-phenol (Okeke *et al.,* 1993) have shown that SMSs are effective in bioremediation strategies of these chemicals. The bioremediation ability depends upon the ability of the fungal species to colonize the substrate and to produce oxidative ligninolytic enzymes such as laccase, manganese peroxidase and lignin peroxidase. These enzymes are responsible for lignin degradation (Buswell *et al.,* 1993, 1996; Silva and Milagres, 2003) along with pollutants having complex molecular structures like that of lignin. Additionally, amendment of contaminated soils with SMS supports the growth of microorganisms and contributes towards change in microbial profiles mainly in rhizospheric zone. After disposal, the SMS is weathered for 1-2 years (Beyer, 1999; Guo *et al.,* 2001) before utilization for obtaining appreciable benefits. A process of passive composting and leaching contribute towards reduction in electrical conductivity (EC), resulted due to salt content. Thus, most studies have been conducted with lower salt-containing weathered SMS. Contrary to this, unweathered SMS, without any post-crop heat treatment, has the advantage of possessing viable mycelia and several extracellular active enzymes produced by the growing fungus during the cropping cycle. Consequently, this fresh SMS may perform more efficiently in remediation activities than the weathered SMS. Therefore, if SMSs are to be used as a manure and bioremediation agent simultaneously, then

their use as fresh (without heat treatment) products should also be considered.Weathering process, if not performed properly, can also results in soil pollution, euthrophization of water resources and underground water pollution.

Although, the literature shows great variability in physico-chemical and microbiological properties of different types of SMSs (Beyer, 1999; Levanon and Danai, 1995; Maher and Magette, 1997), they are widely accepted as good quality substrate for plant growth, particularly for organic farming of horticultural crops. The physical structure of SMS, basically constituted of cereal straw and gypsum holds the property of a good adsorbent material. The chemical constituents like carbon, nitrogen, phosphorus and potassium level along with high cation exchange capacity make it a good nutrient source for supporting the growth of others microbes. The biological component of SMS is also very important, as it is constituted of mushroom mycelial biomass along with high population of heterotrophic bacteria, actinomycetes and the fungi having potential to produce full range of ligninolytic enzymes. The past studies have also supported the role of laccase, lignin peroxidase and manganese dependent peroxidase along with some more enzymes in decolourisation of colouring dyes.

Among edible mushrooms, the ligninolytic system of *Pleurotus* spp. has been extensively studied, and it has been found effective for bioremediation of recalcitrant pollutants (Cohen *et al.,* 2002). Role of extracellular ligninolytic enzymes and microbes from SMS of different mushrooms (Ahlawat *et al.,* 2010) and mushroom mycelia (Kumari *et al.,* 2008), especially of *P. florida*, *P. ostreatus*, *Agaricus bisporus* and *P. sajor-caju*, have been evaluated for their use in dye decolourisation activities (Faraco *et al.,* 2009; Mangat *et al.,* 2008; Neelamegam *et al.,* 2004; Perumal *et al.,* 2007). SMS consists of composted organic material heavily colonized with mushroom mycelia. Highly immobilized microbial cultures in the form of SMS tend to have a higher level of bioremediative activity and are more

resilient to environmental perturbations such as pH and toxic chemicals than the suspended cultures. Besides this, the immobilized cultures offer other advantages like reusability of the same biocatalyst, control of reactions, and the non-contamination of products (Zeroual *et al.,* 2001). Immobilized form of living microorganisms have also been described (Engasser *et al.,* 1988; Hyde *et al.,* 1991) more effective in biological wastewater treatment than the suspended form of microorganisms (Kumar *et al.,* 1997a; Mazmanci *et al.,* 2005).

So the present study was focused on potential uses of microorganisms (especially SMS associated microflora) in degradation / decolourisation of commonly used synthetic colouring dyes. Thus the study was carried out with following under mentioned objectives.

1.2 Objectives of the research work

- To evaluate the ligninolytic enzymes activity and dyes decolourisation potential of spent mushroom substrate of different mushrooms.
- To study the role of cultural conditions on dyes decolourisation.
- To carry out kinetic studies on dyes decolourisation by spent mushroom substrate.

Chapter-2

Review of Literature

2.1 General

2.1.1 Definition of dyes

By definition, dyes can be said to be coloured, ionising and aromatic organic compounds, which show an affinity towards the substrate to which they are being applied. Dyes are applied to numerous substrates such as textiles, leather, plastic, paper, etc. in liquid form. The preparation and application of dyestuffs is one of the oldest forms of human activities, evidences of which were found during excavation at archeological sites from where ancient fabrics were unearthed. There is also mention of it in the Bible and other works of classical antiquity. However, the earliest written records of the use of dyestuffs can be traced back 2600 BC from China.

2.1.2 What makes the dyes coloured?

This is a very common question that comes in every body's mind. The answer to it is explained by the presence of a substance called Chromophore in the dyes. By definition, dyes are basically aromatic compounds. Their structures have an aryl ring that has delocalized electron system. These structures are said to be responsible for the absorption of electromagnetic radiations of varying wavelengths. Rather than making the dyes coloured, the chromophore makes the dyes proficient in their ability to absorb radiations of varied wavelengths. Chromophores act by making energy changes in the delocalised electron cloud of the dyes. This alteration invariably results in absorbing radiation within the visible range of colours and not outside it. The human eyes detect this absorption, and respond to the colours. Another possible explanation is that if the electrons are removed from the electron cloud, it may result in loss of colour. Removing electrons may cause reversion of rest of the electrons to the local orbits. A best example is the Schiff's reagent. In Schiff's reagent,

sulphurous acid reacts with pararosanilin, and during the process, a sulphonic group attaches itself to the compound's central carbon atom. This hampers the conjugated double bond system of the quinoid ring, and causes the electrons to become localised. Consequently the ring ceases to be a chromophore and the dye becomes colourless. To conclude chromophores are the atomic configurations, which have delocalised electrons. Generally they are represented as carbon, nitrogen, oxygen and sulphur. They can have alternate single and double bonds.

2.1.3 Classification of dyes

There are several ways for classifying the dyes. Dyes of each class have a unique chemistry, structure and bonding pattern. Some dyes can react chemically with the substrates forming strong bonds while; others can be held by physical forces. The common property of dyes is to absorb light with the involvement of chromophore, a molecule responsible for its colour. The variation in the structures of dyes is enormous and thousands of different dyes are produced for commercial use. Generally, dyes are classified according to their chemical structures, particularly chromophore, and the methods of application (Corbmann, 1983; Hao *et al.,* 2000).

Among 12 different chromophores, azo and anthraquinone dyes are the major one. Azo dyes, characterized by nitrogen to nitrogen double bond, account for up to 70% of all textile dyestuff produced, and are the most common chromophore of reactive dyes. Anthraquinone dyes are derived from anthraquinone with a quinoid ring acting as the chromophore and either hydroxyl groups or amino groups found attached to the general structure. Triphenylmethane dyes are synthetic organic dyes with a molecular structure based on the hydrocarbon triphenylmethane. These are used in textile applications where light fastness is not important. The phthalocyanines are dyes derived from the macrocyclic compound, which forms a coordination complex with most elements of the periodic table.

They are few in number, but commonly used. Indigo is an organic dye with a distinctive blue colour. Historically, it was extracted from plants; however, nearly all indigo produced today are synthetic. Sulphur dyes are a group of sulphur-containing complex synthetic organic dyes (Hao *et al.,* 2000). Acid dyes are water soluble anionic dyes with different chromophore groups substituted with acidic functional groups such as nitro, carboxyl and sulphonic acid, for the dye to become soluble. Basic dyes are cationic types with chromophores typically having amino groups. Direct dyes are highly water-soluble salts of sulphonic acid of azo dyes. Reactive dyes are highly water-soluble anionic dyes with wet fastness and bind to textile fibres via covalent bonds. Disperse dyes are substantially water-insoluble, non-ionic dyes. These are used for the application to the hydrophobic fibres from aqueous dispersions. Sulphur dyes are dyes applied in two parts. The initial bath consists of the yellow or pale colour, which after treatment with a sulphur compound produce dark black colour. Mordant dyes require a mordant (usually potassium dichromate), which improves dyestuff fastness on a dyeing material in water medium. Many mordants can be hazardous to health. Vat dyes are essentially insoluble in water and are incapable of direct dyeing of fibres. A reduction in alkaline liquor makes them water soluble and attachable to textile fibres, while a subsequent oxidation reforms the originally insoluble dye (Corbmann, 1983).

2.1.4 Adverse effects of dyes

Discharge of the coloured effluents into water bodies' results into reduced dissolved oxygen concentration, thus creating anoxic conditions that are hazardous to inhabiting organisms. Many reports indicate that textile dyes and effluents have adverse effect on the germination rates and biomass concentration of several plant species (Kapustka *et al.,*1993). The toxicity of effluent is due to the presence of dye or its degraded products, which are mutagenic or carcinogenic in nature (Kalyuzhnyi *et al.,* 2000). Therefore, treatment of industrial effluents contaminated with dyes becomes essential prior to their final discharge to the environment.

2.2 Alternative technologies for dye decolourisation

The presence of dyes in wastewaters present a significant problem in the wastewater treatment, mainly because of their complex and varied chemical structures along with other residual chemical reagents and impurities. Such wastewaters used to have high organic content and low BOD/COD ratios, followed by recalcitrant nature of dyes. In addition, the degradative products may be even more toxic. Accordingly, no universal method is in vogue for their treatment. The degradation of synthetic dyes present in waste streams can be performed with various technologies, which can be subdivided into four main groups: 1) physical, 2) chemical and photochemical, 3) electrochemical, and 4) biological processes. The biological processes using fungi and bacteria for dye decolourisation have been reported by several workers (Joshi *et al.,* 2004; Robinson *et al.,* 2001a; Singh, 2006). Regulatory agencies, especially in developed countries, are more concerned with their impact on environment and public health. Consequently, the imposition of the stringent environmental legislations, are increasingly putting pressure on the textile and dyestuff industry. The legislations and colour standards for waste discharge vary in different states. In addition, there are several standard methods for determining the colour standards, which make a comparison of different colour degradation methods from various sources more complex (Hao *et al.,* 2000; Lee *et al.,* 2011; Singh, 2006).

2.2.1 Physical methods

Adsorption has gained a favourable interest due to the efficient pollutant removal, quality product and economical feasibility. It is influenced by many physico-chemical factors such as dye-sorbent interaction, adsorbent surface area and particle size, temperature, pH and contact time. Materials, like activated carbon, peat, wood chips, fly ash and coal, silica gel, microbial biomass, and other inexpensive materials (natural clay, corn cobs, rice hulls), are used, since they do not require regeneration. Sedimentation is a solid-liquid separation

method. In case of dye solutions, it is used in combination with chemical or biological methods producing particles containing dye or dye degradation products due to coagulation/precipitation or with some other chemical methods, or adsorption on various materials. The rate of sedimentation of particles suspended in a fluid can be described with Stoke's law and is influenced by many physico-chemical factors. The disadvantage with this process is high sludge production. Flotation is a foam separation technique. Generally, it is performed by adding a surface active ion of the opposite charge to the ion to be separated from the solution. The solid product, which appears on the gas-liquid surface, is levitated to the surface of the solution by inserting a gentle stream of fine gas bubbles. Coagulation can be induced by an electrolytic reaction at electrode surface or by changing pH or adding coagulants (Shakir *et al.,* 2010). Furthermore, membrane filtration can be used to remove dye molecules. The classification of membranes is done on the basis of their pore size aimed to retain solutes of different molecular weight. The membrane parameter is called molecular weight cut off (MWCO). In case of dye separation, reverse osmosis (MWCO < 1000), nanofiltration ($500 < \text{MWCO} < 15000$) and ultrafiltration ($1000 < \text{MWCO} < 100000$) membranes are used according to the dye characteristics. In addition to the dye solution filtration, membranes are also used for the separation of particles after adsorption or coagulation/precipitation instead of the sedimentation (Hao *et al.,* 2000). Treatment with radiation is also classified as a physical method. However, in case of dye degradation, the radiation treatment of aqueous media leads to the formation of strong oxidizing species such as OH radicals, which react with dye molecules, degrade them and consequently, enhance the degradation process. Therefore, radiation methods are included in the advanced oxidation processes (AOPs) for wastewater treatment (Rauf *et al.,* 2009). During ultrasonic irradiation, the propagation of an ultrasound wave leads to the formation of cavitation bubbles and consequently leading to chemical degradation of dye (Vinodgopal *et al.,* 1998). In general, solid waste disposal is required after the physical methods of separation.

2.2.2 Chemical and electrochemical methods

Chemical oxidation is the most commonly used method of decolourisation, mainly due to its simplicity of application. Hydrogen peroxide is the commonly used oxidizing agent, however, it needs to be activated before use. The way in which H_2O_2 is activated decides the method of oxidation. It removes the dye from effluent by way of aromatic ring cleavage of the dye molecules. A well known activator of H_2O_2 is Fe (II) salt, known as Fenton's reagent. The result of sorption or bonding of dissolved dyes is the sludge generation, which again needs disposal and is therefore disadvantageous. Ozonization can also be used for the activation of H_2O_2. However, major drawback of it is the short half-life of ozone in water and its cost, as it degrades in about 20 minutes and has to be applied continuously. Moreover, its stability is affected by the presence of dyes, salts, pH and temperature. Ultra-violet radiation can also be used for activation of Hydrogen peroxide (H_2O_2). The major advantage of H_2O_2/UV treatment is the requirement of no other chemicals. The wet air oxidation (WAO) process presents a hydrothermal treatment of dissolved and suspended components in water, and has been successfully used for several azo dyes (Rodrigues *et al.,* 2009). Chlorination, using chlorine gas or sodium hypochloride is an inexpensive and effective method. However, its use has become less frequent due to the generation of toxic and carcinogenic compounds. Moreover, the use of chemicals containing chlorine is restricted due to environmental reasons. As already mentioned, photochemical methods are based on the use of UV light, which activates the chemicals and consequently, enhances the chemical reaction and makes the process more efficient. The principle of electrochemical methods is to charge the electric current through electrodes made of different materials (e.g. iron or aluminium) resulting in oxidation process at anode and reduction at the cathode with H_2 production. The resulting processes are known as electrocoagulation, electroflotation, electrooxidation and electroreduction. Majority of the above mentioned methods are the so-

called 'advanced oxidation processes' (AOP), and are essentially based on the generation of highly reactive radical species (Hao *et al.,* 2000; Joshi *et al.,* 2004; Slokar and Majcen, 1998).

2.2.3 Biological methods

A biological treatment presents a process of degradation of organic substances by mixed microbial (Sharma *et al.,* 2002) system or monoculture of microorganisms under aerobic or anaerobic conditions, and has been widely used and researched (Boopathy *et al.,* 2012; Sanayei *et al.,* 2009, 2010). The dyes are generally resistant to oxidative biodegradation. In addition, toxicity, and acclimating ability are the drawback of using microbial cultures. It has been demonstrated that mixed bacterial cultures are capable of decolourizing textile dye solutions. Nevertheless, several studies have showed that little biodegradation actually occurs, while the primary mechanism is adsorption to the microbial biomass (Knapp, 2001; Robinson *et al.,* 2001b; Slokar *et al*., 1998). It has also been reported that few species of algae are capable of degrading azo dyes and utilize them as a sole source of carbon. Some articles on dye decolourisation with yeasts are also there in the literature (Joshi *et al.,* 2004). Several fungal systems have been found to degrade various classes of dyes. More emphasis has been given to the white-rot fungi and azo dyes, the largest class of commercial dyes. A fungal treatment of dyes is an economical and feasible alternative to the present treatment technologies (Knapp, 2001; Singh, 2006).

2.2.4 White rot fungi and role of their ligninolytic enzymes in dye decolourisation

Several studies have demonstrated that white rot fungi are capable of degrading a wide variety of structurally different dyes such as azo, anthraquinone, heterocyclic, triphenylmethane and polymeric dyes (Nigam *et al.,* 2000). The dyes are degraded with the involvement of lignin degrading enzymes namely lignin-peroxidase (LiP), manganese-peroxidase (MnP) and laccase (Buswell *et al.,* 1996; Cripps *et al.,* 1990; Heinfling *et al.,* 1998; Swamy and Ramsay, 1999). The ligninolytic system of *Pleurotus* spp. has been

studied, and it appears to be an effective alternative for the bioremediation of recalcitrant pollutants (Cohen *et al.,* 2002). Submerged cultures of *Pleurotus sajor-caju* have been found to bring about complete degradation of polyphenols from paper mill effluents (Munari *et al.,* 2007).

Several authors have discussed the involvement of the ligninolytic enzymes of fungi in the decolourisation process. (Kumar *et al.,* 1997c ; Liu *et al.,* 2004 ; Nerud *et al.,* 2004 ; Robinson *et al.,* 2001b ; Verma and Madamwar, 2003; Wesenberg *et al.*, 2003 ; Zhao *et al.,* 2005). Considering bioremediation as an environment-friendly and cost-competitive for dye wastewater treatment, researchers have thrust upon microorganisms that are capable to degrade dyes. Ligninolytic fungi seem to be the most promising organisms, characterized by their degradative capabilities toward a broad spectrum of structurally different dyes and their highly oxidative and non-specific enzyme systems (Knapp and Newby, 1995; McMullan *et al.,* 2001; Wesenberg *et al.*, 2003). A great deal of work has been carried out on assessing dye-decolourisation capabilities of ligninolytic fungi. However, it has remained restricted to a few numbers of species.

The most studied organism is the white-rot fungus, *Phanerochaete chrysosporium,* but other species belonging to the genera *Pleurotus, Bjerkandera, Trametes, Polyporus* and *Irpex* have also been investigated (Wesenberg *et al.,* 2003, Yesilada *et al.,* 2003). Ligninolytic fungi especially *Coriolus versicolor, Cerrena unicolor, Daedalea quercina, Funalia trogii, Irpex lacteus, Pleurotus pulmonarius, Schizophyllum commune* and *Pleurotus ostreatus* have been reported for the laccase production and their role in decolourisation of structurally different dyes (Asgher *et al.,* 2006; Baldrian , 2004; Erkurt *et al.,* 2007; Levin *et al.,* 2004; Michniewicz *et al.,* 2008; Svobodova *et al.,* 2008; Tychanowicz *et al.,* 2004; Unyayar *et al.,* 2005). Similarly, *Lentinula edodes, Phanerochaete chrysosporium, Pleurotus ostreatus* and *Schizophyllum commune, Trametes trogii, Trametes versicolor,* etc. have

shown manganese peroxidase production and decolourisation potential of structurally different dyes (Asgher *et al.,* 2006; Boer *et al.,* 2004; Ozsoy *et al.,* 2005; Pazarlioglu *et al.,* 2005; Toh *et al.,* 2003).

2.2.5 Role of bacteria in dye decolourisation

Efforts to isolate bacterial isolates capable of degrading azo dyes started in the 1970s with report of *Bacillus subtilis* (Horitsu *et al.,* 1977; Sharma *et al.,* 2009 b), then *Aeromonas hydrophila* (Idaka and Ogawa, 1978; Ogugbue *et al.,* 2011a), *Bacillus firmus* (Ogugbue *et al.,* 2011b and 2012), followed by *Bacillus cereus* (Wuhrmann *et al.,* 1980). Thereafter, numerous bacteria capable of dye decolourisation, in isolation or in consortia, have been reported (Banat *et al.,* 1996; Coughlin *et al.,* 2002; Hu, 1998; Pearce *et al.,* 2003; Rajaguru *et al.,* 2000; Verma and Madamwar, 2003; Yatome *et al.,* 1991). In similar way, *Pseudomonas* spp. were also tested for decolourisation and COD removal from digested distillery spent wash (Dilbaghi *et al.,* 2002).

Most of the azo dyes are nondegradable under aerobic condition. However, under anaerobic condition, the bacteria can reduce azo linkages in the dye molecule to form colourless aromatic amines. Several bacteria such as *Klebsiella pneumoniae* RS-13 (Wong and Yuen, 1996), *Pseudomonas luteola* (Chang *et al.,* 2001; Hu, 1998), *Pseudomonas* sp. (Mali *et al.,* 2000). *Pseudomonas aeruginosa* NBAR12 (Bhatt *et al.,* 2005), *Acetobacter liquefaciens* S-1 and *Pseudomonas desmolyticum* NCIM 2112 (Kalme *et al.,* 2007) have been reported to have azo dyes degradation abilities.

2.2.6 Immobilization of fungi

Immobilized forms of living microorganisms have been described to be more useful in biological wastewater treatment (Hyde *et al.,* 1991; Shibu *et al.,* 1999; Zeroual *et al.,* 2001). It is widely known that immobilized cells offer a lot of advantages such as reusability

of the same biocatalyst, control of reactions, and the non contamination of products (Engasser, 1988).

2.3 Spent Mushroom Substrate

Spent mushroom substrate (SMS), a by-product of mushroom production is generated in large quantity every year. Commercial mushroom production is a solid-state-fermentation activity and production of 1kg each of *Pleurotus* sp. and *Agaricus bisporus* generate about 3 kg and 5 kg of spent mushroom substrate, respectively (Law *et al.,* 2003; Semple *et al.,* 2001; Singh *et al.,* 2003). Several white rot fungi (*Pleurotus ostreatus, Pleurotus pulmonarius* and *Lentinula edodes*) have been cultivated for mushroom production (Kapoor *et al.,* 2009). The fresh SMS constitutes high level of residual nutrients and extracellular ligninolytic enzymes. When using edible mushrooms, especially white rot fungi for bioremediation, the availability of fungal inocula is a practical consideration and use of SMS could be advantageous due to its low cost and environment-friendly. SMSs have been reported for their ability to degrade some xenobiotic compounds like PAHs (Eggen, 1999; Lau *et al.,* 2003), pentachlorophenol (Law *et al.,* 2003) and benzene (Semple *et al.,* 1998). Since more than 25% of world mushroom production is *Pleurotus*, so it would be advantageous to use *Pleurotus* SMS in bioremediation activities.

On the other hand, the growing mycelium releases abundant extracellular enzymes, especially lignocellulolytic enzymes during its growth and fruiting to utilize the growing substrate. (Hammond, 1981; Sharma *et al.,* 1996; Sharma *et al.,* 2011). The mushroom species does affect the quality and quantity of enzymes immobilized in the spent mushroom compost. Consequently it can be a good source of lignocellulolytic enzymes for biodegradation of various xenobiotics and persistent pollutants, including pentachlorophenol, polyaromatic hydrocarbons, and azo dyes (Chiu *et al.,* 1998; Fermor *et al.,* 2000; Harmsen *et al.,* 1999; Matute *et al.,* 2012).

2.3.1 Physico-chemical properties

Spent mushroom substrate (SMS) normally contains 1.9:0.4:2.4% (N-P-K) before weathering and 1.9:0.6:1.0 (N-P-K) after a weathering period of 8-16 months. Nitrogen and phosphorus do not leach out during weathering but potassium being more leachable is lost in significant amount during weathering. SMS contains much less heavy metals than sewage sludge, which preclude its classification as hazardous substance (Wuest and Fahy, 1991). Weathering causes slow decrease in the organic matter contents (volatile solids) and leads to different characteristics of weathered SMS because of ongoing microbial activity in the field (Beyer, 1999). The SMS, obtained from different commercial sites revealed that except calcium, boron, copper and zinc, coefficient of variability are less than 25% while, the conductivity and pH are consistently high (Devonald, 1987). The sample obtained from 6, 12, 24, 36 and 48 months old decomposed SMS didn't show significant variation in pH and bulk density. However, other parameters like total dissolved solids, conductivity, nitrogen, phosphorus, potassium, calcium, chloride and nitrate content decreased with increasing age of the SMS during natural weathering process (Verma *et al.,* 2004). In a study carried out at Penn State Mushroom Research Laboratory, a little variation in chemical and physical properties of SMS has been ascertained in comparison to earliest study carried out by Wuest and Fahy (1991). There are contradictory reports regarding the pH of fresh SMS and pH after weathering. SMS has an initial pH of around 7.28, which increased during weathering (Wuest and Fahy, 1991). However, pH of the freshly collected SMS ranged from 7.01to 8.04 with ammonia and nitrate content of 174 and 39 ppm, respectively (Devonald, 1987). The pH of well-rotten SMS was found to be little higher (7.70) with NO_3^- content of 15 ppm (Chong and Wickward, 1989). The volume of SMS also decreases (Shrinkage) with time. The fresh SMS obtained from various sources varied in density from 0.15 to 0.24 g/cm^3 in UK (Devonald, 1987), 0.475 g/cm^3 in Ireland and 0.24 to 0.62 g/cm^3 in USA (Maher, 1997). The composting

of SMS in semi-enclosed drum for 6 weeks led to an increase in its bulk density from 0.256 g/cm^3 to 0.293 g/cm^3 (Lohr *et al.,* 1984).

2.3.2 Biological properties

Microbes especially actinomycetes (*Streptomycetes* sp. and *Thermonospora* sp.) present in SMS have strong pentachlorophenol (PCP) catabolizing capability (Semple and Fermor, 1995) which resulted in decrease in level of PCP in contaminated soil after incubation with SMS. The residues obtained after mushroom cultivation can also be utilized for large scale phenoloxidase production, which can later play vital role in environmental protection activities (Steffen *et al.,* 1995).

The SMS is also considered as an inexpensive source of laccase, which has variety of bioremedial applications. In a study carried out by Ahlawat *et al.* (2004), spent mushroom substrate from *Pleurotus* spp. showed higher activity of exoglucanase, endoglucanase, xylanase, laccase and polyphenol oxidase in comparison to spent substrate of *Volvariella volvacea* mushroom. Study further proved that spent substrate from *Pleurotus* spp. is more useful source of enzymes than SMS from paddy straw mushroom. The crude and partially purified extract from SMS has been found to degrade variety of dyes (Ahlawat *et al.,* 2006) and it can be used as bleaching and deinking aid during recycling of paper wastes**.** It has also been observed that SMS stimulates the microbial population of *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., *Aeromonas* sp. and *Bacillus* sp. in soil (Ahlawat *et al.,* 2010).

2.3.3 Adverse effect of SMS in environment

For each metric ton of mushroom produced, at least an equivalent amount of spent mushroom substrate (also called spent mushroom compost) is generated, and this needs to be disposed off. Its disposal can be costly because it is bulky, and in developed countries such as the U.K., disposal charges and taxes on landfill are now implemented (Fermor *et al.,* 2000). Attempts have been made to recycle or reuse this waste (Sharma, 1999). Moreover, the

disposal of SMS is a major problem for mushroom growers. They either discretely burn or discard it (Singh *et al.,* 2003) and thus its exploitation adjuvant has received significant attention (Chiu *et al.,* 2000).

2.4 Mechanism of dyes decolourisation

2.4.1 Mechanism of fungal dye degradation and decolourisation

The mechanisms of fungal dye decolourisation and degradation are listed in figure 2. The accumulation of chemicals with the microbial biomass is termed as biosorption, and it can take place on living or dead biomass. Waste and/or dead microbial biomass can be used as an efficient adsorbent, especially if it contains a natural polysaccharide chitin and its derivative chitosan in the cell walls. Chitosan, a cell wall component of many industrially useful fungi, has a unique molecular structure with a high affinity for many classes of dyes (Joshi *et al.,* 2004). It is known that most of the white-rot fungi produce at least two of the three highly nonspecific enzymes (LiP, MnP and Laccase), which enable the generation of free radicals (Choudhary *et al.,* 2009; Knapp, 2001; Pointing, 2001). The structure of a dye strongly influences its degradability by pure cultures and isolated enzymes. Plenty of data about biodegradation of various synthetic dyes with selected white-rot fungi have been published. Nevertheless, a limited data are available on systematic studies carried out on relation between the structure and biodegradability of a dye, especially for commercial dyes with a complex structure. In order to reach to any conclusion one has to distinguish between dye depletion due to adsorption, depletion and due to enzymatic degradation.

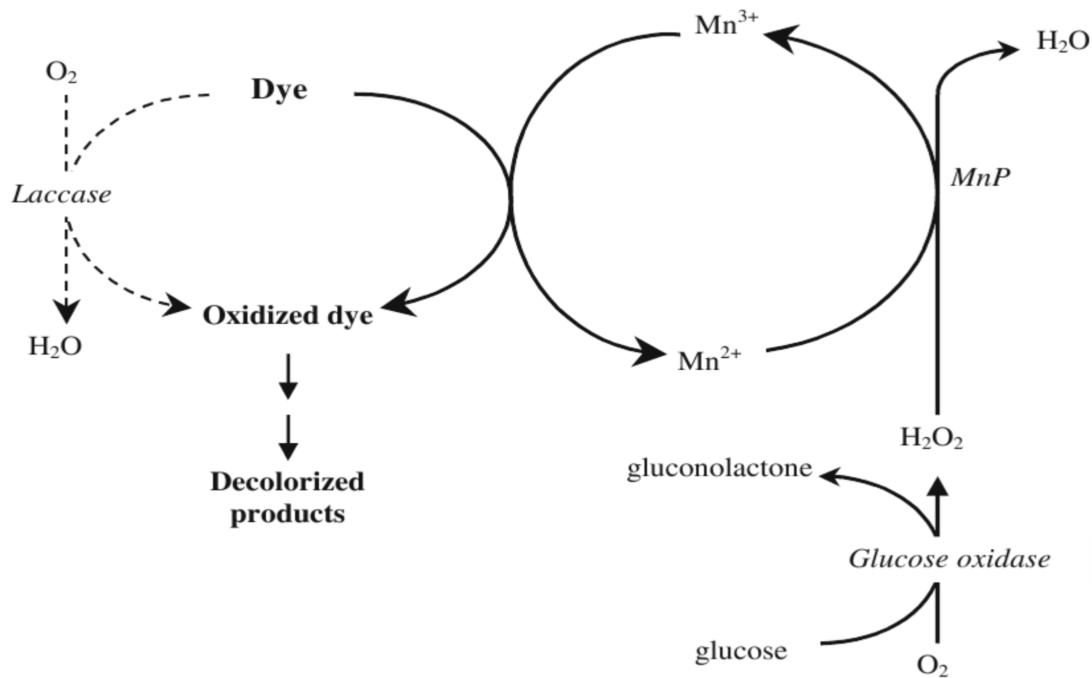


Fig. 2 Proposed mechanism of Amaranth decolourisation by *Trametes versicolor*. Thick and dashed lines represent major and minor reactions when the laccase:MnP ratio (based on units of activity) is less than and greater than 30:1, respectively. At laccase: MnP ratios less than 30:1, MnP would be the main enzyme decolourizing amaranth (Champagne, 2005)

The fungal action rarely leads to the mineralization of dyes and it depends on the chemical structure of the dye. Higher mineralization occurs in dyes with substituted aromatic rings in their structures compared to the unsubstituted rings. A better mineralization is observed also under nitrogen limiting conditions. Some reports on the utilization of dyes as a carbon source have been published in the last decade. Certain bonds in the dye molecule are cleaved and utilized as a carbon source, while, the chromophore remains affected. This mechanism exists preferably with the consortium of microorganisms (Knapp, 2001; Singh, 2006).

2.4.2 Factors affecting decolourisation and degradation of dyes

The fungal growth and enzyme production, and consequently, decolourisation and degradation of dyes are influenced by numerous factors, such as media composition, pH

value, agitation, aeration, temperature and initial dye concentration. Their effects are briefly presented and discussed below.

2.4.3 Media composition

There is no doubt that composition of media has an enormous effect on fungal growth (Postemsky *et al.,* 2006) and production of enzymes leading to decolourisation of dyes. It must be noted that composition of industrial effluents vary with location and time, not to mention that they offer very complex composition with a lack of nutrients, compared to the well defined media used in the research under submerged conditions (Robinson *et al*., 2001a ; Robinson *et al.,* 2001c). Therefore, attention has to be focused on the supply of carbon and nitrogen sources together with mineral nutrients and other additives (Hao *et al.,* 2000; Knapp, 2001; Singh, 2006).

2.4.4 Effect of carbon sources on dyes decolourisation

A carbon source is necessary for fungal growth and to provide oxidants, the fungus requires for decolourisation. Glucose has been used in the majority of research studies (Kumar *et al.,* 1997b). Alternatively fructose, sucrose, maltose, xylose, glycerol, starch and xylen have also been tried and found useful. All the carbon sources added at 1% level were found to stimulate fungal growth, and enhanced dye decolourisation was noted in all flasks. Addition of glucose and fructose enhanced the rate of dyes decolourisation as compared to control (Bhatti *et al.,* 2008). Effluents from dyeing or chemical/dye production usually do not contain usable carbon substrates, while others from distilling or paper pulping may have a range of carbohydrates, useful for certain white-rot fungi. The need to add carbon source depends on the organism used and type of the dye to be treated.

2.4.5 Other media components

Role of growth factors have been studied by many researchers. However, considering their cost, it is not economical to use them in the decolourisation technologies. All microbes

have specific requirements for minerals such as white-rot fungi need iron, copper and manganese. They can be a part of the effluent and if not then must be added to the media. A variety of other compounds, like veratryl alcohol, tryptophan and aromatics (phenol and aniline) can act as low molecular mass redox mediators of ligninolytic activities and therefore promote the decolourisation (Knapp, 2001; Singh, 2006). It is interesting that some components available in wood and straw induce the enzyme production in white-rot fungi. For example, the enzyme activity ratio of Lac/MnP can be regulated using beech wood as the immobilization support and inducer, together with a combination of various concentrations of additional nitrogen and carbon sources in the liquid media during the cultivation of *Ceriporiopsis subvermispora* (Babic and Pavko, 2007). The ligninolytic enzyme production in *Dichomitus squalens* can be substantially induced by adding beech wood and straw particles to the liquid growing media (Pavko and Novotny, 2008).

2.4.6 Effect of pH on dye decolourisation

Majority of the researches on growth and enzyme production by fungi have been performed in batch cultures and usually without any control of pH during fungal cultivation (Sharma *et al.,* 2009a; Sharma *et al.,* 2009b). The role of buffering capacity of the medium is also required to be investigated for studying the influence of initial pH. The majority of filamentous fungi along with white-rot fungi grow optimally at acidic pH values. The change in pH during fungal growth depends upon the type of medium used for growth. The growth on carbohydrate rich media generally causes acidification of the media, which further depends on the available carbon source and the buffering capacity of the medium. The decolourisation can be performed with a whole fermentation broth (mycelia and enzymes) or with isolated enzymes. Distinction has to done between the optimum pH for growth, enzyme production by the fungi and action of isolated enzymes for dye degradation. Therefore, optimum pH depends on the growth medium, fungus and its enzyme system, as well as on the

decolourisation of dye under consideration. Majority of the researchers have suggested optimum pH in the range of 4 to 4.5 (Knapp, 2001). The pH of the medium plays an important role in decolourisation of dyes using white-rot fungi. White-rot fungi have been reported to show highest growth and dye decolourisation in acidic pH range (Asgher *et al.,* 2006; Kapdan *et al.,* 2000).

2.4.7 Effect of temperature on dye decolourisation

Role of temperature has to be considered from diverse angles, its influence on the growth and enzyme production, the rate of enzymatic decolourisation and the temperature of the waste stream (Sharma *et al.,* 2009c). Most of the white-rot fungi are mesophiles with the optimal cultivation temperature of 27 to 30 °C. White-rot fungi exhibit better growth under medium temperature conditions as compared to at higher temperatures (Toh *et al.,* 2003). Temperature optima of 30 to 37 °C have also previously been reported (Asgher *et al.,* 2006; Boer *et al.,* 2004) for different white-rot fungi for decolourisation of chemically diverse dyestuffs. The optimal temperatures for enzyme reactions are usually higher. However, enzyme instability and degradation has to be taken into account at such temperatures. Various textile and dye effluents are produced at temperature range of 50 to 60 °C. Thus the optimal temperature for decolourisation for a particular process has to be selected from case to case basis according to the mentioned parameters (Knapp, 2001; Singh, 2006).

2.4.8 Effect of agitated and stationary conditions on dye decolourisation

Ligninolytic fungi are obligate aerobes and thus need oxygen for their growth and vigour. In addition, lignin degradation system also requires oxygen, either for the mycelial generation of H_2O_2 for peroxidases or for the direct action of oxidases. Oxygen can also act directly on lignin fragments (Chang and Lin, 2000). The oxygen demand depends on the fungus and its ligninolytic system. The oxygen supply to the culture media during the fungal cultivation has remained an interesting research topic for decades and has been covered in

numerous articles (Chang and Lin, 2000; Cheng *et al.,* 2003).The major bottleneck is its low water solubility, which is only 8 mg/L at 20 °C. In order to satisfy the microbial oxygen requirements of fungi during their cultivation and to enhance the oxygen gas-liquid mass transfer, aeration and agitation are necessary. The availability of oxygen might affect the morphology of filamentous fungi and lead to the decreased rate of enzyme synthesis (Znidarsic and Pavko, 2001). As a result various bioreactor types, generally divided into static and agitated configurations were invented. The choice of the reactor depends on a particular system although an appropriate agitation gives as good or even better results as those from static conditions. Studies specifically on the effect of agitation and aeration on the decolourisation process are scanty (Knapp, 2001).

2.4.9 Effect of initial dye concentration on dye decolourisation

It is important to optimize the initial dye concentration for complete colour removal. Dyes are usually toxic to the microorganisms and presence of even low concentrations of dyes in effluent is highly visible and undesirable, reducing light penetration and potentially inhibiting photosynthesis (McMullan *et al.,* 2001), while the toxicity depends on the type of dye. Higher concentrations of dyes are always toxic. The range of initial dye concentrations studied in the literature has varied from 50–1000 mg/L, which depends on the microorganism investigated and type of dye used (Singh, 2006; Xu *et al.,* 2006).

2.5 Kinetics of dye decolourisation

Kinetic model offers a powerful tool to describe biological degradation processes and to elucidate the quantitative degradation behaviours. Furthermore, it provides useful information for optimization of biodegradation processes. It is recognized that, the order of kinetics for dye decolourisation is determined by the rate-limiting step (Van der Zee *et al.,* 2001). First-order kinetics or the same grade reaction such as Michaeli–Menten rate model have been widely used for modeling the decolourisation of azo dyes by mixed cultures, where

the transfer of reducing equivalents presents a rate-limiting step. On the other hand zero-rate kinetics, are frequently used for modeling pure cultures, where the production of reducing equivalents appears to be the rate-determining step (Dubin and Wright, 1975; Sponza and Isık, 2004; Van der Zee *et al.,* 2001). Despite the substantial development in the simulation of dye decolourisation process, some important influencing factors have been underestimated. For example, azo dyes are adsorbed considerably by the anaerobic sludge. However, its kinetics in decolourisation is still not clear. This could lead to an inaccurate modeling of the real decolourisation process (Gonzalez-Gutierrez and Escarmilla-Silva, 2009).

Information about the kinetics of decolourisation and the environmental factors affecting the decolourisation rates is relatively scarce. Monoazo dye decolourisation has been reported to follow first-order kinetics with respect to dye concentration by several workers (Carliell *et al.,* 1995; Hu, 1998; Van der Zee *et al.,* 2001; Wuhrmann *et al.,* 1980), whereas few other have reported zero-order (Harmer and Bishop, 1992; Watanabe *et al.,* 1982) or even half-order kinetics (Yu *et al.,* 2001). Furthermore, for diazo and polyazo dyes, only the first part of the decolourisation profiles has been reported to follow first-order kinetics with respect to the dye concentration (Van der Zee *et al.,* 2001). The various experimental conditions used in the reported studies may have been responsible for these apparently contradictory kinetic results, since the rate-limiting step, of azo dye reduction is highly dependent on these conditions. For example, in pure cultures, the zero-order process of production of reducing equivalents is likely to be the rate-limiting step, whereas in aggregate sludge under anaerobic conditions, the transfer of reducing equivalents, rather than their production, is the probable the rate-limiting step (Van der Zee *et al.,* 2001).

Chapter-3

Materials and Methods

The present investigation entitled "**Studies on decolourisation of synthetic dyes using spent mushroom substrates"** was conducted in the Crop Production Section at Directorate of Mushroom Research (ICAR), Solan, H.P., India. The materials used, experimental details and techniques employed in the present investigation are furnished in this chapter under suitable captions.

3.1 Collection of samples

Spent mushroom substrate (SMS) samples were collected from spent compost released after harvesting the crops of different mushrooms at Directorate of Mushroom Research, Solan (HP), India.

3.1.1 Details of experiments

The present study was based on decolourisation of synthetic dyes by the spent mushroom substrate of different mushrooms and so the study was divided into various experiments. Each treatment was replicated thrice. The principal steps involved in the present study are presented here:

3.1.2 Mushroom species and their dye decolourisation ability on agar plate

Pure cultures of *P. sajor-caju, P. florida, P. ostreatus, P. fossulatus, P. eryngii, P. sapidus, Auricularia polytricha, Lentinula edodes, Agaricus bisporus* (strain S-11 and HU3) were obtained from the culture bank of the Directorate of Mushroom Research, Solan (HP), India. Strains were maintained on Malt Extract Agar (MEA) slants at 4°C and periodically sub-cultured. The above mentioned mushroom species were studied for their dye decolourisation potential against nine structurally different textile dyes on Potato Dextrose Agar (PDA) plates supplemented with respective dye @ 100 ppm. The medium was sterilized

at 15 p.s.i. for 20 minutes. Single mycelial bit (6.0 mm dia.) from each mushroom species was placed at the centre of petri dish (90 mm diameter), followed by incubation at 25 ± 2 °C for 15 days in BOD incubator. The plates were observed at regular time intervals for the diameter of mycelial growth (mm) and the zone of dye clearance around the growing mycelial colony (mm).

3.1.3 Physico-chemical properties of spent mushroom substrate

Spent mushroom substrate was analyzed for its physico-chemical characteristics like moisture, pH, carbon, nitrogen, potassium and calcium content. The standard protocols for their estimation already reported by different workers (Allen, 1974; Black, 1965; Peters, 1965; Walkely and Black, 1934) were adopted for the present study.

3.2 Observation recorded

3.2.1 pH analysis

The pH of the samples was determined using digital pH meter (TPS, Smart CHEM-Lab) (Jackson, 1965). Ten gram (10 g) of sample was taken in 100 ml distilled water and stirred continuously for 30 minutes with a glass rod. The pH of the suspension was recorded after half an hour of settling by the pH meter pre calibrated using standard buffers of 4.0, 7.0 and 9.0 pH.

3.2.2 Total nitrogen

The total nitrogen was measured using micro-Kjeldhal method, which included three steps; digestion, distillation and titration (Subbiah and Asija, 1956). 1.0 g dried and powdered sample was digested with 7.0 g digestion mixture [K_2SO_4 (50 parts): $CuSO_4$. $5H_2O$ (10 part): metallic selenium (1 part)] and 10 to 15 ml concentrated sulfuric acid. The clear solution was distilled with 40% sodium hydroxide (NaOH). The distillate was collected in 25 ml of 4% boric acid solution with a drop of mixed indicator (Bromocresol green and Methyl red). The

distillate was titrated against 0.1 N hydrochloric acid till it became neutralized. The percent nitrogen content was calculated by the following formula.

$$\text{Nitrogen on dry weight basis (\%)} = \frac{(X\text{-}Y) \times N \times 14 \times 100}{W \times 100}$$

X= Volume of HCl used for titration of the sample

N= Normality of acid

Y= Volume of HCl used for titration of blank

W= Dry weight of the sample

3.2.3 Total carbon

Total carbon in spent mushroom substrate samples was estimated by following Walkely and Black (1934) wet digestion method. The carbon was estimated by taking 0.2 g dried sample in 250 ml flask along with 10 ml of 1N $K_2Cr_2O_7$ and 20 ml of concentrated sulfuric acid. The mixture was heated strongly in a fume hood for 30 min and allowed to cool down. In the cold mixture 200 ml of distilled water, 10 ml of concentrated phosphoric acid and 1 ml of diphenyl amine indicator were added. The solution was titrated against N/2 ferrous sulfate till the green colour of the solution disappeared and a purple colour appeared. Calculation was made by the following formula.

$$\text{Carbon on dry weight basis (\%)} = \frac{(X\text{-}Y) \times 0.003 \times 100}{W}$$

W= Weight of the sample

X= Blank reading (Vol. of $FeSO_4$)

Y= Experimental reading

3.2.4 Available Phosphates

The principle employed to estimate the available phosphates in the samples was based upon the fact that phosphates get precipitated as ammonium phosphomolybdate [$(NH_4)_3$ PO_4,

12 M_2O_2], which is dissolved in excess of standard alkali and titrated against standard acid to calculate the available phosphates (Olsen *et al.*, 1954).

1.0 g sample was dissolved in water and filtered after 5 minutes. The residue was washed with warm water and filtrate was collected, and the final volume was made up to 250 ml. 50 ml of the filtrate was added with 10 ml of concentrated HNO_3, 10 g of ammonium nitrate and heated up to 60 $^{\circ}$C, followed by drop by drop addition of 50 ml of 3% ammonium molybdate. The yellow precipitates such appeared were allowed to settle for an hour, followed by decanting of supernatant and filtration through whatmann filter paper no. 44. The precipitates were taken in to a beaker along with filter paper and 10 ml of 0.1 N sodium hydroxide was added accurately along with 1-2 drops of phenolphthalein indicator, followed by titration against 0.1N sulfuric acid. Available phosphates were calculated by following under mentioned formula.

$$\text{Available phosphates (\%)} = \frac{(X\text{-}Y) \times 0.000309 \times 100}{\text{Wt. of sample in 50 ml (1 g)}}$$

X= Volume of NaOH solution taken (10 ml)

Y= Volume of acid utilized

Wt. of sample taken= 1 g

3.2.5 Moisture

Known weight of the sample was taken and dried in oven at 105 $^{\circ}$C for 24 hours. The dried weight of the sample was weighed to calculate the percentage moisture in the sample by following the under mentioned formula.

$$\text{Moisture (\%)} \quad \frac{\text{Fresh weight-Weight of dried sample}}{\text{Fresh weight}} \text{x100}$$

3.2.6 Estimation of calcium (Ca^{2+}) by Flame photometer

The flame photometer was calibrated by using 10, 100 and 1000 ppm of calcium ion concentration in $CaCO_3$ solution. The slope was calibrated by the instrument itself. The number of Ca^{++} ions present in 1 g of the sample was calculated by dipping the electrode in the solution containing 1 g sample and change in potential difference (mV) was read by instrument, which ultimately converted in to ppm/g.

3.2.7 Estimation of sodium (Na^{+}) by Flame photometer

The standards for sodium were 10, 100 and 1000 ppm of sodium in sodium chloride solution. The slope was calibrated by the instrument itself. The number of Na^{+} ions present in 1 g of the sample was calculated by dipping the electrode in the solution containing 1 g sample and change in potential difference (mV) was read by the instrument, which ultimately converted in to ppm/g.

3.2.8 Estimation of potassium (K^{+}) by Flame photometer

Three concentration of K^{+} i.e., 10, 100 and 1000 ppm were prepared in KCl and ion concentration reflected in the flame photometer was calibrated. The concentration of K^{+} ion in ppm was estimated by reading of the change in potential difference (mV) by the instrument.

3.3 Isolation of microflora from SMS of different mushrooms

3.3.1 Preparation of culture media

Various culture media were prepared as per the requirement of the study by following the standard composition and method of preparation with slight modifications like use of Rose Bengal or Streptomycin for isolation of specific type of microbe. For routine purposes, laboratory grade chemicals and reagents were used, while for specific studies analytical grade chemicals were used. The compositions of culture media used are as follow:

Malt Extract Agar (MEA)

Malt extract	25 g
Agar-Agar	20 g
Peptone	5 g
Distilled water	1000 ml
pH	7.0-7.2

Potato Dextrose Broth (PDB)

Potato dextrose	24.0 g
Distilled water	1000 ml
pH	7.2

Potato Dextrose Agar (PDA)

Potato Dextrose	24.0 g
Agar-Agar	20.0 g
Distilled water	1000 ml
pH	7.2

Nutrient Agar

Beef extract	3.0 g
Peptone	5.0 g
Dextrose	3.0 g
Agar-agar	15 g
Distilled water	1000 ml
pH	7.2

Nutrient Broth

Beef extract	3.0 g
Peptone	5.0 g
Dextrose	3.0 g
Distilled water	1000 ml
pH	7.2

Nutrient High Medium (NHM) (Verma *et al.,* 2003)

$MgSO_4$	0.2 g

K_2HPO_4	1.0 g
KH_2 PO_4	1.0 g
$CaCl_2$	0.02 g
$FeCl_3$	0.05 g
NH_4	1.0 g
Yeast extract	1.0 g
Glucose	1.0 g
Distilled water	1000 ml
pH	7.2

The ingredients of the media were dissolved one by one in distilled water in measured quantities and the volume was finally made to one liter. The pH of the medium was adjusted by using 0.1N HCl or 0.1N NaOH as per the requirement of the cultural conditions for the experiment. Prepared media were poured in conical flasks, plugged with non-adsorbent cotton and covered with butter paper. The flasks were steam sterilized in an autoclave at 1.05 kg/cm^3 pressure for 30 minutes.

3.3.2 Isolation of fungi from SMS of different mushrooms

Spent substrate samples of *Agaricus bisporus*, *Pleurotus sajor-caju* and *Volvariella volvacea* collected from Directorate of Mushroom Research, Solan (HP), India were used for isolation of fungi. Isolation of fungi from the SMS samples was done by using dilution plating technique. 10 gram of SMS sample was taken in 250 ml Erlenmeyer flask containing 90 ml sterilized distilled saline water, followed by shaking for 30 minutes at 120 rpm. Serial dilutions were prepared by taking 1 ml of suspension from above, transferring to 9 ml sterilized saline water and making 10^{-2} - 10^{-6} dilutions. To isolate fungi, 10^{-4} and 10^{-5} dilutions were used and 0.1 ml of each dilution was spread on potato dextrose agar in petidishes in triplicates. The inoculated petridishes were incubated at 30 ± 2 °C in BOD incubator for four days. The well isolated fungal colonies appeared on plates were aseptically transferred to the malt extract medium, and were purified by subsequent sub culturing. Purified colonies were

grown on malt extract agar slants and stored thereafter at 4 °C until further use. Purified fungi were identified by sequencing of the ITS region of 5.8S rRNA gene, followed by Nucleotide Basic Local Alignment Search Tool (BLASTn) techniques (Altschul *et al.,* 1990).

3.4 Genotypic characterization of fungal isolates by amplification of the ITS region of 5.8S rRNA gene

All the fungal isolates from SMS were genotypically characterized by amplification the ITS region of 5.8S rRNA gene.

3.4.1 Fungal DNA extraction

All fungal isolates were grown separately on Malt Extract Agar petri plates at 32 ± 2 °C for 7 days. The mycelia from respective isolate plates were scrapped and placed at -85 °C for 2 hours, followed by freeze drying for 16-18 hours. The genomic DNA was extracted from approximately 100 mg of freeze dried fungal mycelia by crushing in 1.5 ml micro-centrifuge tubes using micro-pestles. QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH, D-40724 Hilden) was used for DNA extraction as per the protocol supplied by the manufacturer which was as follow:

1. Four hundred (400) µl of lysis buffer was added in 1.5 ml microcentrifuge tube containing crushed mycelia and mixed well.
2. Five (5) µl of RNAase was added to it and mixed well again. Microcentrifuge tubes were kept in water bath maintained at 65 °C for 10-15 minute with intermittent mixing by inversion of tubes.
3. One hundred (130) µl of AP2 (precipitating buffer) solution was added in each tube and tubes were kept for 15 minutes in ice.
4. The above solution was transferred to Q1A shredder mini column placed in 2 ml collection tube. The whole assembly was centrifuged for 2 minute at 14,000 rpm.

5. The supernatant was taken out without disturbing the cell-debris pellet and transferred it to a new 1.5 ml microcentrifuge tube. Then 675 µl of binding buffer (AP3/E) was added in each tube followed by through mixing by pipetting.
6. Six hundred (650) µl of mixture was transferred to DNeasy column (white) and centrifuged at 8000 rpm for 1 minute.
7. The flow through was discarded and the remaining sample from step 5 was added to the same DNeasy mini column. It was centrifuged again at 8000 rpm for 1 minute.
8. The flow through from column was discarded again and 500 µl of AW buffer was added on column. It was centrifuged at 8,000 rpm for 1 minute.
9. The flow through from column was discarded again and 500 µl of AW buffer was added on column and centrifuged again at 14,000 rpm for 2 minute.
10. The dried column was put back in a new microcentrifuge tube and 50 µl AE buffer (elusion) was added on it and followed by incubation at 65 °C for 5 minute.
11. The whole assembly was centrifuged at 8,000 rpm for one minute (so DNA will dissolve and pass in to the microcentrifuge tube).
12. Without disturbing the tube, another 50 µl of Elusion buffer (AE) was added in inner column, incubated for 5 minutes and centrifuged again at 8,000 rpm for one minute. Collected DNA was stored at 4 °C until further use.
13. Aliquots of 5 µl of DNA solution were placed in 0.8% agarose (Sigma Chemical Co.) gel for quantification by comparison with a quantifying standard (control). Spectrophotometric quantification was also done by taking OD at 260 nm.

3.4.2 Amplification of the ITS region of 5.8 S rRNA gene

The polymerase chain reaction (PCR) primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') developed by White *et al.*

(1990) were used to amplify the ITS region of 5.8S ribosomal DNA. PCR amplification was performed in a reaction mixture of 50 µl, containing 0.2 µl *Taq* DNA polymerase (5 U μl^{-1}), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH-8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2.0 mM each), 1 µl each of ITS-1 and ITS-4 primers (0.01 mM), 1 µl glycerol (5%), 2 µl $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). PCR reaction was performed in Master Cycler Gradient in 36 cycles each of 95 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min 20 sec and final elongation at 72 °C for 10 min with lid heating option at 104 °C. The presence and yield of PCR amplified amplicons was ascertained on 2.0% agarose gel (w/v) prepared in 1% TBE. Gel electrophoresis was carried out at 90 V for 1.30 h in 0.5X TBE buffer. Staining was done with ethidium bromide and the gel was visualized and photographed using Bio Imaging System (Gene Genius, Syngene).

3.5. Isolation and identification of bacteria from SMS of different nushrooms

For isolation of bacteria samples were collected and processed as elaborated under section 3.3.2. The extract was serially diluted 10^{-1} to 10^{-8}. For isolation of bacteria 10^{-6}, 10^{-7} and 10^{-8} dilutions were surface spread on nutrient agar plates in triplicate. The inoculated plates were incubated at 30±2 °C in BOD incubator for 48 hrs. The cultures were purified by subsequent sub culturing. Purified cultures were grown of nutrient agar medium slants and stored thereafter at 4 ºC until further use. Biochemically, all the bacterial isolates were identified at Microbial type culture collection, Institute of Microbial Technology, Chandigarh India. Purified bacteria were also identified using 16S rRNA gene sequencing, followed by Nucleotide Basic Local Alignment Search Tool (BLASTn) techniques (Altschul *et al.,* 1990).

3.5.1. Genotypic characterization of bacterial isolates by 16S rRNA gene amplification

3.5.1.1 Isolation of genomic DNA

All bacterial isolates were grown separately in 10 ml nutrient broth in 20 ml screw caped tubes at 30±2 ºC for 2 days. For extraction of total genomic DNA, 1.5 ml log phase

broth culture was used and DNA was extracted using Bacterial DNA Isolation Kit from Molecular Research Centre, Inc., Cincinnati OH, (USA) with protocol supplied by the manufacture:

The kit included three reagents:

i) 10X Bactozol Enzyme solution ™

ii) Bactozol Enzyme Dilution Buffer

iii) DNAzol ^{R}

An aliquot of 10X Bactozol Enzyme solution was diluted with nine volumes of the Bactozol Enzyme Dilution Buffer to obtain the required 1X Bactozol Enzyme solution.

(A) LysisA

i) Bacterial suspension was centrifuged at 6,000 g for 4 minutes at 25 °C.

ii) The supernatant was discarded and the pellet was resuspended in 100 µl of 1X Bactozol Enzyme solution by vortexing to achieve a homogeneous suspension.

iii) The bacterial suspension was lysed by incubating at 50 °C for 30 min.

(B) Lysate solubilization

i) The resulting bacterial lysate (0.1 ml) was mixed with four volumes of DNAzol (0.4 ml) by hand for 30 second to obtain a homogeneous solution.

ii) The lysate was incubated for 15 min at 50 °C to improve DNA quality and recovery.

(C) DNA precipitation

i) DNA from the DNAzol-lysate solution (0.5 ml) was precipitated by adding 0.6 volume of 100% ethanol (0.3 ml).

ii) The lysate was mixed by inversion for 15 second and stored at room temperature for 5 min. Bacterial DNA formed a white thread like precipitate.

iii) DNA was sedimented by centrifugation at 3000 g for 4 min at 25 °C.

(D) DNA wash

(i) Following centrifugation, supernatant was decanted carefully and residual fluid was removed with the help of a micropipette.

(ii) DNA pellet was washed twice with 1 ml of 75% ethanol by vortexing.

(iii) Sample was stored for 2 min to allow the pellet to sediment and ethanol was drained out.

(iv) Residual ethanol was removed and DNA was solubilised when it was still wet.

(E) DNA solubilization

(i) 100 µl of TE buffer was added to the DNA pellet for solubilisation and was stored at 4 °C.

Extracted DNA samples from different bacterial isolates were run on 1.2% agarose gel (w/v) in 1.0% TBE buffer at 90 V for 1 hr along with quantification marker in one lane. Gel was visualized using Bio Imaging System (Gene Genius, Syngene).

3.5.1.2 Spectrophotometric quantification of DNA

The concentration of DNA in suspension was estimated by spectrophotometric measurement at $A_{260.}$ For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path is equal to a concentration of 50 µg/ml H_2O. The quality of DNA was determined by measurement of the A_{260}/A_{280}. Ideally, the A_{260}/A_{280} should be $\geq$ 1.8. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA. The concentration was calculated assuming that one A_{260} unit is equal to 50 µg of double stranded DNA/ml H_2O.

3.5.2 16S rRNA gene amplification

Amplicon of 16S rDNA was obtained for each bacterial isolate by PCR amplification employing forward primer (5'-GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and reverse primer (5'-CCG TCA ATT CMT TTG AGT TT-3'). PCR reactions were performed in a reaction mixture of 50 µl containing 0.2 µl *Taq* DNA polymerase (5 U μl^{-1}), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH-8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2 mM each), 1 µl each of forward primer and reverse primer (0.01 mM), 1 µl glycerol (5%), 2 µl of $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). The reactions were performed in a Master Cycler Gradient with initial denaturation at 95 °C for 3 min, followed by 35 cycles each of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and final elongation at 72 °C for 10 min with lid heating option at 104 °C. The presence and yield of specific PCR product (16S rRNA gene) was visualized as per the protocol adopted for the ITS region of 5.8S rRNA gene.

3.5.3 PCR product cleaning up, sequencing, annotation and blasting

PCR amplified amplicons of the ITS region of 5.8S rDNA and 16S rDNA were cleaned up by using RCB kit (Banqiao City, Taipei County 220, Taiwan) for removing of, if any undesired DNA fragments. The cleaned up PCR products were got sequenced using 3730Xl (96 capillary) electrophoresis instruments from Bioserve Biotechnologies, Hyderabad, India. The received sequences in ab1/SCF format were cleaned up to remove the misleading sequences and were improved upon using Chromas software (Technelysium Pty Ltd.). The improved consensus sequences were blasted using BLASTn (Altschul *et al.,* 1990) of NCBI (http://blast.ncbi.nlm.nih.gov/Blast) and the specie against which highest total score was exhibited, was considered as the species identified.

3.6 Different dyes used and decolourisation potential of different fungi isolated from SMS

All the dyes (Table 4.1) were procured from Sigma-Aldrich. Seven fungi belonging to 5 different species [*Aspergillus fumigatus* (Ab), *Aspergillus fumigatus* (Ps), *Schizophyllum*

commune (Ps), *Pezizomycotina* sp. (Ps), *Aspergillus fumigatus* (Vv), *Paecilomyces variotii* (Vv) and *Pichia guilliermondii* (Vv)] isolated from spent substrate of three different mushrooms (given in parenthesis), were used for dye decolourisation. The dye decolourisation potential was studied against 100 ppm concentration of the dye in Potato Dextrose Broth (PDB). Five day old fungal culture was inoculated separately in dye mixed 100 ml PDB in 250 ml Erlenmeyer flasks and incubated at 25 ± 1 °C for 12 days in BOD incubator. Three replications were kept for each treatment keeping un-inoculated flasks as the control treatment. The decolourisation if any, was measured as per the protocol elaborated under heading 'measurement of decolourisation extent.

3.7 Dye decolourisation potential of different bacteria from SMS

Eight bacteria belonging to 5 different species [*Sphingobacterium multivorum*-1 (Ab), *Sphingobacterium multivorum*-2 (Ab), *Bacillus pumilus* (Ab), *Rummelibacillus stabekisii* (Ps), *B. licheniformis* (Ps), *B. subtilis* (Ps), *B. pumilus*-1 (Vv) and *B. pumilus*-2 (Vv)] isolated from spent substrate of three different mushrooms (given in parenthesis) were screened for their dye decolourisation potential against 100 ppm concentration of Orange II Sodium salt in PDB. Forty eight (48) hrs old cultures ($\times 10^6$/ml) of different bacteria were inoculated @ 0.1 ml separately in dye mixed 100 ml PDB filled in 250 ml Erlenmeyer flasks and the flasks were incubated at 30 ± 1 °C for 8 days in BOD incubator. Three replications were kept for each treatment by keeping un-inoculated flasks as the control treatment. Decolourisation was recorded like in case of earlier steps. The potential bacteria (*B. licheniformis* and *B. pumilus*) isolated from *P. sajor-caju* spent substrate were further evaluated for their dye decolourisation potential against 2 additional dyes (Azure B and Methyl blue). The decolourisation potential was studied against 100 ppm concentration of each dye in 100 ml PDB filled in 250 ml Erlenmeyer flasks. Two day old broth culture of each bacterial culture ($\times 10^6$/ml) was inoculated separately in each flask and the inoculated flasks were incubated at

25 ± 1 °C for 8 days in BOD incubator. Three flasks were kept for each treatment by keeping flasks devoid of bacterial culture inoculation as the control treatment. Decolourisation was recorded like in case of earlier steps.

3.7.1 pH optima for dye decolourisation with *B. licheniformis*

pH range of 4.0 to 10.0 was used for studying the pH optima of Orange II sodium salt decolourisation with the potential bacterium, *B. licheniformis*. Prepared nutrient broth was distributed in 250 ml Erlenmeyer flasks @ 100 ml each and sterilized at 15 p.s.i. pressure for 20 min. The 0.1 ml stock solution (1 g/10 ml) of the dye was added in 100 ml sterilized broth in flasks to give a final concentration of 100 ppm and 0.1 ml of two day old bacterial broth ($\times 10^7$) was inoculated separately in each flask. Flasks devoid of bacterial broth but with PDB supplemented with dye and with different pH were kept as control treatments. Three replications were kept for each treatment and all flasks including control were incubated at 25 ± 1 °C for next 8 days. Decolourisation was recorded like in case of earlier steps.

3.7.2 Temperature optima for dye decolourisation with *B. licheniformis*

Five different incubation temperatures (20, 25, 30, 35 and 40 °C) were used for studying the temperature optima of Orange II Sodium salt decolourisation with potential bacterium, *B. licheniformis*. Protocol for media preparation and setting up of experiment was same as was for pH studies, excepting medium pH which was 7.0. Experiment was carried out in triplicate and all flasks including control were incubated at 5 different temperatures for next 10 days. Decolourisation was recorded like in case of earlier steps.

3.8 Extracellular ligninolytic enzymes

The majorities of mushroom species come under basidiomycetes are saprophytic in their mode of nutrition and hence use their hydrolytic enzymes system to breakdown the complex organic matter in to simpler forms and their use as food for their mycelial growth and fruiting. The same set of enzymes which are used for the breakdown of complex organic

compounds like lignin are used for the breakdown of other recalcitrant compounds like synthetic dyes. The spent mushroom substrate released after harvest of mushroom crop also contains good quantity of these extracellular enzymes with possibilities of their utility in dyes decolourisation.

3.8.1 Screening of fungi for dye decolourisation potential

Screening for laccase producing fungus was done on plates using PDA supplemented with 0.02% guaiacol (Vishwanath *et al.,* 2008). Initially the fungi were screened for laccase activity using guaiacol as indicator compound. In the presence of guaiacol, intense reddish brown colour is produced in the medium around the fungal colonies and it is taken as the positive reaction for the presence of laccase activity as previously reported. Seven different fungi were inoculated separately in sterile petri plates containing the above medium and were incubated at 30 °C for a period of 7 days. *Schizophyllum commune* and *Pezizomycotina* sp. showing definite colour changes around their mycelial colonies were considered laccase producing fungi and these were selected further for synthetic dyes decolourisation potential.

3.8.2 Inoculum preparation for fungi and bacteria

Inoculums of the test fungi were prepared by taking a small mycelial bit from growing margin of a pure culture and transferring to petridishes (90 mm dia) containing 30 ml of malt extract agar medium each and incubating at 30 ± 2 °C for seven days. Similarly pure cultures of bacteria were obtained by streaking on the nutrient agar medium petri dishes and incubation at 30 ± 2 °C for 2 days. The well isolated colonies were taken and inoculated on nutrient agar medium slants, followed by incubation at 30± 2 °C for next 2 days. The pure culture of each bacterium was used for raising the broth culture in 10 ml nutrient broth taken in test tube after incubation at 30 ± 2 °C for 2 days.

3.8.3 Ligninolytic enzyme extraction from fungi

Wheat straw was soaked overnight in water to achieve sufficient moisture level. Flasks containing wet substrate @ 50 g / flask were autoclaved at 121 °C for 1 hour and were incubated at 30 °C after inoculation with respective fungal mycelium (2 bits each of 4 mm dia.). After 12 days of incubation, whole substrate colonized with fungal mycelium was used for enzyme extraction. Crude enzymes extract was prepared by extraction with 0.1 M sodium acetate buffer of pH 4.5 under magnetic stirring conditions for 30 min at room temperature (1 g substrate/ 5 ml buffer) and filtration through muslin cloth sheet. The filtrate was then centrifuged at 3,000 rpm for 10 min at 4 °C to make the final enzyme source.

3.8.4 Ligninolytic enzyme extraction from bacteria

The ligninolytic enzymes activity in different bacteria was studied by first growing different bacteria in nutrient rich medium as given by (Verma and Madamwar, 2003). The bacterial broth was then centrifuged at 3,000 rpm for 10 min at 4 °C to obtain the cell free supernatant, which was used as enzyme source. All enzymes activities were determined spectrophotometrically at 30 °C. Lignin peroxidase (LiP) activity was measured by the oxidation of veratryl alcohol to veratrylaldehyde and corrected for veratryl alcohol oxidase activity (Tien and Kirk, 1984). One unit of activity represents 1 µmol of veratryl alcohol oxidized to veratrylaldehyde per minute. Activity of extracellular laccase was determined by monitoring the increase in absorbance at 425 nm in the reaction mixture. Laccase activity was assayed according to Bourbonnais *et al.* (1990) by monitoring the oxidation of ABTS. One unit was defined as the amount of enzyme that oxidize 1 µmol of substrate per minute and the activity was expressed in Ul^{-1}. Manganese peroxidase (MnP) activity was measured by the method of Paszczynski *et al.* (1992) using Mn^{2+} as the substrate. One unit is defined as the amount of enzyme that oxidizes 1 µmol of substrate per minute.

3.8.5 Ligninolytic enzyme extraction from SMS

Pleurotus sajor-caju was grown under conditions of solid state fermentation at Directorate of Mushroom Research, Solan (HP), India. Crude enzymes extract from SMS (10 g homogenously mixed SMS) was prepared by extraction with 0.1M sodium acetate buffer, pH 4.5 under magnetic stirring for 30 min at room temperature (1 g solid substrate/ 5 ml buffer) and filtration through muslin cloth sheet. The filtrate was then centrifuged at 3,000 rpm for 10 min at 4 $^{\circ}$C. All enzymes activities were determined spectrophotometrically at 30 $^{\circ}$C.

3.8.6 Decolourisation through crude enzyme extract

Crude enzymes extract extracted out of the spent substrate of *P. sajor-caju* was used in different ratios with the dye supplemented malt extract broth to ascertain the effective ratio for decolourisation of different dyes. The ratios varied from 1: 4, 1:5, 1:6, 1:7, 1:9, 1:12, 1:19 and 1: 39 were tested against 100 ppm of Rhodamine B, Chicago sky blue, Starch azure and Methyl violet 2B for highest decolourisation at different time intervals (upto 3 days).

3.9 Role of cultural conditions on dyes decolourisation

The role of cultural conditions *viz*., medium, temperature, pH, initial concentration of dye, carbon and nitrogen sources, heavy metals, additives (veratryl alcohol and manganese sulphate), agitated/stationary conditions and pellet/intact forms of mycelia on decolourisation of different dyes was studied, the details of which is elaborated under the proceeding sections.

3.9.1 Effect of initial dye concentration on dye decolourisation

Five concentrations (25, 50, 100, 150 and 200 ppm) of Methyl violet 2B were used for evaluating the effect of initial dye concentration on its decolourisation. The PDB medium was distributed in 250 ml Erlenmeyer flasks (100 ml/flask) and sterilized. The fresh spent substrate of *P. sajor-caju* was added aseptically @ 1%, w/v to dye mixed (100 ppm) sterilized PDB and mixed thoroughly. Flasks devoid of spent substrate but with dye were

kept as control. Samples were taken at different time intervals and analysed for dye decolourisation, if any.

3.9.2 Effect of cultural Media

Two different growing media [malt extract broth (MEB) and potato dextrose broth (PDB)], procured from Hi-Media Labs, were used for studying effect of media composition on decolourisation of Rhodamine B, Methyl violet 2B, Chicago sky blue and Quinaldine red with spent substrate of *Pleurotus sajor-caju*, which is considered as potential dye decolorizer among basidiomycetous fungi. Media (100 ml each) of MEB (malt extract, 25 g; distilled water, 1000 ml; pH, 7.2) and PDB (potato dextrose, 24 g; distilled water, 1000 ml; pH, 7.2) was poured in Erlenmeyer flasks (250 ml) and sterilized at 15 psi for 20 min. The 0.1 ml of stock solution (1 g/10 ml water) of each dye was added in pre-sterilized growing media flasks to make up 100 ppm concentration of each dye, followed by addition of 1 g spent substrate of *P. sajor-caju* in each flask using sterilized forcep. Experiment was carried out in triplicate and all flasks were incubated at 25 ± 1°C for next 3 days. Decolourisation, if any, was recorded by recording decrease in optical density at λmax from 0 day up to 3 days of incubation by keeping flasks devoid of SMS as control treatment.

3.9.3 Effect of additional carbon sources

Six sources of carbon, namely fructose, glucose, lactose, sucrose, starch and cellulose were used to study the effect of carbon sources on decolourisation of Methyl violet 2B. All were used at a concentration of 0.5% in PDB medium and spent substrate of *Pleurotus sajor-caju* (PSC) @ 1.0%, w/v was used as potential dye decolourizing agent. For further confirmation of the role of carbon sources in dye decolourisation, glucose and starch (carbon source) were studied at 3 different concentrations (25, 50 and 100 ppm). Flasks devoid of any additional carbon source but with dye @ 100 ppm and *P. sajor-caju* spent substrate @ 1.0%, w/v were used as control.

3.9.4 Effect of veratryl alcohol on Methyl violet 2B (MV2B) decolourisation

Veratryl alcohol, a natural secondary metabolite of *Phanerochaete chrysosporium* as well as a substrate for Lignin peroxidase (LiP), is considered as an inducer of the ligninolytic enzymes system under *in vitro* conditions. For studying the effect of veratryl alcohol on dye decolourisation four concentrations (0.025, 0.05, 0.075 and 0.1%) of veratryl alcohol were used in PDB medium and its effect on dye decolourisation was recorded at different time intervals. Flasks devoid of veratryl alcohol but with dye and spent substrate were kept as control.

3.9.5 Effect of Manganese sulphate ($MnSO_4$) on Methyl violet 2B decolourisation

The effect of five concentrations of $MnSO_4$ was studied by adding 25, 50, 75, 100 and 125 mg of $MnSO_4$ in 100 ml of sterilized PDB medium in 250 ml flasks, each containing dye and spent substrate @ 100 ppm and 1.0%, w/v, respectively. Flasks without of Manganese sulphate but with 100 ppm concentration of dye in PDB and 1.0% *P. sajor-caju* spent substrate were used as control (Vahabzadeh *et al.,* 2004).

3.9.6 Effect of heavy metals on Methyl violet 2B (MV2B) decolourisation

Effect of 6 heavy metals (cadmium acetate, lead nitrate, mercuric iodide, cobaltous sulfate, zinc sulfate and nickel chloride) on decolourisation of MV2B was studied using 0.5% concentration of each heavy metal, 100 ppm concentration of dye and 1.0%, w/v of spent substrate of *P. sajor-caju* in PDB. The rest of the experimental protocol was similar to earlier steps. Flasks without of any heavy metal but with 100 ppm concentration of dye and 1.0%, w/v of spent substrate of *P. sajor-caju* were used as control.

3.9.7 Effect of agitated/stationary growing conditions on Methyl violet 2B decolourisation by *P. sajor-caju*

Experiment was conducted by keeping dye mixed and *P. sajor caju* inoculated PDB medium flasks at 2 sets of conditions; one at stationary condition of 25 ± 1 °C for 15 days in

BOD incubator, while another in incubator shaker at 25 ± 1 °C and 50 rpm for 15 days. Flasks devoid of *P. sajor caju* inoculation were kept as control for both sets of treatments.

3.9.8 Effect of intact/pellet form of mycelia of *P. sajor-caju*

Dye decolourisation potential of intact and pellet forms of mycelia of *P. sajor caju* was studied by inoculating pre-grown intact and homogenized forms of mycelia in 2 different sets of PDB medium flasks, pre-mixed with of MV2B @100 ppm. Flasks devoid of *P. sajor-caju* inoculation were kept as control for both sets of treatments. Both sets of flasks were incubated at 25 ± 1 °C for next 15 days in BOD incubator.

3.10 Dyes decolourisation through spent mushroom substrate

3.10.1 Effect of pH on dye decolourisation with spent mushroom substrate

Three different pH levels (4.0, 7.0 and 10.0) were used for studying the effect of pH on decolourisation of different dyes with spent mushroom substrate generated mainly by *P. sajor-caju* and *Agaricus bisporus*. PDB was prepared by adjusting its pH with 0.1N solution of HCl or NaOH. Prepared broth was distributed in 250 ml Erlenmeyer flasks @ 100 ml per flask and sterilized. The 0.1 ml stock solution (1 g/10 ml) of each dye was added seperately in 100 ml sterilized PDB in flasks to give a final concentration of 100 ppm and 1.0 g of spent substrate of *P. sajor caju* and *Agaricus bisporus* was added separately using sterilized forcep. Flasks devoid of SMS but with different dyes were kept as control. Three replications were kept for each treatment and all flasks including control were incubated at 25 ± 2 °C for next 120 hrs in BOD incubator. Before recording optical density, 3 ml sample from each flask was withdrawn and centrifuged (Sigma centrifuge) at 5000 rpm for 10 min and clear supernatant obtained, was used for recording optical density. Decolourisation, if any, was recorded by

recording decrease in optical density at λ_{max} (Table 4.1) using UV-Visible double beam Spectrophotometer (Unico-3802) starting from 0 to 120 hrs of incubation.

3.10.2 Effect of temperature on dye decolourisation with spent mushroom substrate

Three different temperatures (15, 25 and 35 °C) were used for studying the effect of temperature on decolourisation of different dyes with SMS of *P. sajor-caju* and *Agaricus bisporus*. Protocol for media preparation and setting up of experiment was same as was for the pH studies and all flasks including control were incubated at 3 different temperatures (15, 25 and 35 °C) for next 120 hrs. Flasks devoid of SMS were kept as control.

3.10.3 Effect of pH on dye decolourisation with fungi isolated from SMS of *P. sajor-caju*

Seven different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) values were used for studying the effect of pH on decolourisation of Rhodamine B and Methyl violet 2B with *S. commune* and *Pezizomycotina* sp. isolated from SMS of *P. sajor-caju*. PDB was prepared by adjusting pH with 0.1N solution of HCl or NaOH. Prepared broth was distributed in 250 ml Erlenmeyer flasks @ 100 ml each and sterilized at 15 psi for 20 min. The 0.1 ml stock solution (1 g/10 ml) of each dye was added in 100 ml sterilized broth in flasks to give a final concentration of 100 ppm and a bit of mycelia (3 mm dia.) of each fungus was added separately and incubated at 30±1 °C for next 18 days. Flasks devoid of culture but with different dyes were kept as control treatments. Before recording optical density, 3 ml sample from each flask was withdrawn and centrifuged (Sigma centrifuge) at 10,000 rpm for 10 min and clear supernatant was used for recording optical density. Decolourisation if any was recorded by recording decrease in optical density at λ_{max} using UV-Visible Spectrophotometer (Unico-3802) starting from 0 day to 18 days of incubation.

3.10.4 Effect of temperature on dye decolourisation with fungi isolated from SMS of *P. sajor-caju*

Three different temperatures (15, 25 and 35 °C) were used for studying the effect of different temperatures on decolourisation of Rhodamine B and Methyl violet 2B using *S. commune* and *Pezizomycotina* sp. isolated from SMS of *P sajor-caju*. Protocol for media preparation and setting up of experiment was same as was for pH studies, excepting medium pH, which was kept 7.2. Experiment was carried out in triplicate and all flasks including control were incubated at 3 different temperatures (15, 25 and 35 °C) for next 18 days. Protocol for sample processing for recording of dye decolourisation, if any, was similar as in earlier step.

3.10.5 Infrared spectrum analysis

The controls and samples were dried and mixed with KBr (1:20; 0.02 g of sample with 0.4 g of KBr). The samples were then ground, desorbed at 60 °C for 24 h and pressed to obtain IR-transparent pellets. The absorbance FT-IR spectra of the samples were recorded using an FT-IR Spectrum 2000 Perkin–Elmer spectrophotometer. The spectra were collected within a scanning range of 400–4000 cm^{-1}. The FT-IR was first calibrated for background signal scanning with a control sample of pure KBr, followed by scanning of the experimental samples. The FT-IR spectrum of the control was finally subtracted from the spectra of the non-degraded and degraded dyes.

3.11 Decolourisation of textile effluent

Textile effluent was collected from a commercial unit located at industrial area Baddi, Solan (HP), India. It was analyzed for pH and electrical conductivity by using Smart Chem autoanalyzer and HPG System, respectively, both before and after treating with different combinations of bacteria and fungi. SMS used for isolation of effective bacteria and fungi, and agricultural residues used as immobilizing agents were obtained from Directorate of Mushroom Research (DMR), Solan, India.

3.11.1 Screening of immobilizing agents for textile effluent decolourisation using fungi from SMS

Agricultural residues *viz.*, wheat straw, paddy straw, soybean straw, saw dust and peat moss were used for immobilization of the mycelial cultures of potential fungi isolated from spent substrate of *P. sajor-caju*. All the substrates were separately soaked overnight in water to achieve moisture content of 65 - 70% and pH was adjusted to 7.0 using 0.1 N HCl and NaOH. Wet substrates were filled in flasks (@ 50g/flask) and autoclaved at 121 ºC for 60 min. It was followed by inoculation with two fungi separately and incubation at 30 ± 2 °C for 12 days. Immobilized cultures of *S. commune* and *Pezizomycotina* sp. were added separately @ 0.1 g/flask in 100 ml textile effluent filled in 250 ml Erlenmeyer flask using sterilized forcep. Flasks containing only textile effluent were kept as first control, while flasks with different agricultural residues separately along with effluent were kept as second control. Three replications were kept for each treatment and all flasks including both controls were incubated at 30 ± 2 ºC for next 6 days (144 h). The decolourisation, if any was measured using the protocol and equation elaborated under heading measurement of decolourisation extent.

3.11.2 Preparation of bacterial consortia

All selected potential bacteria *viz.*, *Bacillus pumilus*, *B. licheniformis* and *Pseudomonas fluorescens* (Ahlawat and Singh, 2011) were grown separately in 100 ml sterilized nutrient broth (NB) of pH 7.2, filled in 250 ml Erlenmeyer flask. The inoculated NB flasks were incubated at 30 ± 2 ºC for 2 days. The 0.1 ml of broth culture (cfu× 10^{7-8}/ml) of different bacteria was used for decolourisation studies.

3.11.3 Effect of cultural conditions on textile effluent decolourisation

The textile effluent released from textile industries is a heterogeneous waste containing diverse types of chemicals dominated by synthetic dyestuff. The physico-chemical

conditions including the nutrient composition of the effluent, pH, temperature, etc do contribute towards its decolourisation with SMS or its associated microorganisms.

3.11.3.1 Effect of carbon sources for textile effluent decolourisation

Five carbon sources (glucose, fructose, sucrose, maltose and starch) in their three different concentrations (0.25, 0.5 and 1%) were used for studying their effect on decolourisation of textile effluent using immobilized form of two potential fungi and broth cultures of three bacteria in their isolation and different combinations. Eight treatments including six of different microorganisms and two of controls were used with 100 ml of effluent filled in 250 ml Erlenmeyer flask. Three replications were kept for each treatment. The two controls included, one, effluent devoid of microbial inoculum but with wheat straw, while second, effluent devoid of both wheat straw and microbial inoculum. All flasks including controls were incubated at 30 ± 2 °C for 4 days and decolourisation of effluent, if any, was recorded after 48 h and 96 h of incubation by the method elaborated under the heading measuring the decolourisation extent.

3.11.3.2 Effect of temperature on textile effluent decolourisation

Four different temperatures (20, 25, 30 and 35 °C) were used for studying the effect of temperature on decolourisation of textile effluent with immobilized form of two fungi and broth cultures of three bacteria. Protocol for media preparation and setting up of experiment was same as was for the carbon sources study and all flasks including controls were incubated at 4 different temperatures for next 6 days. Two controls were kept, one, effluent devoid of microbial inoculum but with wheat straw, while second devoid of both wheat straw and microbial inoculum. The decolourisation, if any, was recorded by following the protocol mentioned under heading measuring the decolourisation extent.

3.12 Measurement of decolourisation extent

Sample (3 ml) collected each time from each replication and centrifuged at 10,000 rpm for 10 min was used for measuring decolourisation extent by measuring absorbance of supernatant at specific λ_{max} for each dye by using UV-Visible double beam Spectrophotometer (Unico-3802). Decolourisation extent was calculated as:

Decolourisation extent (%) = $[100 \times (OD_1 - OD_t)]/OD_1$

Where OD_1 is initial absorbance at 0 day, OD_t is absorbance after incubation for different periods under different experimental conditions, t is incubation time (Kaushik *et al.,* 2009).

3.13 Kinetics of dye decolourisation by spent mushroom substrate

An understanding on kinetics of physico-chemical process is essential for optimization of the process. In present case the data obtained from the dye decolourisation experiments involving the spent mushroom substrtae of *P. sajor-caju* and the potential fungi isolated from *P. sajor-caju* SMS at different pH and temperature was further used for the applicability of the sultable kinetics model to explain the decolourisation process.

3.13.1 Kinetics of Methyl violet 2B decolourisation by spent substrate of *Pleurotus sajor-caju*

Three different temperature (15, 25 and 35 ºC) and pH (4.0, 7.0 and 10.0) were used for studying the highest decolourisation of Methyl violet 2B with spent substrate of *P. sajor-caju.* Data obtained from the dye decolourisation experiment at different pH and temperature with spent substrate of *P. sajor-caju* was further analyzed for the applicability of the sultable kinetics model to explain the decolourisation process. Optical density recorded at different time intervals and corresponding temperature and pH was used to determine the kinetics of the decolourisation process.

3.13.2 Kinetics of Methyl violet 2B by *Schizophyllum commune* and *Pezizomycotina* sp. isolated from SMS of *P. sajor-caju*

Two potential fungi namely *Schizophyllum commune* and *Pezizomycotina* sp. isolated from spent substrate of *P. sajor-caju* were tested for decolourisation of Methyl violet 2B. Seven different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) and five different temperatures (15, 20, 25, 30 and 35 °C) were tested for Methyl violet 2B decolourisation with *Schizophyllum commune* and *Pezizomycotina* sp. in potato dextrose broth. Data obtained from the dye decolourisation experiment at different pH and temperature with *Schizophyllum commune* and *Pezizomycotina* sp. was further analyzed for the applicability of the sultable kinetics model to explain the decolourisation process. Optical density recorded at different time intervals, and corresponding temperature and pH was used to determine the kinetics of the decolourisation process.

Chapter-4

To Evaluate the Ligninolytic Enzymes Production and Dyes Decolourisation Potential of Spent Mushroom Substrates

4.1. Dye decolourisation potential of different edible mushroom species

Eleven different strains belonging to ten different species and four genera of commonly grown edible mushrooms were screened for their radial mycelial growth and dye decolourisation potential against nine structurally different dyes (Table 4.1). The edible mushroom species used in the study were, *Pleurotus sajor-caju, P. florida, P. ostreatus, P. fossulatus, P. eryngii, P. sapidus, P. flabellatus, Auricularia polytricha, Lentinula edodes, Agaricus bisporus* (strain S-11), *A. bisporus* (strain HU3). A simple agar-plate test was performed for determining the decolourisation capability of different mushroom species against different dyes (Table 4.2) after 15 days of incubation at 25 °C. All mushroom species grew on media plates supplemented with different dyes but majority of the *Pleurotus* spp. exhibited higher decolourisation against all the 9 dyes (Fig. 4.1). Out of different mushroom species, *P. sajor-caju* and *P. flabellatus* exhibited higher mycelial growth in presence of different dyes (Table 4.2 and Fig. 4.1) along with higher decolourisation of these dyes.

Among different *Pleurotus* spp., highest mycelial growth against Chicago sky blue was of *P. sajor-caju* (88.30 mm), followed by *P. flabellatus* (83.60 mm) after 15 days of incubation at 25 °C. Amongst different dyes, Methyl violet 2B, Rhodamine B and Orange II sodium salt supported lesser mycelial growth of different mushroom species compared to remaining six dyes. In presence of these three dyes the mycelial growth was less than in the control plates, while in presence of rest six dyes the mycelial growth was higher than in the control plates (Fig. 4.1). The results indicate that azo dyes were more resistant to decolourisation than anthraquinone dyes.

Table 4.1 Dyes used in decolourisation studies involving mycelial cultures of different mushroom species and spent substrate of *Pleurotus* spp. and other edible mushrooms

Dye type	Dye	Chemical formula	Concentration used (ppm)	Wavelength (λmax in nm)
Azo	Methyl violet 2B	$C_{25}H_{30}ClN_3$	100	584
Anthraquinone	Quinaldine red	$C_{21}H_{23}IN_2$	100	528
Azo	Orange II sodium salt	$C_{16}H_{18}ClN_3S$	100	550
Azo	Rhodamine B	$C_{28}H_{31}N_2O_3Cl$	100	543
Azo	Azure B	$C_{15}H_{18}ClN_3S$	100	648
Diazo	Methylene blue	$C_{16}H_{18}ClN_3S$	100	655
Azo	Chicago sky blue	$C_{34}H_{24}Na_4O_{16}S_4$	100	650
Azo	Starch Azure	$C_{22}H_{16}N_2Na_2O_{11}S_3$	100	650
Azo	Reactive blue	$C_{29}H_{20}ClN_7O_{11}O_3$	100	645

The decolourisation process in *Pleurotus* spp. started very early (on 3rd day of inoculation) and complete decolourisation was recorded after 12 to 18 days of mycelial culture inoculation with higher mycelial growth on dyes supplemented media.

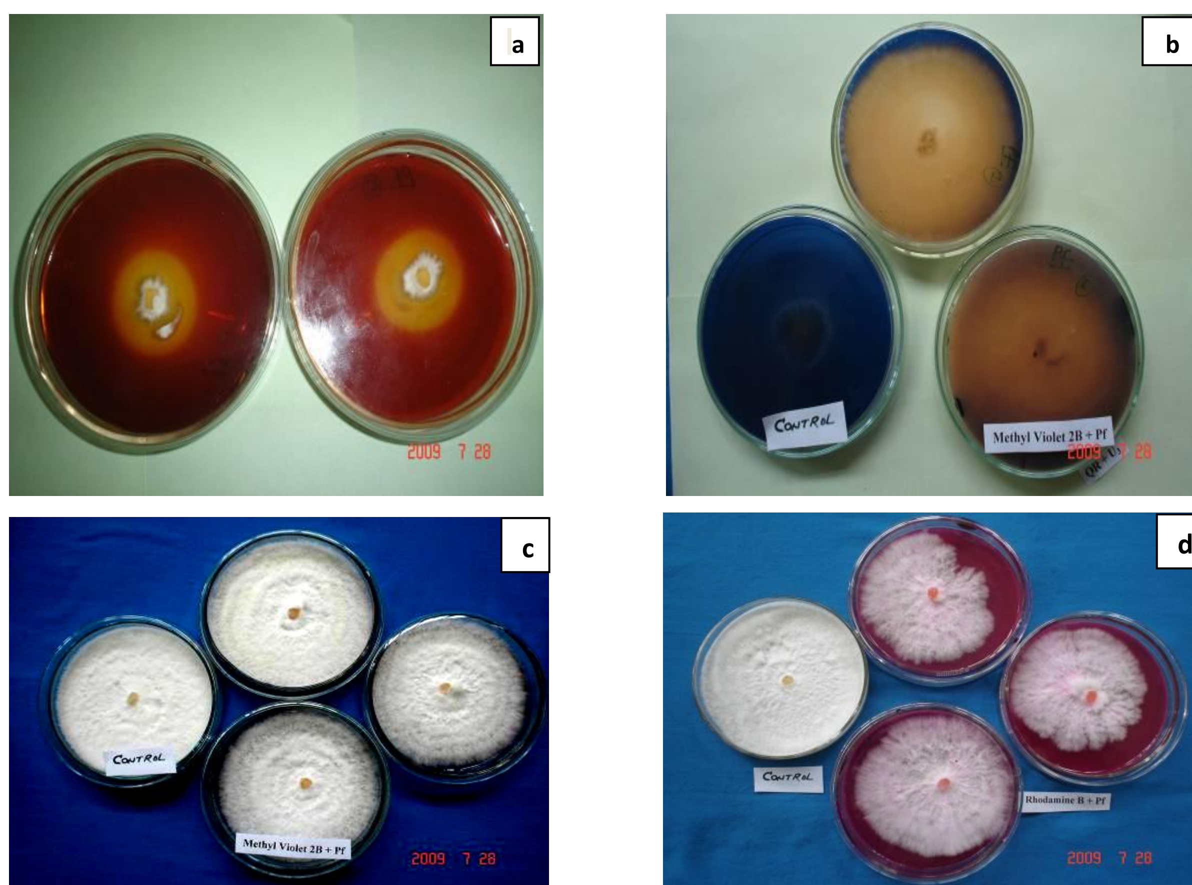


Fig 4.1 Agar Plate Assay: a) Decolourisation of Quinaldine red in agar plate by *Pleurotus sajor-caju*; b) Whole plate decolourisation of Methyl violet 2B by *Pleurotus flabellatus*; c) Mycelial growth of *P. sajor-caju* in the presence of Methyl violet 2B; d) Mycelial growth of *Pleurotus flabellatus* in the presence of Quinaldine red

Table 4.2 Mycelial growth of different edible mushroom species on dye supplemented potato dextrose agar medium

Mushroom species	Mycelial Growth on dye supplemented potato dextrose agar (mm)									
	Dye									
	Control	RB	MV 2B	CSB	AB	QR	Reactive blue	SA	Orange II Sod. salt	MB
Pleurotus ostreatus	53.50	62.00	50.60	74.70	85.50	70.00	86.20	60.00	55.70	68.00
P. flabellatus	75.50	72.30	70.00	83.60	37.80	70.30	88.00	82.60	70.60	75.90
P. florida	63.75	52.00	53.70	86.00	53.70	51.80	86.00	74.00	66.00	78.00
P. fossulatus	35.25	23.80	26.20	35.50	27.80	22.35	25.00	29.20	24.50	28.80
P. sapidus	70.00	68.50	67.00	88.00	60.10	72.50	82.00	78.00	69.00	72.30
P. eryngii	36.50	33.00	32.70	41.60	40.00	38.00	44.80	32.30	29.00	40.40
P. sajor-caju	71.00	75.50	70.60	88.30	80.00	70.50	90.00	83.60	65.00	78.50
Lentinula edodes	65.00	41.00	42.10	80.50	79.10	44.50	81.00	60.00	56.20	52.00
Auricularia polytricha	51.00	52.00	50.00	61.00	68.50	49.00	53.50	62.00	54.00	55.00
Agaricus bisporus (HU3)	43.50	22.20	21.00	24.25	22.00	25.70	24.00	25.00	24.40	22.50
Agaricus bisporus (S-11)	40.50	24.00	22.00	20.00	23.00	24.60	26.00	25.00	24.20	23.00
SE±	0.2828	0.2581	0.2588	0.1020	0.0559	0.0279	0.0774	0.1991	0.0922	0.059
$CD_{0.05}$	0.578	0.527	0.529	0.208	0.115	0.057	0.157	0.409	0.196	0.117

Where, RB-Rhodamine B; MV2B-Methyl violet 2B; CSB-Chicago sky blue; AB-Azure B; QR- Quinaldine red; SA-Starch Azure; MB-Methylene blue and control without dye

Lentinula edodes showed highest mycelial growth of 80.50 mm in presence of Chicago sky blue compared to 65.00 mm in control plates. Amongst different mushroom species, both strains of *Agaricus bisporus* i.e. HU3 and S-11 exhibited least mycelial growth and dyes decolourisation potential on potato dextrose agar medium supplemented with different dyes. In presence of Azure B, highest mycelial growth of 85.50 mm was recorded for *Pleurotus ostreatus*, followed by 80.00 mm and 79.10 mm in case of *P. sajor-caju* and *Lentinula edodes,* respectively. In case of Reactive blue, the highest mycelial growth of 90.00 mm was recorded in *P. sajor-caju,* followed by 88.00 mm in *P. flabellatus*, 86.20 mm in *P. ostreatus,* 86.00 mm in *P florida,* 82.00 mm in *P. sapidus* and 81 mm in *L. edodes.* The mycelial growth was lowest in *Agaricus bisporus* (strain S-11) (20.00 mm). In presence of Rhodamine B, *L. edodes* exhibited lesser mycelial growth of 41.00 mm compared to 65.00 mm in control plates. However, it was just reverse in presence of Chicago sky blue, where the mycelial growth in presence of dye was significantly higher (80.50 mm) compared to its growth in control plates (Fig. 4.2).

Against Chicago sky blue, all *Pleurotus* spp., exhibited higher mycelial growth and whole plate decolourisation compared to control plates. Out of the tested species, highest decolourisation ability was recorded with *P. sajor-caju* and *P. flabellatus* against majority of the dyes. Considering mycelial growth and dyes decolourisation potential in presence of different dyes in agar plate studies as an important criterian, out of seven different *Pleurotus* spp. used in the initial study, *P. sajor-caju* was selected for further studies. Similarly, out of two strains of *A. bisporus* used in the initial study, strain HU3 was selected for further studies. Both these edible mushroom species are cultivated at industrial scale in India, which led to generation of huge quanity of spent mushroom substrate annually. The spent mushroom substrate of *P.*

sajor-caju and *A. bisporus* strain HU3 were also studied further for different textile dyes decolourisation.

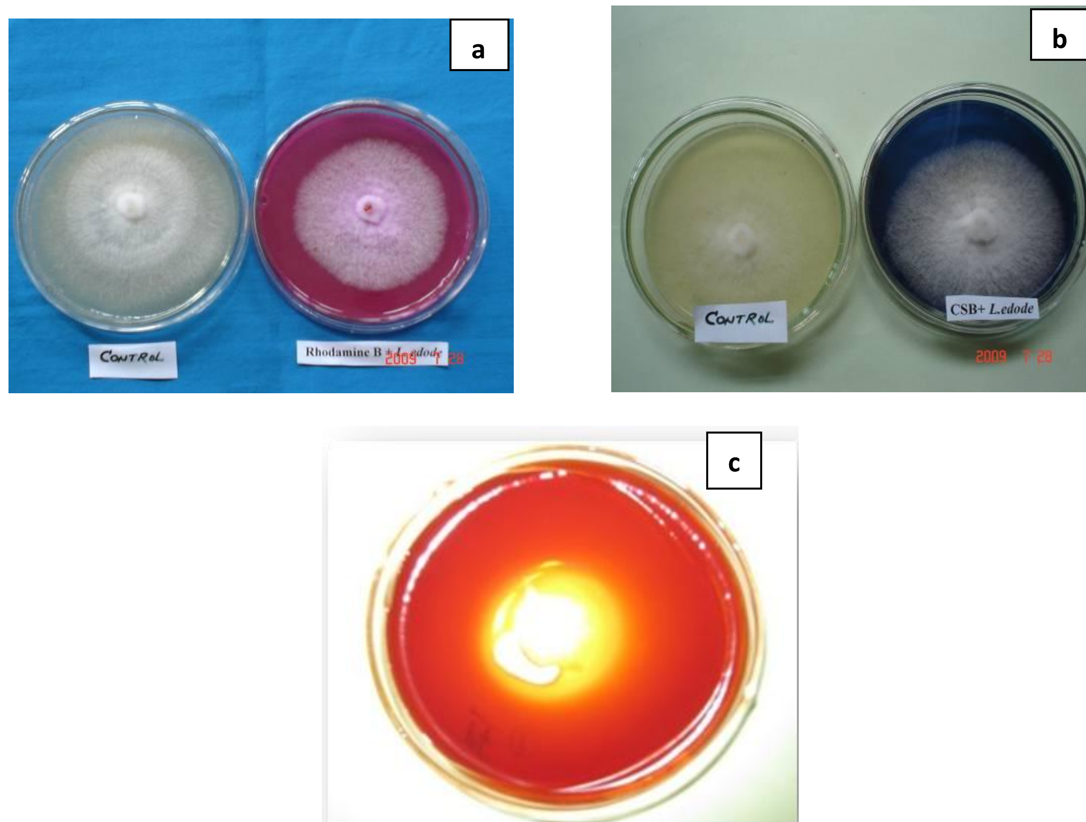


Fig 4.2 Agar Plate Assay: a) Mycelial growth of *Lentinula edodes* on Rhodamine B; b) Mycelial growth of *L. edodes* in the presence of Chicago sky blue; c) Decolourisation zone in presence of *P. sajor-caju* against Rhodamine B

4.1.1 Effect of pH of the medium on decolourisation of different dyes with spent subatrate of *P. sajor-caju*

The effect of pH of the medium on decolourisation of different dyes with spent subatrate of *P. sajor-caju* is presented in figure 4.3. The result revealed that spent substrate of *P. sajor-caju* has the ability to decolourize different dyes over a wide range of pH (4.0 to 10.0). The decolourisation of Reactive blue reached at highest level (100%) at pH 10.0 after 72 hrs of incubation. Contary to this the highest decolourisation of Methyl violet 2B (96.3%), Chicago sky blue (96.00%) and Quinaldine red (90.17%) was recorded at pH 4.0 after 72 hrs of incubation. In case of Rhodamine B, highest decolourisation (74.4%) was recorded at pH 7.0, while in

Starch azure the highest decolourisation (54.0%) was at pH 10.0 after 72 hrs of incubation.

4.1.2 Effect of temperature of incubation on decolourisation of different dyes with spent subatrate of *P. sajor-caju*

The study to determine temperature optima for decolourisation of different dyes was performed at three different temperatures (15, 25 and 35 °C). Highest decolourisation of Rhodamine B (58%), Methyl violet 2B (98%), Chicago sky blue (95%), Quinaldine red (91%) and Reactive blue (100%) was recorded at 25 °C after 72 hrs of incubation. However, in starch azure, the highest decolourisation (99%) was recorded at 35 °C. In case of Methyl violet 2B and Reactive blue, the decolourisation was at par at 25 to 35 °C after 72 hrs of incubation (Fig. 4.4). The lower temperature (15 ºC) supported lower decolourisation compared with the temperatures of 25 and 35 ºC.

4.1.3 Effect of pH of the medium on decolourisation of different dyes with spent substrate of *Agaricus bisporus*

The study was performed at three different pH values (4.0, 7.0 and 10.0) by using spent substrate of *A. bisporus* strain HU3 and six different dyes. Highest decolourisation of Methyl violet 2B (100%) was recorded at pH 7.0 after 72 hours of incubation. Similarly, highest decolurization (95%) of Rhodamine B was recorded at pH 7.0 after 72 hrs. Contrary to this, highest decolourisation (90 %) of Quinaldine red and Chicago sky blue (84%) was recorded at pH 4.0. Decolourisation of Reactive blue (74%) and Starch azure (48%) was highest at pH 10.0 (Fig. 4.5). The preferential pH for decolourisation of different dyes varied from 4.0 to 10.0, but majority dyes exhibited higher decolourisation at neutral pH.

4.1.4 Effect of temperature of incubation on decolourisation of different dyes with spent substrate of *A. bisporus*

Three different temperatures (15, 25 and 35 °C) of incubation were used for studying for their effect on decolurization of six different dyes with spent substrate of *A. bisporus*. For almost all dyes, the lower temperature (15 °C) of incubation was recorded to support lesser decolourisation compared with the temperatures of 25 and 35 °C. Highest decolourisation of Chicago sky blue (98%), Methyl violet 2B (97%), Quinaldine red (95%) was recorded at 35 °C, followed by Reactive blue (88%) and Rhodamine B (85%) at 25 °C with the spent substrate of *A. bisporus* after 72 hours of incubation (Fig. 4.6). Starch azure exhibited lowest decolourisation and it was highest of 50% at 35 °C, followed by 48% at 25 °C. In case of Quinaldine red, the difference between decolourisation at different temperatures of incubation was insignificant and same was the case with Chicago sky blue at themperatures of 15 and 25 °C and Rhodmine B at temperatures of 15 and 35 °C.

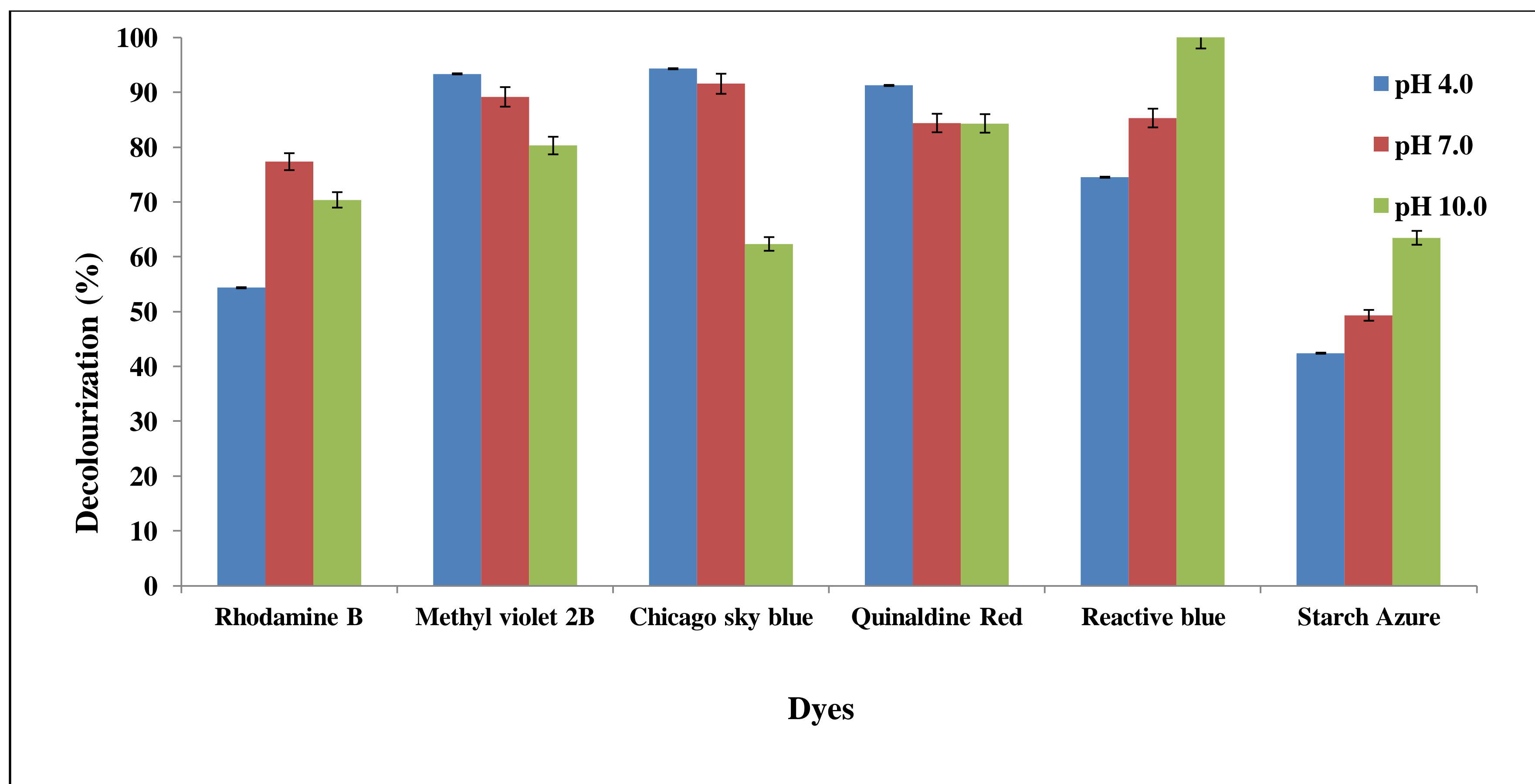


Fig 4.3 Effect of pH of the medium on dye decolourisation with spent substrate of *P. sajor-caju* after 72 hrs

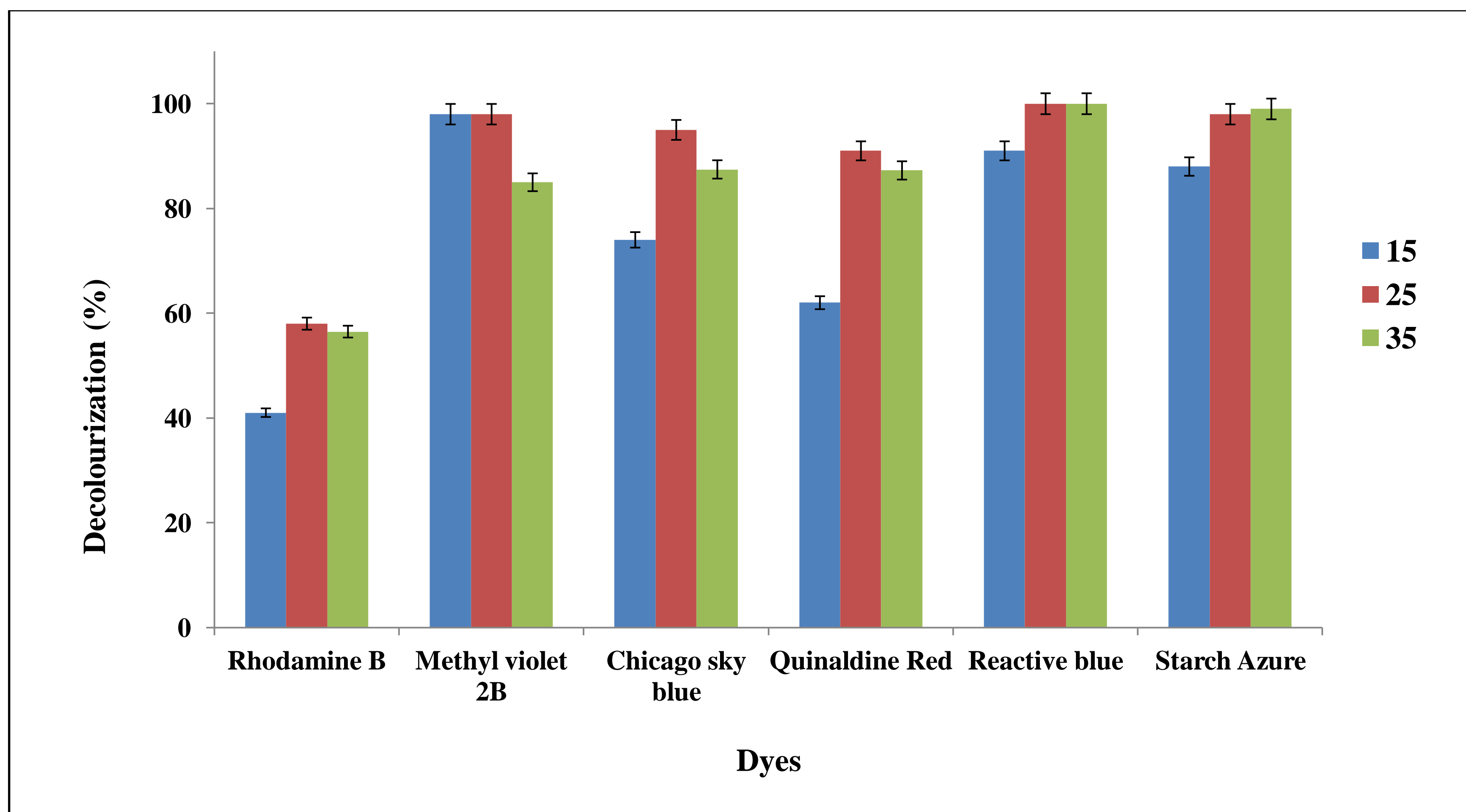


Fig. 4.4 Effect of temperature of incubation on dye decolourisation with spent substrate of *P. sajor-caju* after 72 hrs

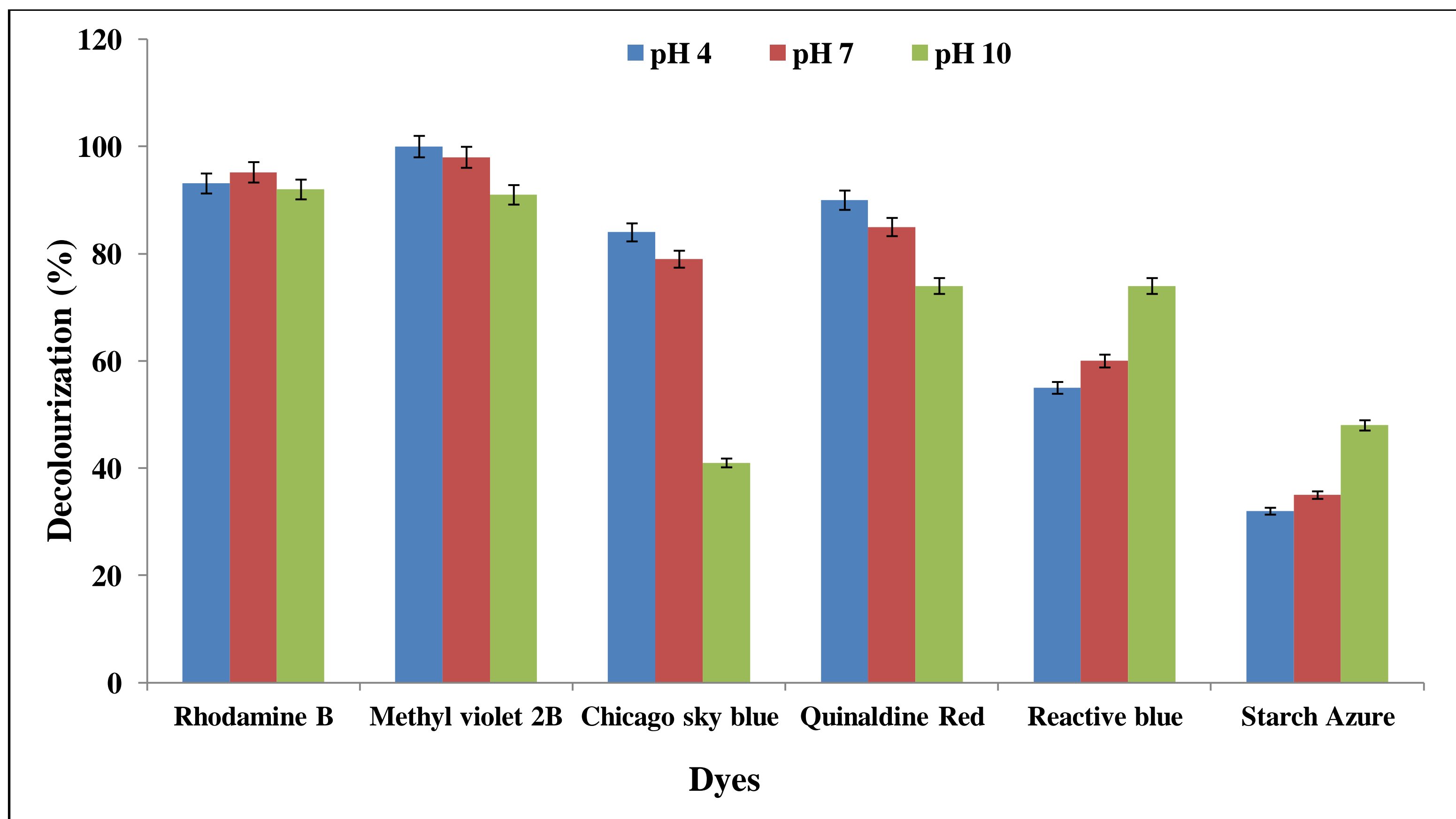


Fig. 4.5 Effect of pH of the medium on dye decolourisation with spent substrate of *Agaricus bisporus* after 72 hrs

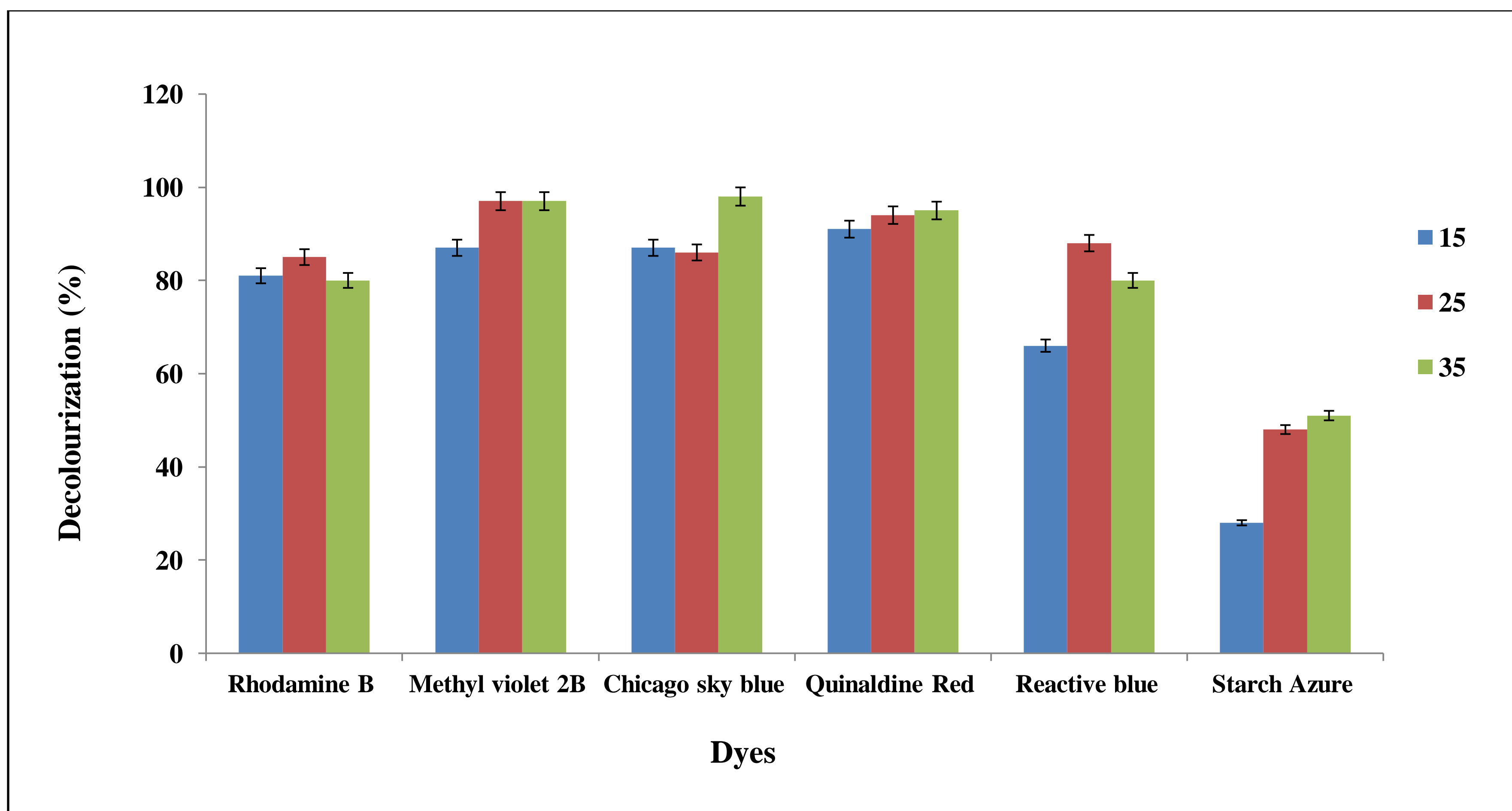


Fig. 4.6 Effect of temperature of incubation on dye decolourisation with spent substrate of *Agaricus bisporus* after 72 hrs

4.2 Physico-chemical properties of spent mushroom substrate (SMS) of different mushrooms

The major physico-chemical properties of spent mushroom substrate of different mushrooms were determined and the pH of spent substrates varied between 7.7 in *Agaricus bisporus* to 6.8 in *Pleurotus sajor-caju* SMS. Moisture content was higher (64 %) in SMS of *Agaricus bisporus*, followed by spent substrate of *Pleurotus sajor-caju* (55%). Moisture content (64%), Nitrogen (1.8 %), Sodium (105 ppm), Potassium (275 ppm) and Calcium (1045 ppm) were higher in spent substrate of *A. bisporus* (Table 4.3) compared to SMS of *P. sajou-caju* with an exception of Carbon (33.2 %). The values of Carbon, Nitrogen, Sodium, Potassium and Calcium were 42%, 1.7%, 48 ppm, 134 ppm and 896 ppm, respectively in spent substrate of *Pleurotus sajor-caju.* These results indicate that spent substrate of both *Agaricus bisporus* and *Pleurotus sajor-caju* provides good nutrient source for the growth of different bacteria and fungi.

Table 4.3 Physico-chemical properties of spent mushroom substrate (SMS) of different mushrooms

Physico-chemical properties	**Spent mushroom substrate**	
	Agaricus bisporus	***Pleurotus sajor-caju***
Moisture (%)	64.00	55.00
pH	7.70	6.80
Total Nitrogen (%)	1.8	1.70
Total Carbon (%)	33.2	42
Sodium (ppm)	105.00	48.00
Potassium (ppm)	275.00	134.00
Calcium (ppm)	1045.00	896.00

4.3 Biological properties of spent substrate of different mushrooms

4.3.1 Macroscopic and microscopic studies of potential fungi isolated from spent mushroom substrate

Both the potential fungal isolates (DMRF-7 and DMRF-8) were examined for their morphological and microscopic characteristics. Morphologically, the mycelial colony of isolate DMRF-7 appeared white initially and turned blakish with time when grown on malt extract agar plates. The mycelial colony of isolate DMRF-8 appeared white coloured initially and then quickly became white thick covering on the malt extract agar plates (Fig. 4.7).

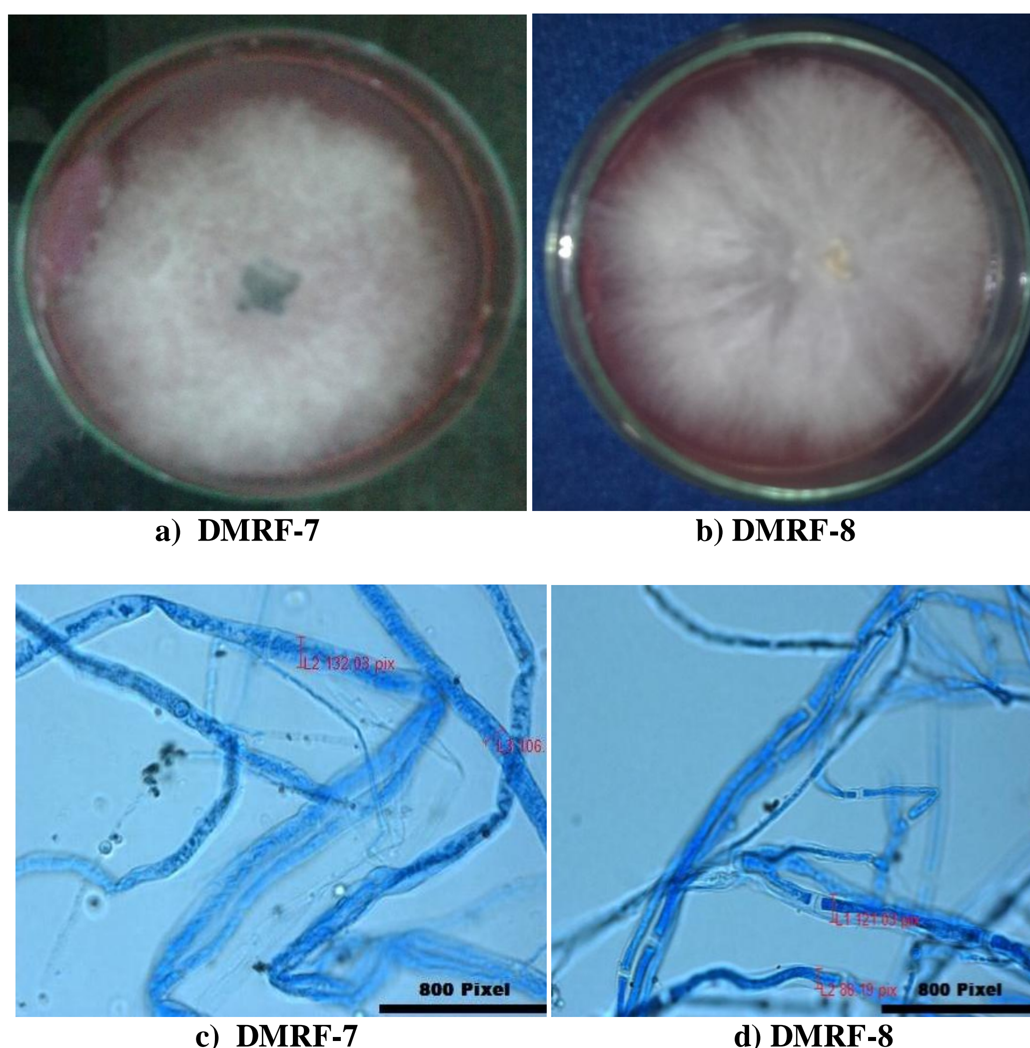


Fig. 4.7 Mycelial growth features of potential fungi: a) DMRF-7; b) DMRF-8; Microscopic features: c) DMRF-7; d) DMRF-8

Microscopically, the mycelium of isolate DMRF-7 appeared inamyloid, hyaline, and cylindrical in shape (Fig 4.7), whereas mycelium of DMRF-8 appeared septate with clamp connection.

4.3.2 PCR amplification of ITS region of 5.8S rRNA gene of fungal isoaltes

After critical examination of both morphological as well as microscopic characteristics of two potential fungal isolates isolated from SMS, all eight fungal isolates were also identified using molecular tools like sequencing of the ITS regions of their 5.8S r RNA genes. The ITS region of 5.8S rDNA from genomic DNA of all eight isolates (DMRF-1, DMRF-2, DMRF-3, DMRF-4, DMRF-5, DMRF-6, DMRF-7 and DMRF-8) was amplified by the PCR using ITS1 and ITS4 primers (White *et al*., 1990). Resultant PCR amplicons were viewed after electrophoresis in agarose gel. The results showed that the ITS amplicons of fungal isolates produced a single band of approximate 600-700 bp (Fig 4.8). The eight fungal isolates were found to belong to 5 different species (Table 4.4). The three isolates were identified as *Aspergillus fumigatus* (DMRF-1, DMRF-2 and DMRF-6), while the rest were identified as *Paecilomyces variotii*, *Pichia guilliermondii Schizophyllum commune* and *Pezizomycotina* sp. (Table 4.4).

Spent substrate from three mushrooms varied both quantitatively as well as qualitatively with respect to fungal population. Spent substrate from *Pleorotus sajor-caju* harbored highest fungal population, dominated by *Aspergillus fumigatus*, followed by spent substrate of *Agaricus bisporus*, harbored next highest population exclusively dominated by *A. fumigatus*. Spent substrate from *Volvariella volvacea* although harbored three dominating fungi like spent substrate of *P. sajor-caju*, but it exhibited two very distinct fungi (*Paecilomyces variotii* and *Pichia guilliermondii*) along with a common fungus (*A. fumigatus*) (Table 4.5).

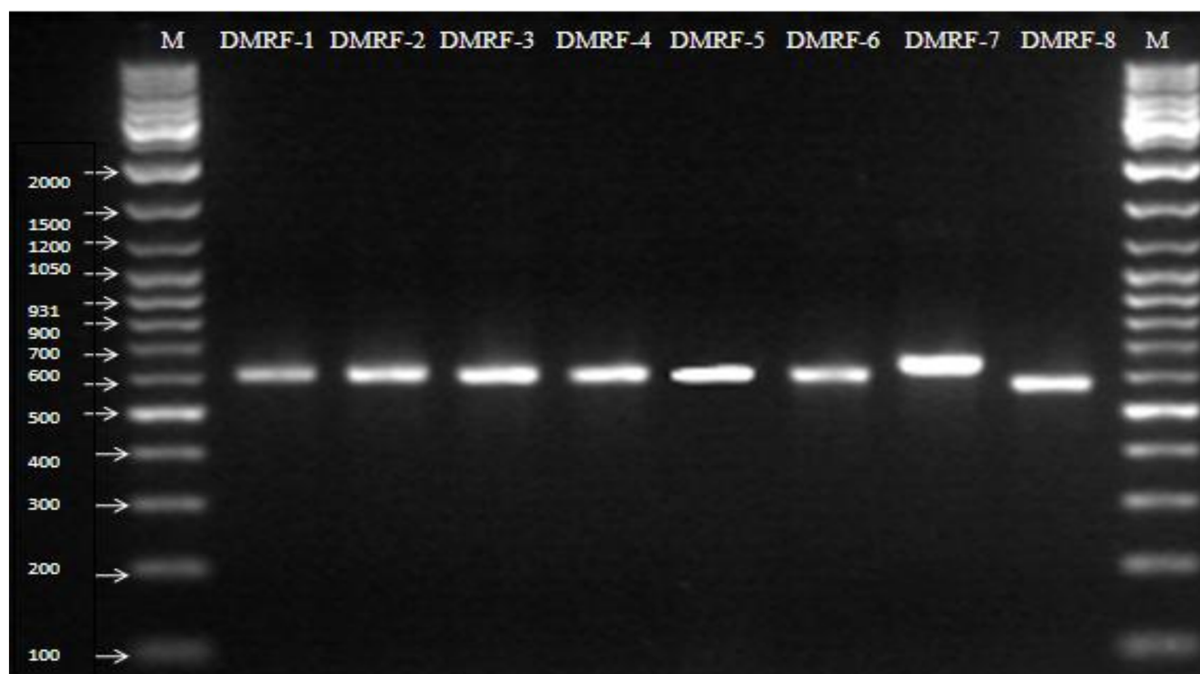


Fig. 4.8 ITS profile of isolated fungal isolates: 1) DMRF-1; 2) DMRF-2; 3) DMRF-3; 4) DMRF-4; 5) DMRF-5; 6) DMRF-6; 7) DMRF-7; 8) DMRF-8

Table 4.4 Identification of fungal isolates inferred from sequencing of ITS region of 5.8S rRNA gene by BLAST

Name of isolates	Maximum score	Maximum identity	Query coverage	Description name	Gene bank accession Number
DMRF-1	1098	100%	100%	*Aspergillus fumigatus*	KC237291
DMRF-2	977	100%	100%	*Aspergillus fumigatus*	KC237292
DMRF-4	1086	99%	100%	*Paecilomyces varioti*	KC237293
DMRF-5	1127	100%	100%	*Pichia guilliermondii*	KC237294
DMRF-6	881	100%	100%	*Aspergillus fumigatus*	KC237295
DMRF-7	1149	99%	99%	*Schizophyllum commune*	KC237296
DMRF-8	1109	100%	100%	*Pezizomycotina* sp.	KC237297

Table 4.5 Fungi isolated from spent substrate of different mushrooms

Spent substrate	Fungal isolates
Agaricus bisporus CFU[(5x10^4)/ml]	DMRF-1
Pleurotus sajor-caju CFU[(14.5x10^4)/ml]	DMRF-2
	DMRF-7
	DMRF-8
Volvariella volvacea CFU[(4x10^4)/ml]	DMRF-6
	DMRF-4
	DMRF-5

4.3.3 Evolutionary relationship of spent mushroom substrate fungal isolates DMRF-7 and DMRF-8

The blasting of the sequences of ITS region of 5.8S rRNA gene from two fungal isolates, DMRF-7 and DMRF-8 using NCBI BLAST(n) tool helped them to be identified as *Schizophyllum commune* and *Pezizomycotina* sp., respectively. The ITS sequences of these two fungi were funther aligned against other closely matching ITS sequences of *Schizophyllum commune* (AM493689.1, FJ426395.1, JX848644.1 and HQ331059.1) and *Pezizomycotina* sp. (GU212422.1, GU212416.1, HQ607902.1 and EU003047.1) available in NCBI genebank using Mega 5.05 software. The resultant phylogenetic tree was analysed for evolutionary relationship between different isolates. The isolate DMRF-7 (*S. commune*) showed a bootstrap value of 99 along with other four *S. commune* isolate ITS sequences obtainded from NCBI genebank, proving the closeness of the present isolate to the earlier reported isolates of this fungus. The other isolate DMRF-8 (*Pezizomycotina* sp.) again showed a bootstrap value of 98 with the ITS sequences of other related fungi or of *Pezizomycotina* sp. obtained from NCBI genebank, elucidating the close relationship of present isolate with other isolates of same or related species, sequences of which are already available in public domain (Fig. 4.9).

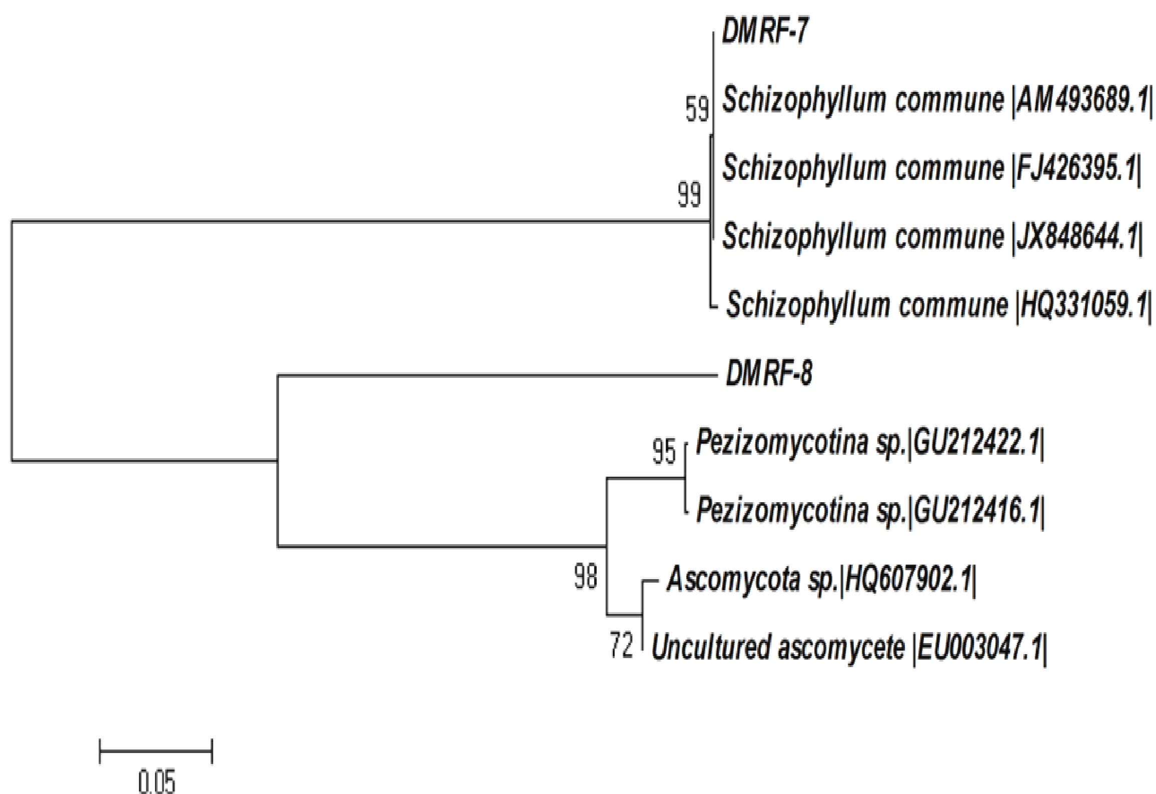


Fig. 4.9 Maximum parsimony phylogenetic tree derived from ITS sequences of 5.8S rRNA gene of potential fungal isolates from spent mushroom substrate of *P. sajor caju* and related sequences obtained from NCBI genebank

4.3.4 Isolation of bacteria from SMS of different mushrooms and their identification

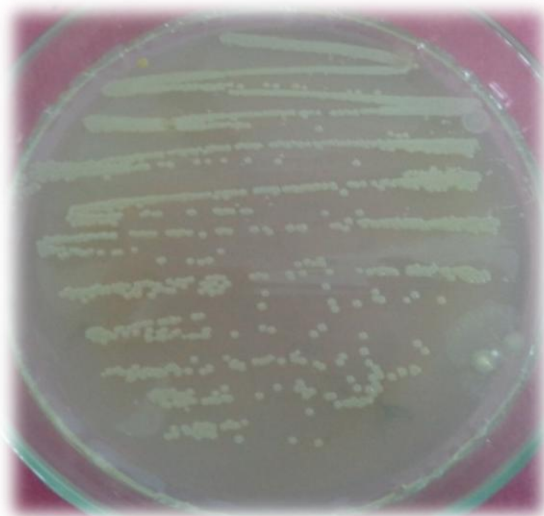
The collected spent mushroom substrate samples of *Agaricus bisporus*, *Pleurotus sajor-caju* and *Volvariella volvacea* were found to contain 16.1×10^7, 35.7×10^7 and 29.6×10^7 cfu (colony forming unit) in 1.0 g of fresh SMS, respectively (Table 4.6). All the isolates were sub-cultured at least thrice to confirm their purity. These isolates were designated as DMRB-1 to DMRB-11.

Table 4.6 Bacterial population in spent substrate of different mushrooms

Spent substrate	Bacteria Isolates
A. bisporus CFU[(16.1×10^7)/ml]	DMRB-3
	DMRB-1
	DMRB-6
	DMRB-7
P. sajor-caju CFU[(35.74×10^7)/ml]	DMRB-2
	DMRB-4
	DMRB-9
	DMRB-10
	DMRB-11
V. Volvacea CFU[(29.6×10^7)/ml]	DMRB-3

4.3.4.1 Biochemical characterization of spent mushroom substrate bacteria

All the bacterial isolates were screened for their dye decolourisation potential and the isolates DMRB-1, DMRB-2 and DMRB-10 showing highest dye decolourisation potential were characterized for their morphological, physiological and biochemical characteristics. Isolates DMRB-1 and DMRB-2 showed positive reaction to Gram stain, while isolate DMRB-10 showed negative reaction (Table 4.7a). Colony margins of the isolates were entire with raised elevation and the cells were rod shaped (Fig. 4.10). Isolates DMRB-1 and DMRB-2 showed presence of endospores, while isolate DMRB-10 showed its absence. The cells of isolates DMRB-1 and DMRB-10 showed mobility, while it was absence in isolate DMRB-2. All isolates were aerobic in their mode of growth. Optimum growth temperature in all three isolates was 25 to 37 $^{\circ}$C, while the pH requirement varied from 5.0 to 11.0 in isolates DMRB-1 and DMRB-10 and 5.0 to 8.0 in isolate DMRB-2 (Table 4.7 b). All isolates were positive for catalase, oxidase and gelatin hydrolysis. Only isolate DMRB-1showed positive reaction for methyl red and Vogas Proskauer tests. Acid production was noticed in all three isolates in presence of sorbitol, xylose, mannose and cellobiose. It was also recorded in presence of fructose, sorbitol and raffinose in case of isolates DMRB-1 and DMRB-10. Isolate DMRB-1 also produced acid in presence of salicin (Table 4.7 b).



DMRB-10

DMRB-2

DMRB-1

Fig. 4.10 Colony morphology of potential bacterial isolates

Table 4.7a Physiological characteristic of the potential bacterial isolates having dyes decolourisation potential

Test	**DMRB-1**	**DMRB-2**	**DMRB-10**
Colony Morphology			
Configuration	Circular	Circular	Flat irregular
Margin	Entire	Entire	Entire
Elevation	Raised	Raised	Raised
Surface	Rough	Rough	Irregular
Pigment	Off-white	Off-white	White yellow
Opacity	Opaque	Opaque	Opaque
Gram’s reaction	Positive	Positive	Negative
Cell shape	Rods	Rods	Rods
Spore (s)	+	+	–
Motility	Motile	Non-Motile	Motile
Anaerobic Growth	–	–	–

+ = Presence
– = Absent

Table 4.7b Biochemical characteristic of the potential bacterial isolates having dyes decolourisation potential

Growth at temperature	DMRB-1	DMRB-2	DMRB-10
4 °C	–	–	+
25 °C	+	+	+
30 °C	+	+	+
37 °C	+	+	+
42 °C	+	–	+
55 °C	–	–	–

Growth at pH	DMRB-1	DMRB-2	DMRB-10
pH 5.0	+	+	+
pH 6.0	+	+	+
pH 7.0	+	+	+
pH 8.0	+	+	+
pH 9.0	+	–	+
pH 10.0	+	–	+
pH 11.0	+	–	+
pH 11.0	+	–	–

Biochemical Test	DMRB-1	DMRB-2	DMRB-10
Methyl red test	+	–	–
Vogas Proskauer test	+	–	–
Indole	–	–	–
Gelatin hydrolysis	+	+	+
Starch hydrolysis	–	–	–
Catalase test	+	+	+

Acid Production from	DMRB-1	DMRB-2	DMRB-10
Sorbitol	+	+	+
Xylose	+	+	+
Mannose	+	+	+
Cellobiose	+	+	+
Fructose	+	–	+
Sorbitol	+	–	+
Raffinose	+	–	+
Salicin	+	–	–
Dulcitol	–	–	–

Where + = Presence

– = Absent

4.3.4.2 Identification of bacterial isolates isolated from SMS of three mushrooms using 16S rDNA sequencing technique

The eleven isolates (DMRB-1 to DMRB-11) were identified by using BLAST (n) software of NCBI using the partial sequences of 16S rDNA and were identified as *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis*, *Sphingobacterium multivorum*, *Sphingobacterium multivorum*, *Bacillus subtilis*, *Rummeliibacillus stabekisii*, *Pseudomonas fluorescens* and *Pseudomonas fluorescens*, respectively (Table 4.8). The potential isolate DMRB-1 sequence showed 100 % identity to accession numbers JX912979.1, HF586420.1, JQ283972.1 and JX841107.1 of *Bacillus pumilus*, whereas, DMRB-2 closely matched to accession numbers KC441869.1, KC441823.1, KC441817.1 and KC441863.1 of *Bacillus licheniformis* and DMRB-10 was identical to accession numbers FN666563.1, GU198111.1, JN872543.1, JN872541.1 and JN872539.1 of *Pseudomonas fluorescens* (Table 4.8).

Table 4.8 Identification of bacterial isolates inferred from 16S rRNA gene sequences by BLAST

Name of isolates	Maximum score	Maximum identity	Query coverage	Description name	Gene bank accession Number
DMRB-1	1541	100%	100%	*Bacillus pumilus*	KC237298
DMRB-2	1533	100%	100%	*Bacillus licheniformi*	KC237299
DMRB-3	1411	99%	100%	*Bacillus pumilus*	KC237300
DMRB-4	1552	100%	100%	*Bacillus subtilis*	KC237301
DMRB-6	1452	98%	100%	*Sphingobacterium multivorum*	KC237302
DMRB-7	1410	98%	100%	*Sphingobacterium multivorum*	KC237303
DMRB-8	1567	100%	100%	*Bacillus subtilis*	KC237304
DMRB-9	1544	100%	100%	*Rummeliibacillus stabekisii*	KC237305
DMRB-10	1537	100%	100%	*Pseudomonas fluorescens*	KC237306
DMRB-11	1519	100%	100%	*Pseudomonas fluorescens*	KC237307

Spent substrate of *P. sajor-caju* did harbor highest population as well as diversity of bacteria comprised of three different Species (*B. licheniformis*, *B. subtilis*, *Rummeliibacillus stabekisii* and *Pseudomonas fluorescens*) compared with spent substrate of *A. bisporus* and *V. volvacea*, which harbored three and one species, respectively (Table 4.6). Spent substrate of *V. volvacea*, harboured second highest population, dominated by only one species (*B. pumilus*).

4.3.4.3 Evolutionary relationship of potential bacterial isolates (DMRB-1, DMRB-2 and DMRB-10) isolated from spent mushroom substrate

The partial sequences of 16S rDNA from three potential bacterial isolates (DMRB-1, DMRB-2 and DMRB-10) were used for deducing the neighbour joining phylogenetic tree using Mega 5.05 software (Fig. 4.11). The tree could be differentiated into three different clusters each represented by isolate DMRB-1, DMRB-2 and DMRB-10, and the related isolates, sequences of which were retrieved from NCBI genebank and used for studying the evolutionary relationship with the present isolates. Isolate DMRB-2 as was identified as *Bacillus licheniformis* and formed a clade with bootstrap value of 100 with other *Bacillus licheniformis* isolates sequences of which were used as the reference sequences. The isolate DMRB-1 although identified as *Bacilus pumilus* but showed very low bootstrap value indicating a very diverse origin from the existing isolates, sequences of which are availbe in public domain. The isolate DMRB-10, actually identified as *Pseudomonas fluorescens* formed a clade with bootstrap value of 87 with other isolates of *Pseudomonas fluorescens*, sequences of which are already available in gene bank.

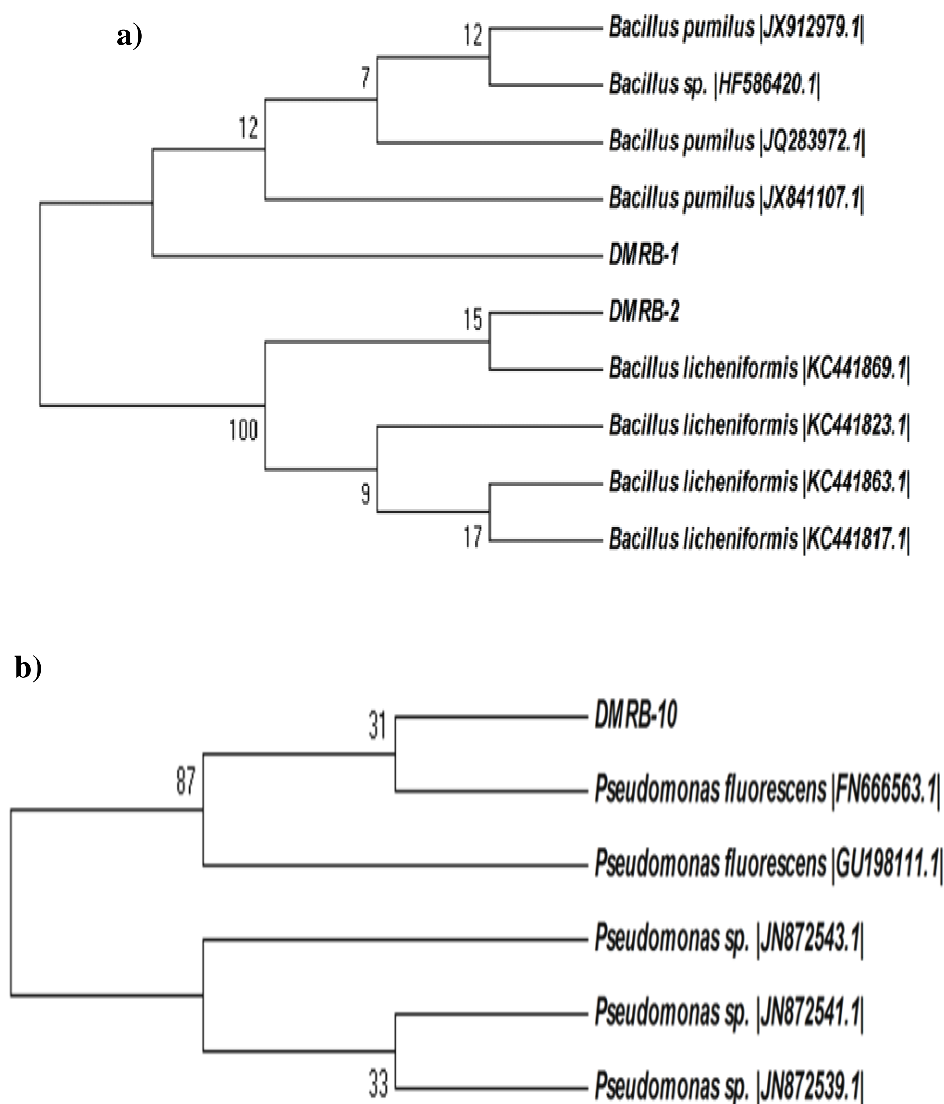


Fig.4.11 Maximum parsimony phylogenetic tree derived from 16S rRNA gene sequence of bacterial isolates of spent mushroom substrate (SMS) of different edible mushrooms showing genetic relationship with nucleotides sequences available in NCBI genebank a) DMRB-1 and DMRB-2; b) DMRB-10

4.4 Dye decolourisation by different fungi

In agar plate assay, *Schizophyllum commune* (DMRF-7) exhibited whole plate decolourisation against Chicago sky blue, Quinaldine red, Rhodamine B and Methyl violet 2B (Fig. 4.12). Similarly, under broth culture study, out of seven fungi from spent substrate of three different mushrooms, highest decolourisation of Chicago sky blue (95.0 %) after 12 days of incubation was with *Schizophyllum commune* isolated from spent substrate of *P. sajor-caju*, followed by *Pezizomycotina* sp. again from the

same SMS (Table 4.9). It was followed by three isolates of *A. fumigatus* isolated from three different spent mushroom substrates (33.6 to 49.0 %). Decolourisation was completely absent in *Pichia guilliermondii* isolated from spent substrate of *V. volvacea*. Decolourisation increased with time of incubation and become near static after 10 days of incubation. The potential dye decolourizing fungus, *Schizophyllum commune* was further evaluated for its decolourisation potential against 6 additional dyes for 18 day of incubation. This fungus exhibited significantly higher decolourisation of Starch azure (100%), followed by Reactive blue (92.5%), Rhodamine B (81.90%), Orange II sodium salt (73.40%) and Methylene blue (67.8%). Negligible decolourizarion was recorded in case of Azure B (Table 4.10).

4.5 Dye decolourisation by bacteria isolated from spent mushroom substrate of different mushrooms

Out of eight different bacteria studied for their dye decolourisation potential against Orange II sodium salt for 8 days of incubation, highest decolourisation (66.1%) after 8 days of incubation was with *B. licheniformis* isolated from *P. sajor-caju* SMS, followed by *B. pumilus* (58.0%) isolated from *V. volvacea* SMS (Table 4.11). Lowest decolourisation was with *B. subtilis* and *Rummelibacillus stabekisii* both isolated from *P. sajor-caju* SMS. The three isolates of *B. pumilus* isolated from SMS of three different mushrooms also varied in their dye decolourisation potential. In majority of the dyes, highest decolourisation was achieved up to 6 days of incubation, after which it remained almost static. The two potential bacteria, *B. licheniformis* and *B. pumilus* isolated from *P. sajor caju* SMS were restudied from their decolourisation potential against two more recalcitrant dyes (Azure B and Methylene blue) in agar plate (Fig. 4.13) as well as in broth culture, where in *B. pumilus* decolourized two dyes up to 44.6 and 91.3%, respectively (Fig. 4.14a). Decolourisation of these two dyes was comparatively lower (15.0 and 75.8%) in case of *B. licheniformis* (Table 4.12).

Table 4.9 Decolourisation of synthetic dye Methyl violet 2B with different fungi isolated from spent mushroom substrate

Fungi	Dye decolourisation (%) at different interval of time (days)						
	0th	**2nd**	**4th**	**6th**	**8th**	**10th**	**12th**
Aspergillus fumigatus (Ab)	0.00	6.70	20.9	28.70	30.30	31.80	33.61
Aspergillus fumigatus (Vv)	0.00	12.31	37.0	41.80	43.72	47.10	49.00
Paecilomyces variotii (Vv)	0.00	0.00	11.26	25.70	27.30	29.44	29.73
Pichia guilliermondii (Vv)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aspergillus fumigatus (Ps)	0.00	11.0	32.10	36.00	34.95	36.20	40.54
Schizophyllum commune (Ps)	0.00	0.00	60.40	79.80	90.77	95.30	95.00
Pezizomycotina sp. (Ps)	0.00	0.00	53.50	54.70	73.80	87.80	92.00
$CD_{0.05}$	-	0.096	0.064	0.075	0.141	0.039	0.081

Name given in parentheses are of abbreviated form of mushroom, SMS of which the bacteria were isolated; Ab- *Agaricus bisporus*; Vv- *Volvariella volvacea*; Ps- *Pleurotus sajor-caju*

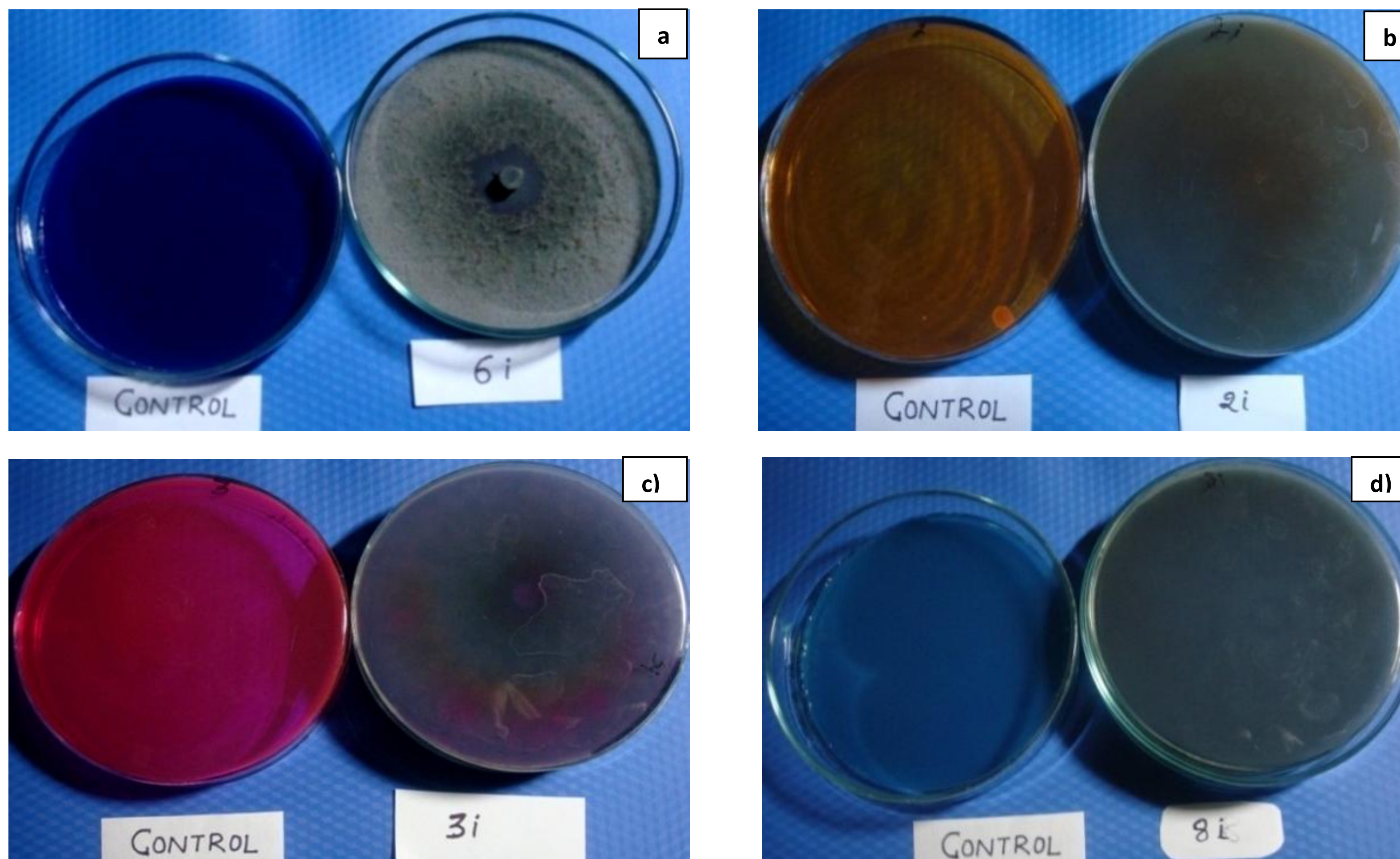


Fig 4.12 Agar Plate Assay: a) Decolourisation of Chicago sky blue in agar plate by *Schizophyllum commune*; b) Decolourisation of Quinaldine red by *S. commune*; c) Decolourisation of Rhodamine B by *S. commune*; d) Decolourisation of Methyl violet 2B by *S. commune*

Table 4.10 Decolourisation of different dyes by promising fungus *Schizophyllum commune* isolated from spent substrate of *Pleurotus sajor-caju*

Dye	Dye decolourisation (%) at different interval of time (days)						
	0th	**2nd**	**4th**	**6th**	**8th**	**10th**	**12th**
Methylene blue	0.00	0.00	15.9	29.6	46.2	67.2	67.8
Orange II sodium salt	0.00	0.00	16.9	34.4	59.7	73.1	73.4
Rhodamine B	0.00	0.00	13.6	14.3	18.6	27.8	81.6
Chicago sky blue	0.00	0.00	2.2	2.5	2.2	2.2	2.2
Azure B	0.00	0.00	4.1	4.1	4.1	4.0	4.0
Methyl violet 2B	0.00	0.00	20.5	22.5	44.6	81.0	92.5
Starch Azure	0.00	0.00	100	100	100	100	100
$CD_{0.05}$	--	--	0.075	0.041	0.069	0.060	0.079

Table 4.11 Decolourisation of synthetic dye Methyl violet 2B with different bacteria isolated from spent mushroom substrate of three mushrooms

Bacteria	Dye decolourisation (%) at different interval of time (days)				
	0th	2nd	4th	6th	8th
Bacillus pumilus (Vv)	0.00	0.00	48.4	57.7	58.0
Sphingobacterium multivorum-1 (Ab)	0.00	10.0	30.4	32.9	33.0
Bacillus pumilus (Vv)	0.00	22.0	28.6	44.7	45.9
Sphingobacterium multivorum-2 (Ab)	0.00	23.1	40.5	41.0	42.1
Rummelibacillus stabekisii (Ps)	0.00	12.7	15.9	11.6	10.0
Bacillus pumilus (Ab)	0.00	26.8	40.3	40.2	40.0
B. licheniformis (Ps)	0.00	17.3	17.8	62.7	66.1
B. subtilis (Ps)	0.00	1.1	1.3	1.4	1.4
$CD_{0.05}$	-	0.146	0.228	0.129	0.109

Name given in parenthesis is of abbreviated form of mushroom, SMS of which the bacteria were isolated; Ab- *Agaricus bisporus*; Vv- *Volvariella volvacea*; Ps- *Pleurotus sajor-caju*

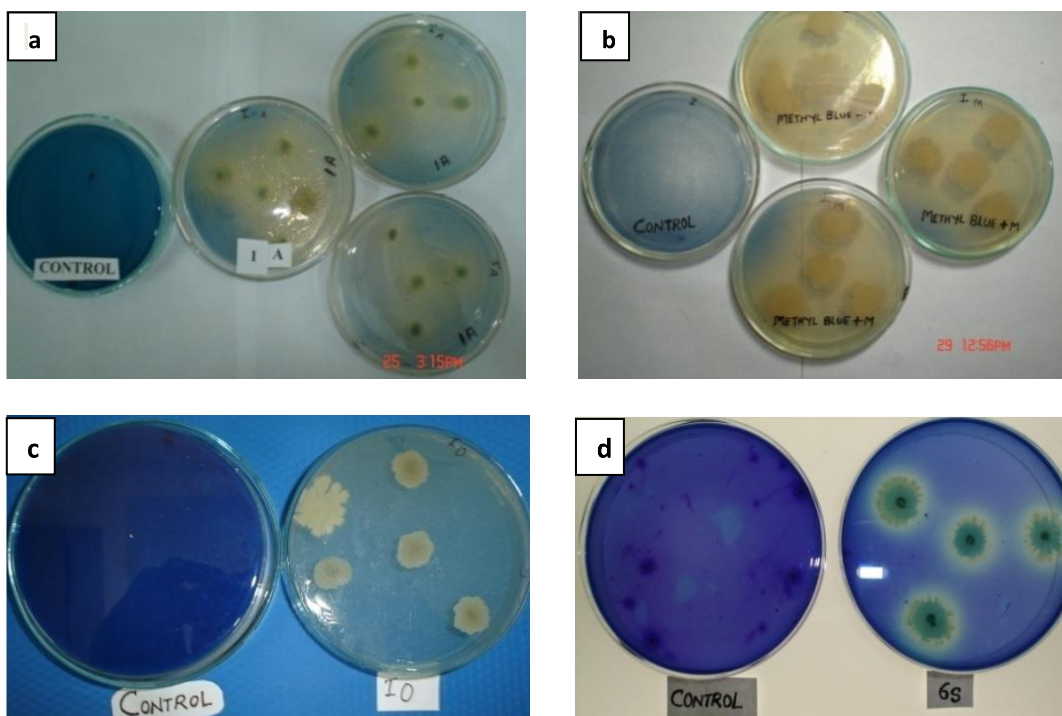


Fig 4.13 Agar Plate Assay: a) Partial decolourisation of Methylene blue by *Bacillus pumilus* isolated from spent substrate of *Volvariella volvacea*; b) Whole plate decolourisation of Azure B by *Sphingobacterium multivorum*; c) Decolourisation of Methylene blue by *Bacillus licheniformis*; d) Decolourisation of Methylene blue by *Bacillus pumilus*

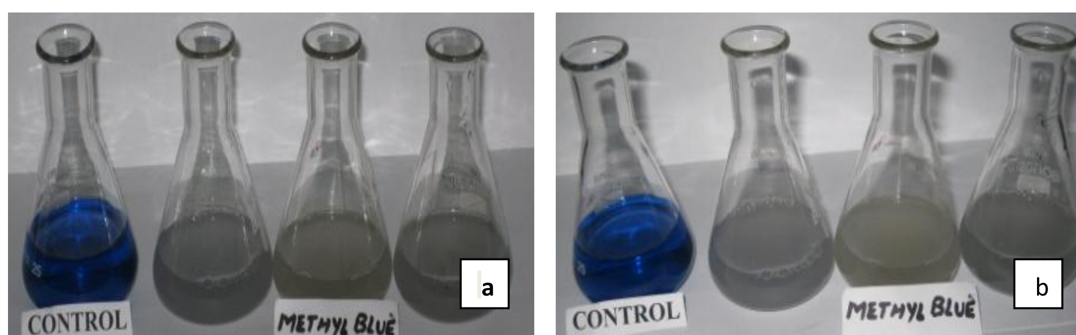


Fig. 4.14 a) Decolourisation of Methylene blue by *Bacillus licheniformis*; b) decolourisation of Methylene blue by *B. pumilus* isolated from spent substrate of *P. sajor-caju*

4.6 pH and temperature optima for dye decolourisation with *B. licheniformis*

The most efficient dye decolourizing bacterium *B. licheniformis* was studied for its decolourisation potential against Orange II Sodium salt at seven different pH levels *viz*., 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, where in highest decolourisation (85.6 and 81.1%) was recorded at pH levels of 6.0 and 7.0, followed by 8.0. Decolourisation was almost similar at pH 5.0 and 9.0, while lowest at 10.0 (Table 4.13). The decolourisation was almost negligible up to first 4 days of inoculation, which suddenly increased on day 6 of incubation.

Table 4.12 Decolourisation of Azure B and Methylene blue with two potential bacteria *B. licheniformis* and *B. pumilus*

Bacteria	Dye	Dye decolourisation (%) at different interval of time (days)				
		0-day	2-day	4-day	6-day	8-day
B. licheniformis	Azure B	0	1.7	24.2	25.1	25.0
	Methylene blue	0	9.8	43.1	52.9	75.8
B. pumilus	Azure B	0	2.5	36.4	40.3	44.6
	Methylene blue	0	43.7	58.0	66.0	91.3
$CD_{0.05}$		--	0.045	0.064	0.194	0.113

Table 4.13 Effect of pH of the growing medium on decolourisation of Orange II Sodium salt with potential bacterium, *B. licheniformis*

pH	Dye decolourisation (%) at different interval of time (days)				
	0-day	2-day	4-day	6-day	8-day
4.0	0	0	0	56.6	74.1
5.0	0	3	5.5	3.0	75.4
6.0	0	1.6	1.9	78.0	85.6
7.0	0	0	1.4	71.8	81.1
8.0	0	0	0	62.6	79.4
9.0	0	0	3.5	74.1	75.9
10.0	0	0	0	43.2	59.9
$CD_{0.05}$	--	0.026	0.053	0.089	0.064

Like pH, temperature of incubation has direct role is sustaining enzymatic activities and growth of different microorganisms in a cultural medium, which ultimately influences the dye decolourisation process. The temperature optima of *B. licheniformis* for decolourisation of Orange II Sodium salt was studied at 5 different temperatures *viz.*, 20, 25, 30, 35 and 40 °C. Highest decolourisation (62.2% and 61.7%) was recorded at 25 °C, closely followed by 20 °C. The decolourisation level decreased with increasing temperature of incubation (Table 4.14).

Table 4.14 Effect of temperature of incubation on decolourisation of Orange II sodium salt with potential bacterium, *B. licheniformis* isolated from spent substrate of *P. sajor-caju* under nutrient deficient conditions

Temperature (°C)	Dye decolourisation (%) at different interval of time (days)					
	0-day	2-day	4-day	6-day	8-day	10-day
20	0	33.9	45.7	57.7	58.4	61.7
25	0	35.9	51.3	60.0	58.4	62.2
30	0	43.3	53.4	56.0	53.0	51.0
35	0	48.3	43.3	54.7	50.0	45.0
40	0	49.6	49.0	44.1	39.0	35.0
$CD_{0.05}$	--	0.149	0.071	0.067	0.194	0.081

4.7 Screening of potential laccase producing fungal isolates

Screening for laccase producing fungi was done on petriplates using Potato dextrose agar (PDA) supplemented with 0.02% guaiacol (Vishwanath *et al.,* 2008; Wang *et al.,* 2010). Seven different fungi were inoculated in sterile petridishes containing the above medium separately and were incubated at 30 °C for a period of seven days. *Schizophyllum commune* and *Pezizomycotina* sp. showing definite colour change around growing mycelial colony were considered as laccase producing fungi (Fig. 4.15) and these were selected further for synthetic dyes decolourisation studies.

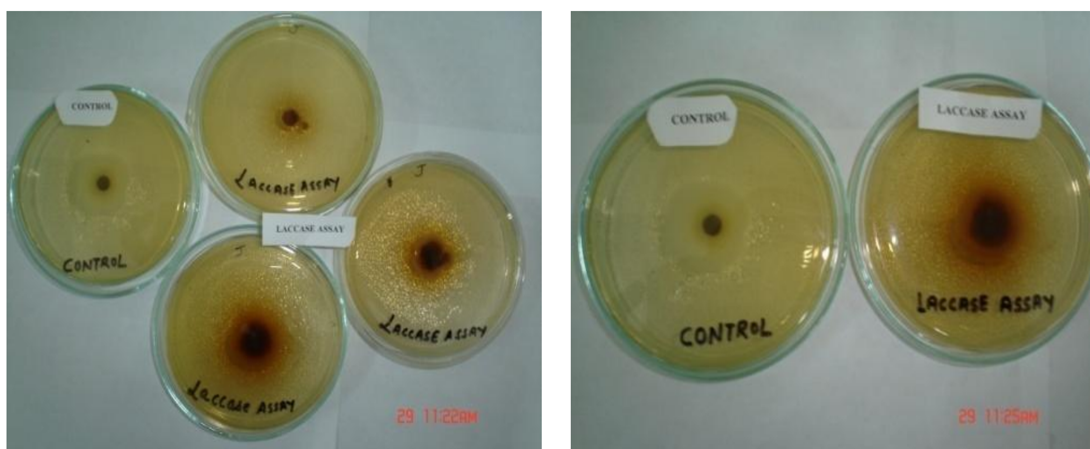


Fig. 4.15 Screening of fungi for laccase production potential: a) (*Schizophyllum commune*); b) (*Pezizomycotina* sp.) for laccase producing ability in PDA plate supplemented with guaiacol as enzyme substrate

4.7.1 Extracellular ligninolytic enzymes activity

Out of different fungi and bacteria isolated from SMS of different mushrooms, the two potential fungi and three bacteria were studied for their dye decolourisation potential based on their extracellular ligninolytic enzymes activity. Highest activity of laccase was recorded in *S. commune* (11.8 U mL^{-1}), followed by *Pezizomycotina* sp. (8.32 U mL^{-1}) in the natural medium containing wheat straw as the main substrate. The activity of laccase in three bacteria was almost at par with each other and nearly 3 to 4 folds lesser than the two fungi (Fig. 4.16 a). Contrary to this, the activity of Manganese peroxidase (MnP) was highest in *P. fluorescens* (513 U L^{-1}), followed by *B. licheniformis* (457 U L^{-1}) and *Pezizomycotina* sp. (417.5 U L^{-1}). Lignin peroxidase (LiP) activity was highest (339 U L^{-1}) in *S. commune*, followed by *Pezizomycotina* sp. (322 U L^{-1}) and *P. fluorescens* (318 U L^{-1}). Activity of LiP was lowest in *B. pumilus* (196 U L^{-1}) (Fig. 4.16 b).

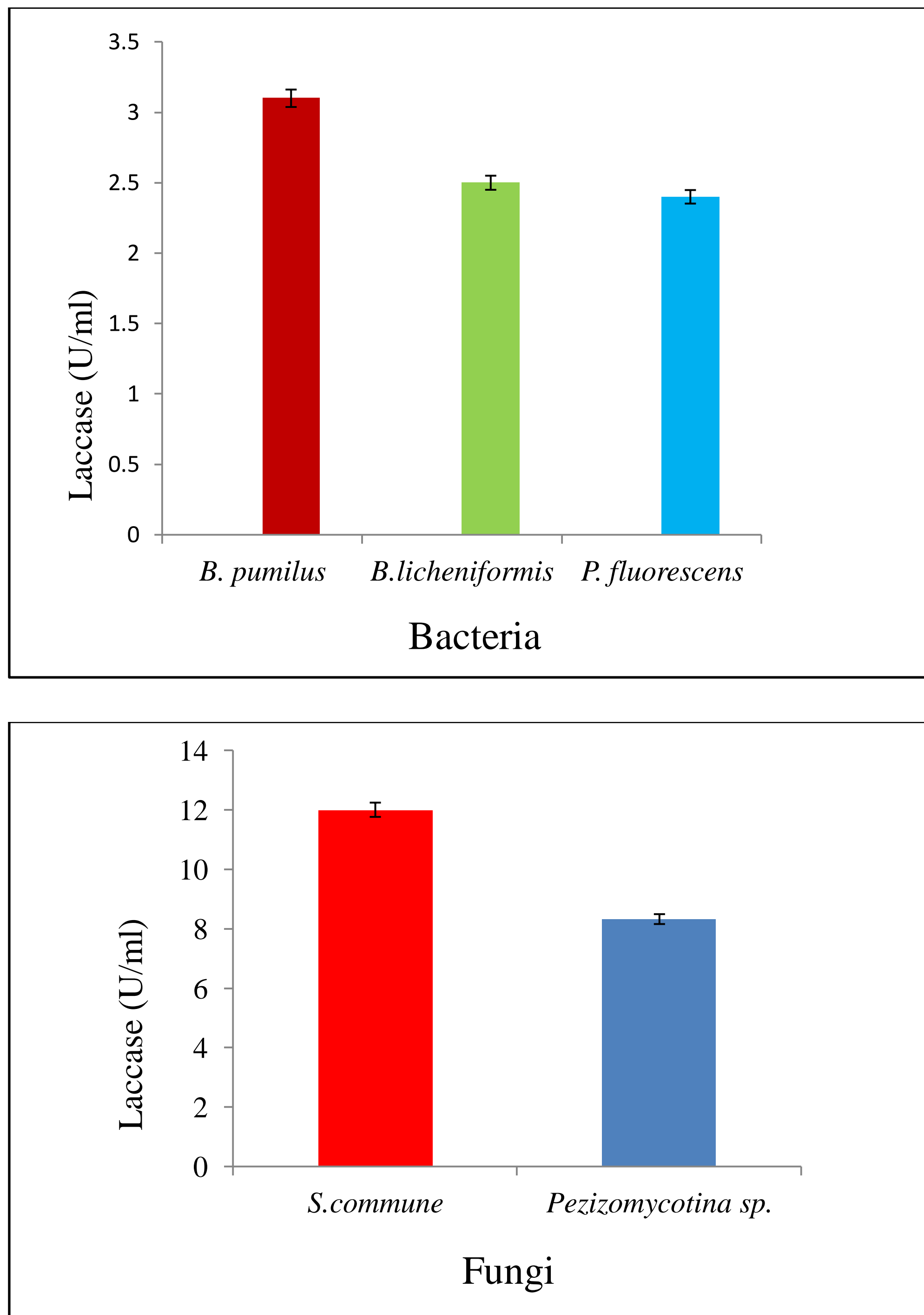


Fig.4.16a Laccase activity of potential fungi and bacteria isolated from spent substrate of different mushrooms

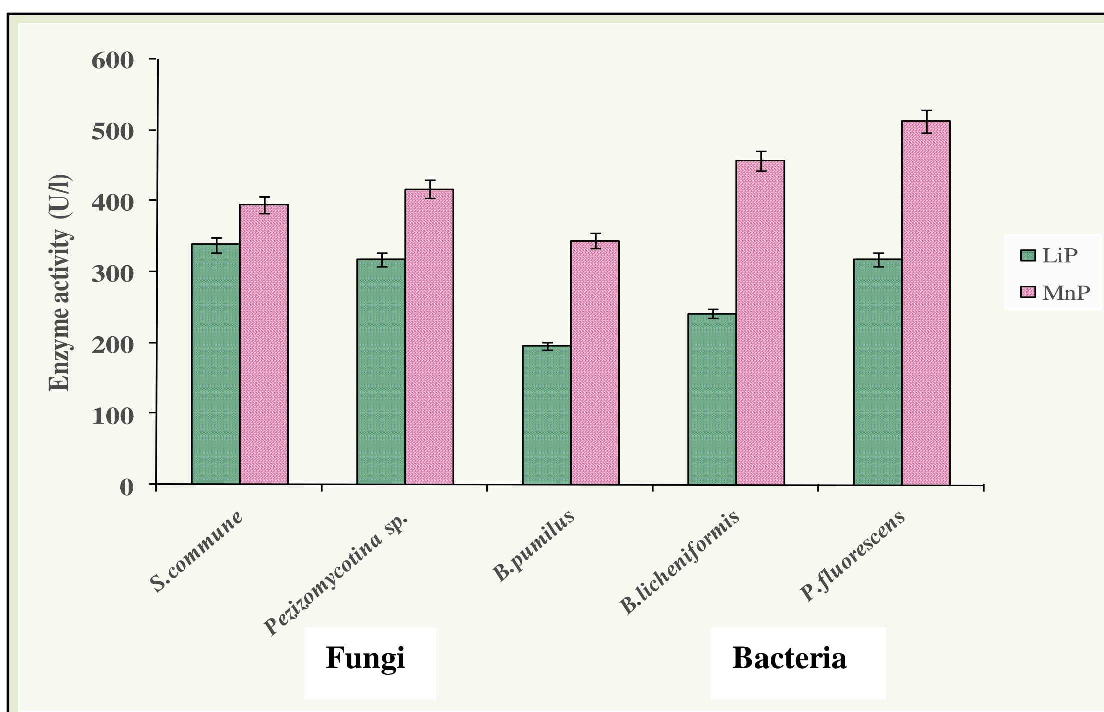


Fig. 4.16b Lignin peroxidase and Manganese peroxidase activity of potential bacteria and fungi isolated from spent substrate of different mushrooms

4.7.2 Extracellular ligninolytic enzyme activity in sub-merged conditions

The activities of three extracellular ligninolytic enzymes including LiP, MnP and Laccase, related to textile dyes decolourisation, were assayed under sub-merged conditions. The figure 4.17 depicts changes in the activities of MnP, LiP and Laccase during growth of different fungi in nutrient rich medium. *Schizophyllum commune* exhibited highest activity of all three enzymes compared with *Pezizomycotina* sp. The activity of MnP in *S. commune* (56 Uml^{-1}) was almost double to that in *Pezizomycotina* sp. (27 Uml^{-1}). The difference in activities of laccase and LiP was not that much between two fungi. Compared to other two enzymes, the activity of LiP was lowest in two fungi (Fig. 4.17). The study proves the higher utility of *S. commune* in dye decolourisation studies compared with *Pezizomycotina* sp.

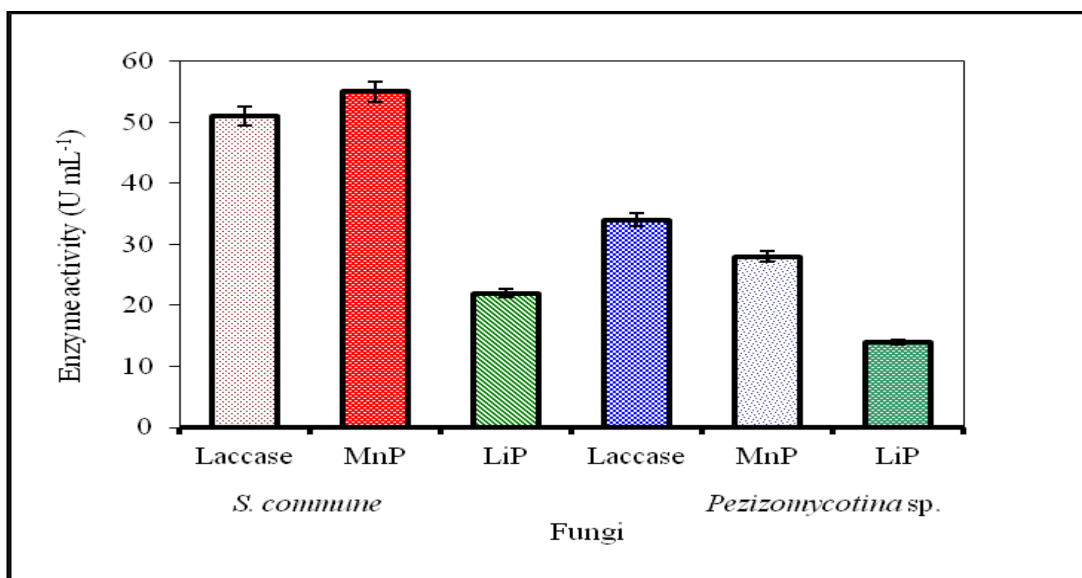


Fig. 4.17 Extracellular ligninolytic enzymes activity of potential dye decolourizing fungi, *S. commune* and *Pezizomycotina* sp.

4.7.3 Extracellular ligninolytic enzymes activity in SMS

Spent substrate of *P. sajor-caju* exhibited highest activity of MnP (55 UmL^{-1}), followed by laccase (47 UmL^{-1}). Lower activity of LiP was recorded as compared to other two enzymes in both the spent substrates (Fig. 4.18). Fungal ligninolytic enzymes are commercially in use in textile industry and have potential for more industrial applications. The present study envisages that out of three enzymes, activity of MnP was higher in two SMSs.

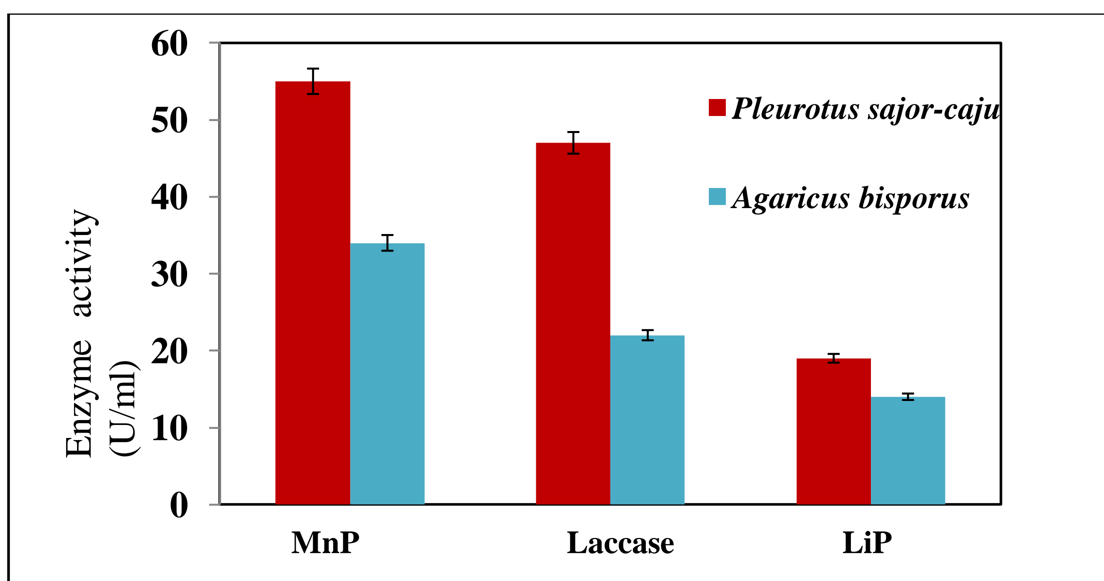


Fig. 4.18 Extracellular ligninolytic enzymes activity in spent substrate of *P. sajor-caju* and *Agaricus bisporus*

4.7.4 Dye decolourisation potential of crude enzymes extract from spent substrate of *Pleurotus sajor-caju*

Crude enzymes extract extracted out of the spent substrate of *P. sajor-caju* was used in different ratios with the dye supplemented malt extract broth to ascertain the effective ratio for decolourisation of different dyes. The ratios varied from 1: 4 to 1: 39 and out of these, the ratio of 1: 4 to 1: 6 exhibited highest decolourisation and the decolourisation reached up to 100% of Rhodamine B after 2 days of crude enzymes extract mixing in the dye supplemented malt extract broth (Fig. 4.19). In case of Chicago sky blue, the effective ratios were 1: 4, 1: 5 and 1: 6 and the decolourisation was 100% even after 1 day of crude enzymes extract mixing in the dye supplemented malt extract broth (Fig 4.20). Compared to other dyes, the starch azure exhibited higher decolourisation in wider range of ratios (1:4 to 1:9) between crude enzymes extract and dye supplemented malt extract broth and nearly 100% decolourisation was recorded after 2 days of incubation (Fig. 4.21). In Methyl violet 2B, effective ratios were 1:4 and 1:5, which resulted in 100% dye decolourisation after one day of crude enzyme extract mixing. It was followed by 1:6 ratio, which also exhibited 100% dye decolourisation after 2 days of extract mixing (Fig. 4.22).

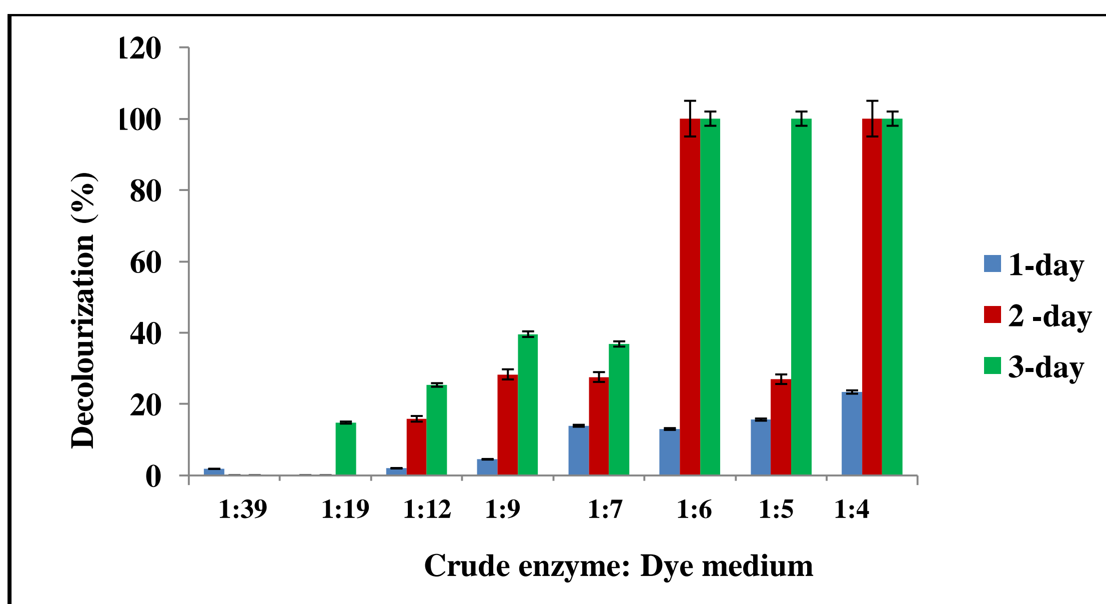


Fig 4.19 Effect of different ratios of crude enzyme extract from *P. sajor-caju* spent substrate with dye decolourizing medium on decolourisation of Rhodamine B

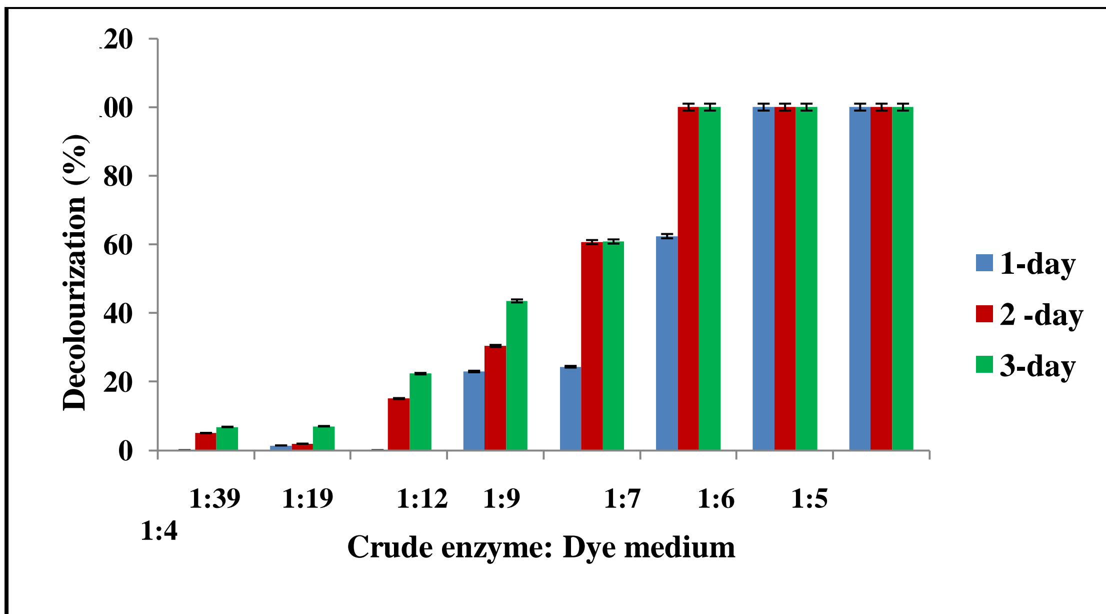


Fig. 4.20 Effect of different ratios of crude enzyme extract from *P. sajor-caju* spent substrate with dye decolourizing medium on decolourisation of Chicago sky blue

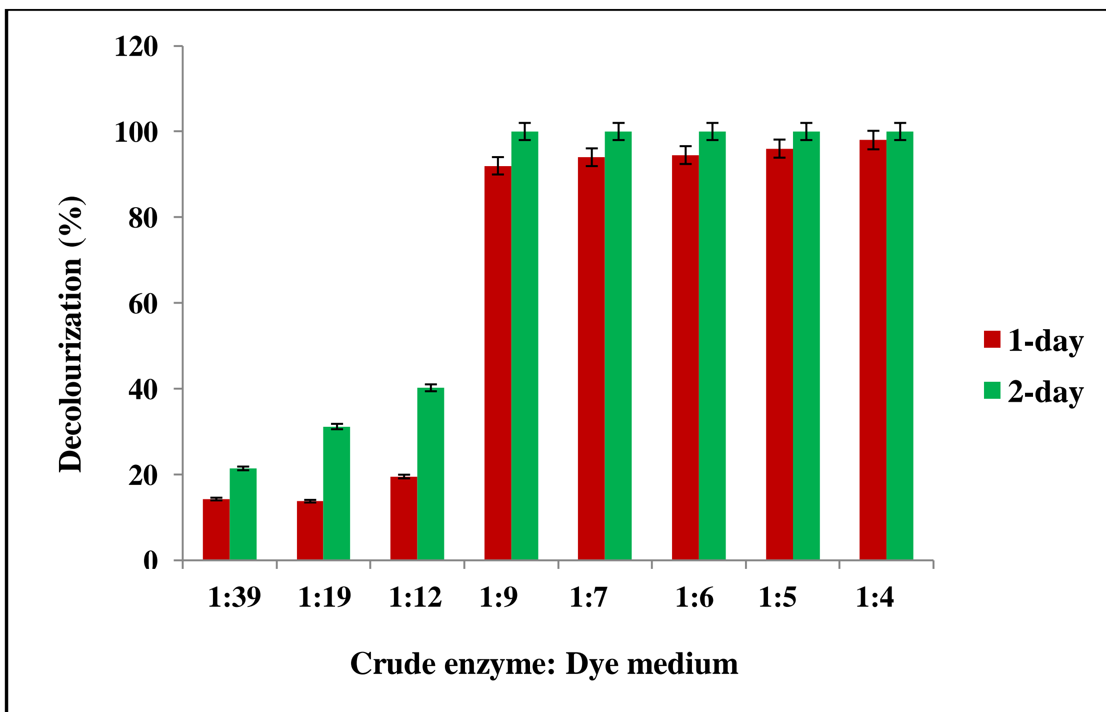


Fig 4.21 Effect of different ratios of crude enzyme extract from *P. sajor-caju* spent substrate with dye decolourizating medium on decolourisation of Starch azure

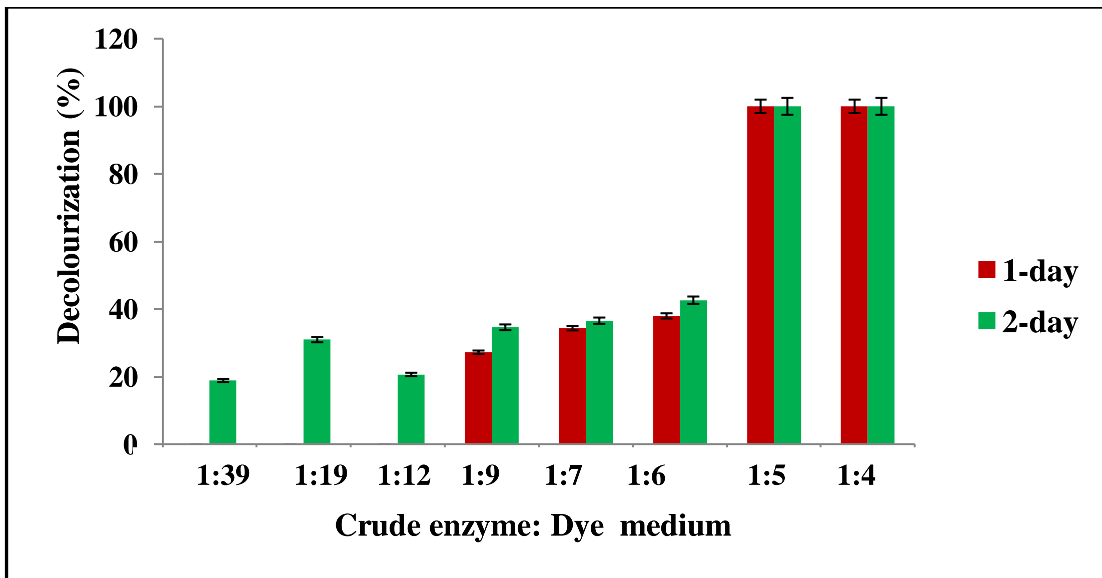


Fig 4.22 Effect of different ratio of crude enzymes extract from *P. sajor-caju* with dye decolourizing medium on decolourisation of Methyl violet 2B

Chapter-5

To Study the Role of Cultural Conditions on Dyes Decolourisation

5.1 Effect of initial concentrations of dyes on their decolourisation

5.1.1 Effect of different initial concentrations of Rhodamine B on its decolourisation with spent substrate of *P. sajor- caju*

Experiment conducted with different concentrations of Rhodamine B revealed that starting from day 1 to day 4 of spent substrate mixing; highest decolourisation (%) was in 25 and 50 ppm initial dye concentration. However, after 4 days of SMS mixing, the relationship was inversely proportional between initial dye concentration and its decolourisation, as it was near 100% in lowest concentration (25 ppm), while only 90% in highest dye concentration (150 ppm) (Fig. 5.1).

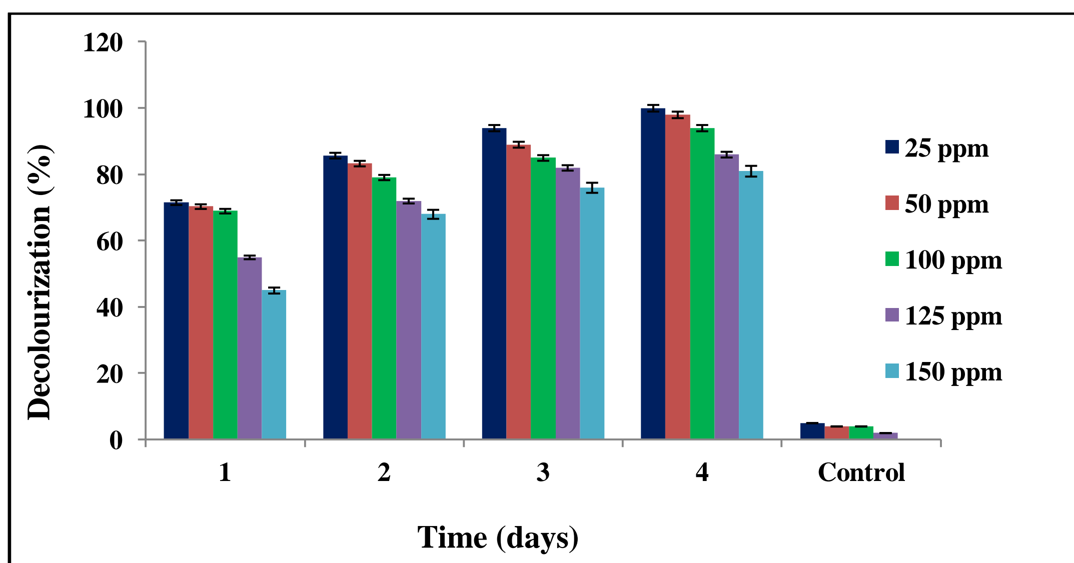


Fig. 5.1 Effect of initial concentration of Rhodamine B on its decolourisation w spent substrate of *P. sajor- caju*

5.1.2 Effect of different initial concentrations of Azure B on its decolourisation with spent substrate of *P. sajor- caju*

In case of Azure B, the role of initial dye concentration was quite significant and from day 1, it was highest in treatment with lowest initial dye concentration (25

ppm), followed by next higher initial dye concentration (50 ppm) and so on (Fig 5.2). On completion of the experiment, it was significantly higher in the lowest initial dye concentration than the highest initial dye concentration (150 ppm). In case of Azure B, the trend of dye decolourisation in different initial concentrations of dye was similar to that in Rhodamine B (Fig 5.2). The decolourisation at the end of day 5 was more than 2 folds higher in lowest initial dye concentration (25 ppm) than it was there in highest initial dye concentration (150 ppm).

5.1.3 Effect of different initial concentrations of Methyl violet 2B on its decolourisation with spent substrate of *P. sajor- caju*

Similar trend of decolourisation was recorded in case of Methyl violet 2B as in case of Rhodamine B and Azure B; highest in lowest initial dye concentration (25 ppm), which decreased with increasing initial concentration of dye upto 150 ppm. Control containing dye with plain wheat straw exhibited only 5-6% decolourisation of Methyl violet 2B (Fig. 5.3).

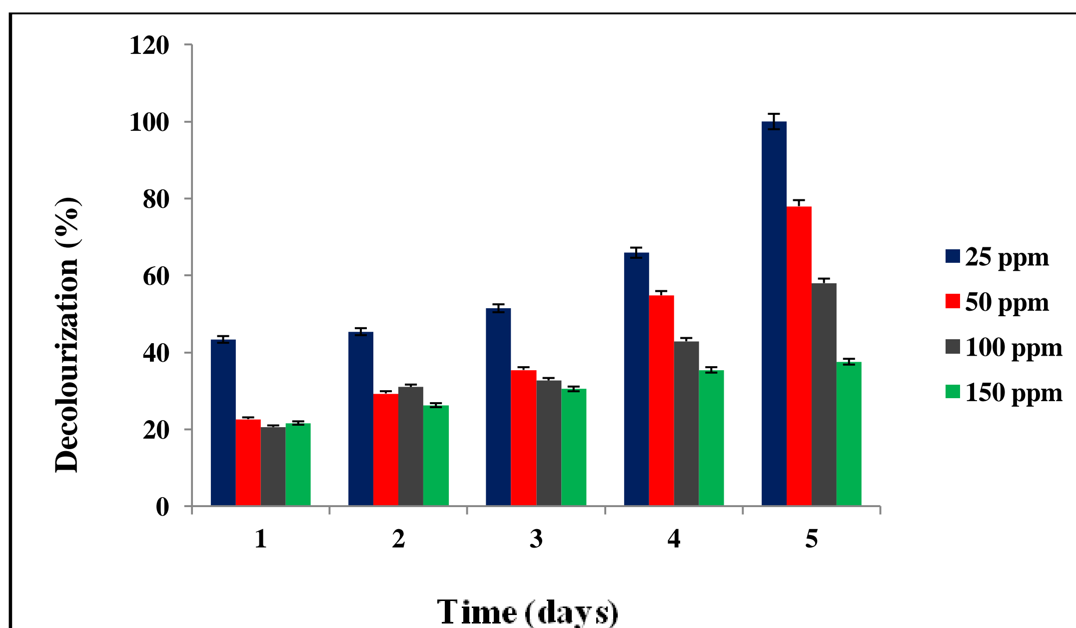


Fig. 5.2 Effect of initial concentration of dye on decolourisation of Azure B with spent substrate of *Pleurotus sajor-caju*

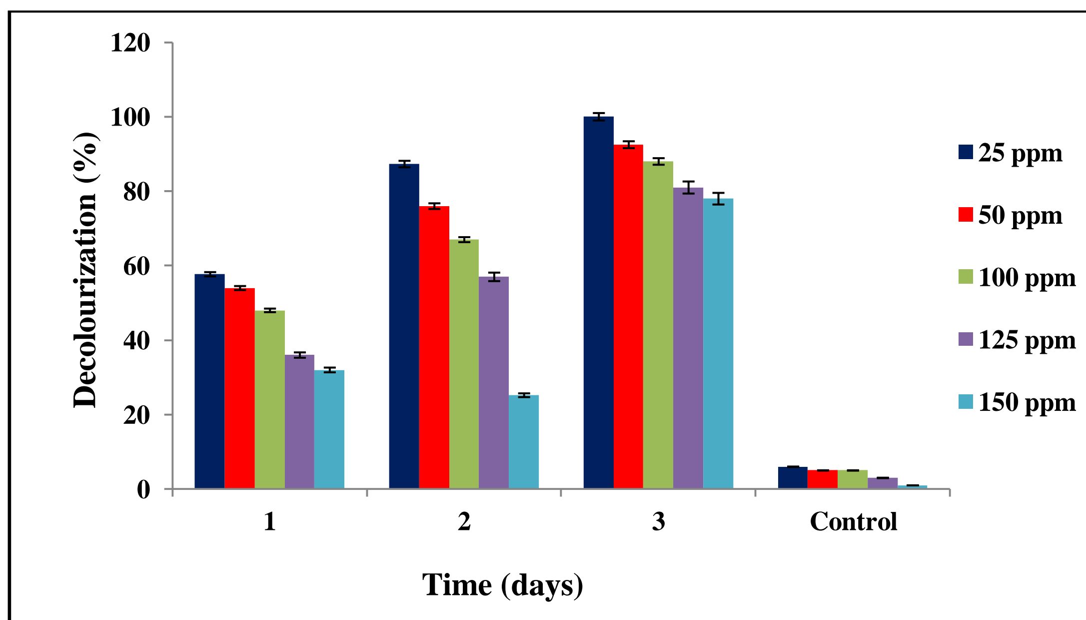
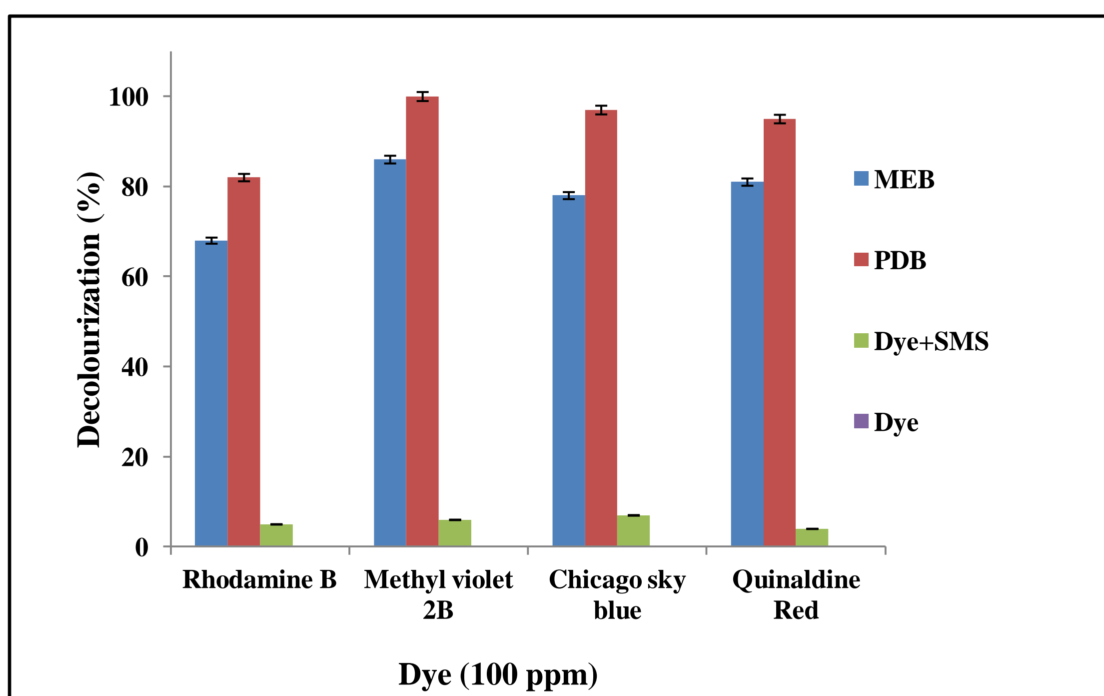


Fig. 5.3 Effect of initial concentration of dye on decolourisation of Methyl violet 2B with spent substrate of *Plerotus sajor-caju*

5.2 Effect of media on dyes decolourisation with spent substrate of *P. sajor-caju*

Three growth media *viz*., Potato dextrose broth, Malt extract broth and plain water were used to study the effect of media composition on decolourisation of four different dyes using spent substrate of *P. sajor-caju*. Highest decolourisation of Chicago Sky blue 6B (46 %) and Rhodamine B (17 %) after one day of SMS mixing was in Potato dextrose broth. Although decolourisation of dyes increased with time up to 4 days of incubation, however, major enhancement (Rhodamine B 82%, Methyl violet 2B, 100%, Chicago Sky Blue 6B, 97% and Quinaldine red, 95%) was recorded up to 3 days of incubation (Fig. 5.4). Preferences toward culture media remained same throughout the 5 days of incubation. In present study, difference in decolourisation of 4 dyes on using different culture media is attributed to variation in nutritional composition of three media; as this variation also contributes towards variation in their ability to sustain growth and multiplication of different types of microorganisms contributed by *P. sajor-caju* spent substrate. In some cases, higher decolourisation in

Potato dextrose broth can be ascribed to residual extracellular ligninolytic enzymes and microbes contributed by spent substrate, and least chances of enzymatic inhibition compared with other culture media. Dyes supplemented with spent substrate of *P. sajor-caju* in plain distilled autoclaved water showed only 4-7% decolourisation in all the 4 synthetic dyes. In second control (dyes in distilled autoclaved water without *P. sajor-caju* spent substrate inoculation), there was no decolourisation.



Where, MEB= Malt extract broth; PDB= Potato dextrose broth; SMS= Spent mushroom substrate

Fig. 5.4 Effect of culture medium on decolourisation of different dyes with spent substrate of *Pleurotus sajor-caju*

5.3 Effect of different carbon sources on decolourisation of Methyl violet 2B with spent substrate of *P. sajor-caju*

The data on effect of different carbon sources mixed in decolourisation medium on decolourisation of Methyl violet 2B is presented in figure 5.5. From the results it is evident that decolourisation of Methyl violet 2B at different stages was higher in presence of different carbon sources, except cellulose, where it was lesser than control. Decolourisation stimulatory effect was more pronounced at early stage

(after 1 and 2 days) of SMS mixing, which became lesser significant at later stages (after 3 and 4 days). At the end of experiment, sucrose and starch added media exhibited highest decolourisation of Methyl violet 2B. Highest decolourisation was recorded with sucrose (87.75%), followed by starch (87.48%), while only 80.29% in control.

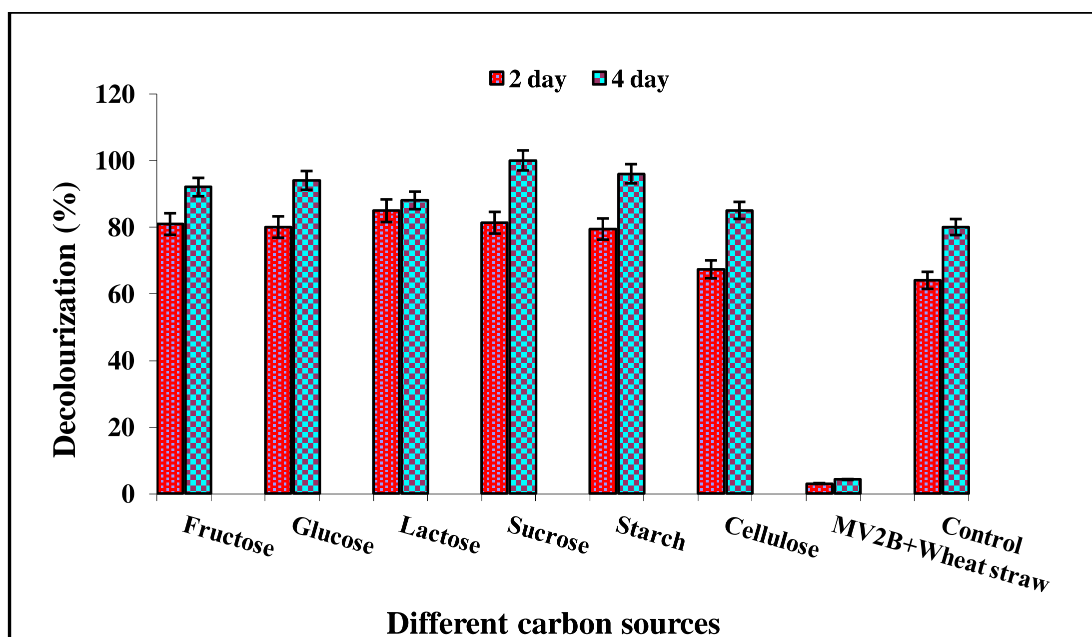


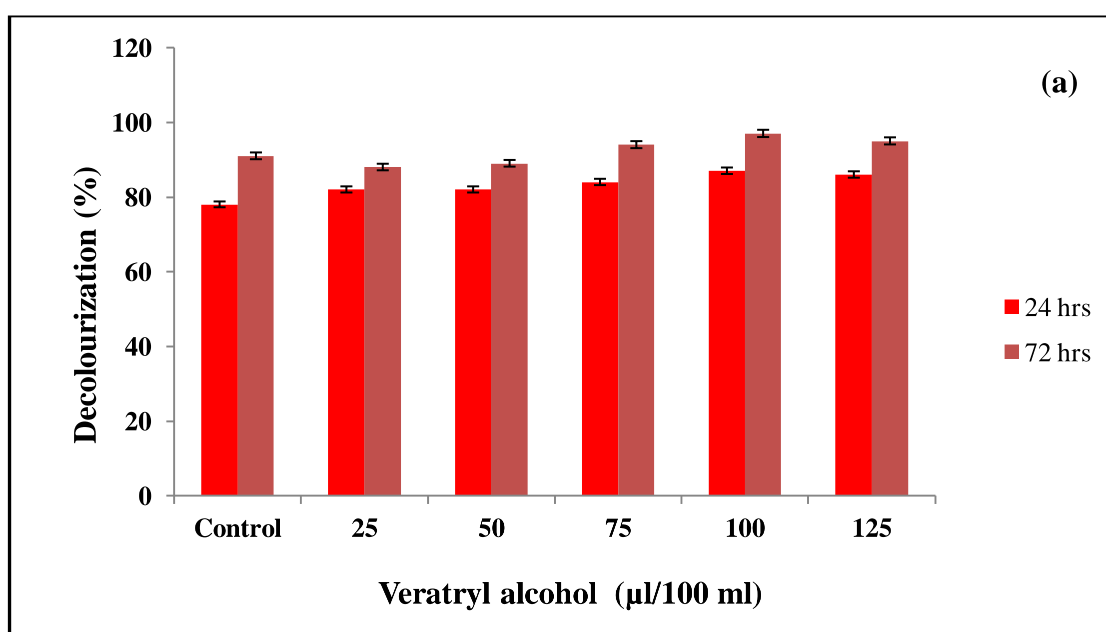
Fig. 5.5 Effect of different carbon sources in growing medium on decolourisation of Methyl violet 2B using spent substrate of *P. sajor-caju*

5.4 Effect of different concentrations of Veratryl alcohol (VA) and Manganese sulfate on decolourisation of Methyl violet 2B with spent substrate of *P. sajor-caju*

For studying effect of veratryl alcohol on dye decolourisation five concentrations (25, 50, 75, 100 and 125 µl/100 ml) were used in Potato dextrose broth medium by inoculating spent substrate of *P. sajor-caju* (1%). At an early stage, lower concentration of veratryl alcohol (25 µl) supported highest decolourisation of Methyl violet 2B, while at later stages, highest concentration (100 µl) supported higher decolourisation. Effect of 5 concentrations of manganese ions (25, 50, 75, 100 and 125 mg/100ml) was studied by adding 25, 50, 75, 100 and 125 mg of manganese ions in 100 ml of sterilized Potato dextrose broth medium in 250 mL flasks, each

containing dye and spent substrate @ 100 ppm and 1.0%, w/v, respectively. Manganese ions also exhibited similar trend, as at initial stage, slightly enhanced decolourisation of Methyl violet 2B was recorded with lower concentration of manganese ions as compared to other tested concentrations including control. However, at a later stage (5 day), all manganese ions treatments supported almost equal level of decolourisation but slightly higher than control. Compared to control, both veratryl alcohol and manganese ions supported higher decolourisation, though more with lower concentration (25 mg/100 ml) at initial stage and with higher concentrations at later stages (Fig 5.6a and b).

The presence of Mn (II) in medium has been reported to induce higher activity of MnP (Urek and Pazarlioglu, 2005; Vahabzadeh *et al.*, 2004), while reduced activity of LiP (Vahabzadeh *et al.*, 2004). Under such circumstances the rate of decolourisation precedes the rate of veratryl alcohol oxidation (Singh *et al.*, 2010). In presence of Veratryl alcohol, both the Veratryl alcohol and dyes compete for the catalytic sites of LiP and with Veratryl alcohol being a preferred substrate for LiP as compared to dyes, the oxidation of Veratryl alcohol to Veratryl aldehyde precedes the oxidation of dyes resulting in a lower rate of decolourisation (Singh *et al.*, 2010).



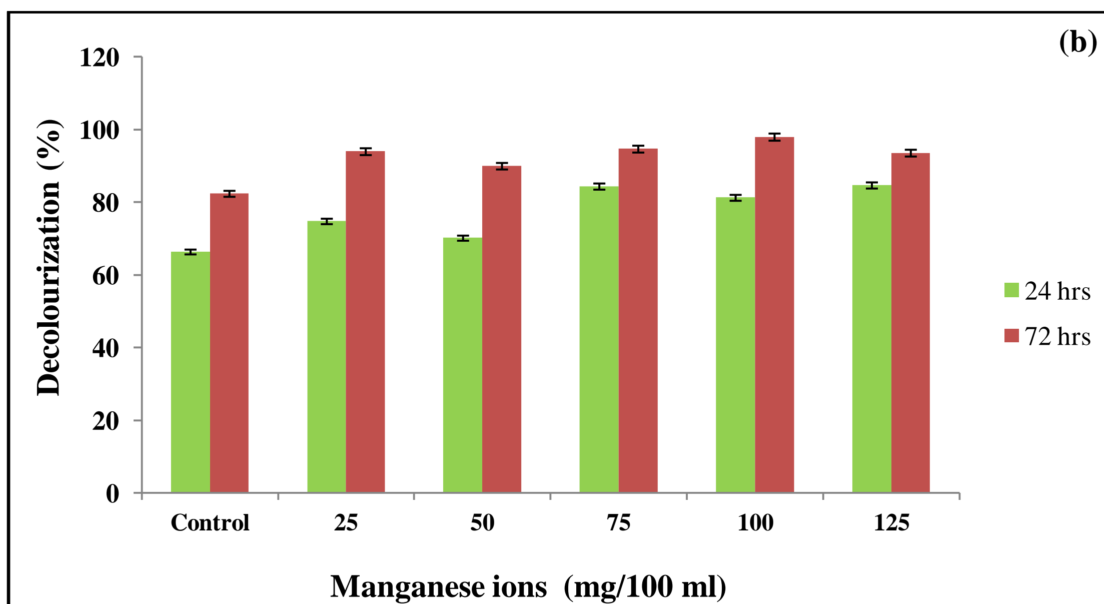


Fig. 5.6 Effect of different concentrations of mediator and co-factor on decolourisation of Methyl violet 2B through *P. sajor-caju* spent substrate: a), Veratryl alcohol; b), Manganese ions

5.5 Effect of heavy metals on decolourisation of Methyl violet 2B using spent substrate of *P. sajor-caju*

Effect of six heavy metals ions (Cadmium, Lead, Mercuric, Cobaltous, Zinc and Nickel) on decolourisation of Methyl violet 2B (MV2B) was studied using 0.01% concentration of each heavy metal, 100 ppm concentration of dye and 1.0%, w/v of spent substrate of *P. sajor-caju* in PDB. The present study revealed that out of 6 heavy metals, presence of only mercuric ions and zinc ions decreased the decolourisation of MV2B compared to control (Fig 5.7). Contrary to this, presence of lead ions and cadmium ions @ 0.01% in growing medium separately enhanced dye decolourisation and 94.6 to 100% decolourisation of MV2B was recorded after 4 day of mixing of spent substrate along with two heavy metals seprately. Decolourisation of MV2B in the presence of different heavy metal ions indicated that the spent mushroom substrate contains enough strength of diverse bacteria, fungi and active mushroom mycelia to counter the toxic effect of heavy metals.

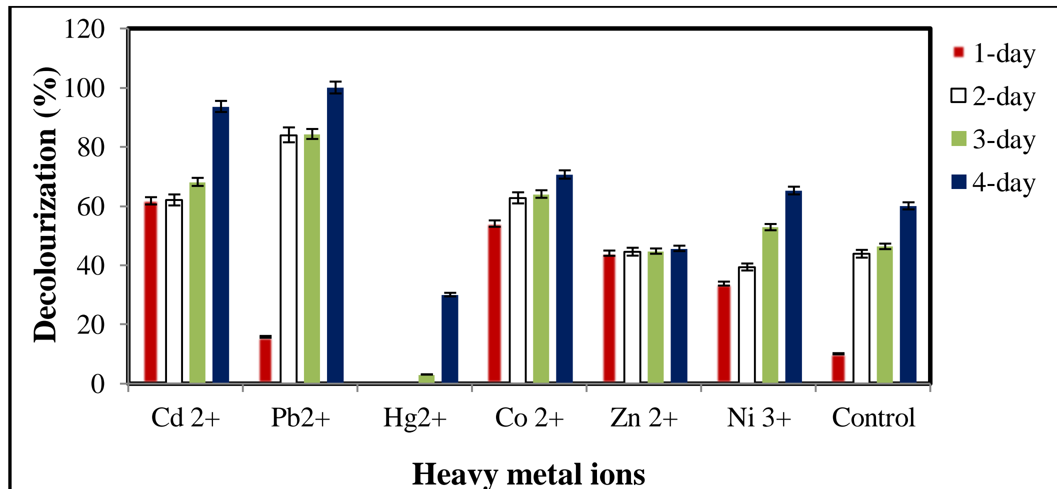


Fig. 5.7 Effect of heavy metals on decolourisation of Methyl violet 2B using spent substrate of *P. sajor-caju*

5.6 Effect of different growth conditions (intact and pellet form of mycelia) on decolourisation of Methyl violet 2B through *P. sajor-caju*

Again under 2 different growth conditions (intact and pellet forms of mycelia), inoculation of dye supplemented medium with pellet form of mycelia supported higher decolourisation from very beginning than inoculation with intact form of mycelia and same trend was maintained up to end of the experiment (15 days). Difference in decolourisation was more significant in middle of the experiment (9 days). Nearly 100% decolourisation of dye was recorded in pellet form of mycelia treatment after 15 days of inoculation compared with 96% in intact form of mycelia (Fig. 5.8).

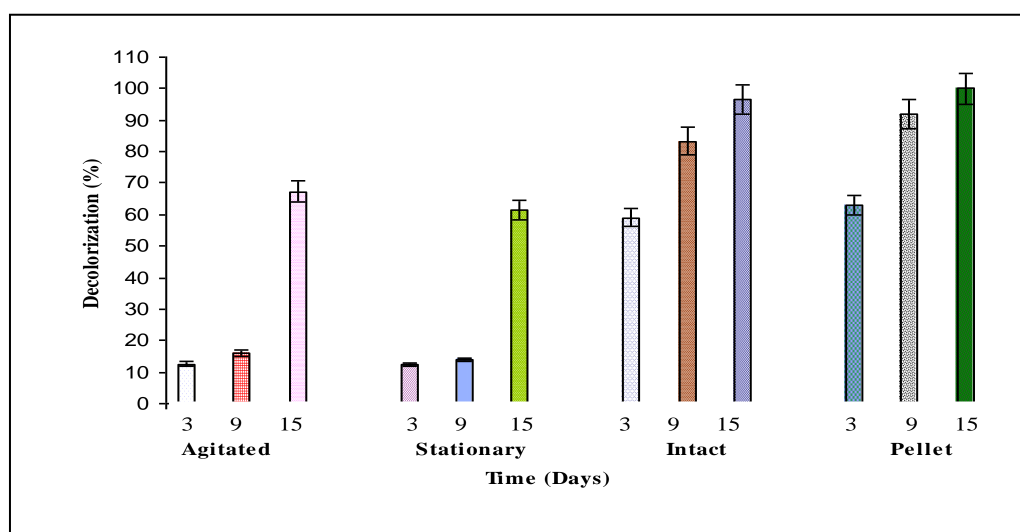


Fig. 5.8 Effect of cultural conditions on decolourisation of Methyl violet 2B through *P. sajor-caju* in agitated, stationary, intact and pellet forms of mycelia

5.7 Effect of different growing conditions (agitated and stationary) on decolourisation of Methyl violet 2B through *P. sajor-caju*

Under 2 different growing conditions (agitated and stationary), decolourisation of MV2B was almost similar after 3 d of inoculation of *P. sajor-caju*. However, after 3rd day onward decolourisation under agitated condition was higher than stationary growth condition (Fig. 5.8). Difference in decolourisation at initial stage was less significant mainly because of the lag phase of growth of mushroom mycelia, as it took few days to attain appreciable mycelial growth and to show its effect on dye decolourisation.

5.8 Dye decolourisation using potential fungi isolated from *P. sajor-caju* SMS

5.8.1 Effect of different pH and temperature on decolourisation of Rhodamine B and Methyl violet 2B by *Schizophyllum commune* and *Pezizomycotina* sp.

The two fungi, *Schizophyllum commune* and *Pezizomycotina* sp. isolated from SMS of *P sajor-caju* and showing significant dyes decolourisation potential in initial studies, were used for studying the effect of temperature and pH of the medium on decolurization of comparatively more recalcitrant dyes using them. Out of seven pH values tested for decolourisation of two dyes with *Schizophyllum commune*, pH from 5.0 to 10.0 showed appreciable level of decolourisation (90-100%) after 18 days of incubation. Initially, decolourisation was not recorded because inoculated fungus reached at an exponential phase on 6th day onward and than it started decolourising the dyes. But pH from 7.0 to 10.0 was recorded for complete decolourisation of

Rhodamine B using *S. commune*. The second fungus *Pezizomycotina* sp. also exhibited appreciable level of decolourisation of Rhodamine B at a pH range of 7.0 to 10.0, but highest decolourisation was recorded at pH of 10.0, which was also isolated from spent substrate of *P. sajor-caju* (Fig. 5.9). In case of Methyl violet 2B, highest decolourisation was recorded at pH range from 7.0 to 10.0 after 18th day of inoculation of *Schizophyllum commune. Pezizomycotina* sp. showed a narrower pH range (9.0 to 10.0) for complete decolourisation of Methyl violet 2B. Out of two fungi, *S. commune* was found to have higher decolourisation ability of two dyes over a wide pH range (Fig. 5.10).

Out of three temperature levels (15, 25 and 35 $^{\circ}$C) tested for decolourisation of two dyes with two potential fungi, highest decolourisation at different stages of observations was recorded at 35 $^{\circ}$C, followed by 25 $^{\circ}$C and 15 $^{\circ}$C (Fig. 5.11). At all stages of observations at all temperature levels, the decolourisation was higher with *S.commune* than *Pezizomycotina* sp. Nearly 100% decolourisation of Rhodamine B was recorded at 35 $^{\circ}$C after 18 days of incubation. Almost similar trend of decolourisation of Methyl violet 2B was recorded at different temperature levels with two fungi (Fig. 5.12).

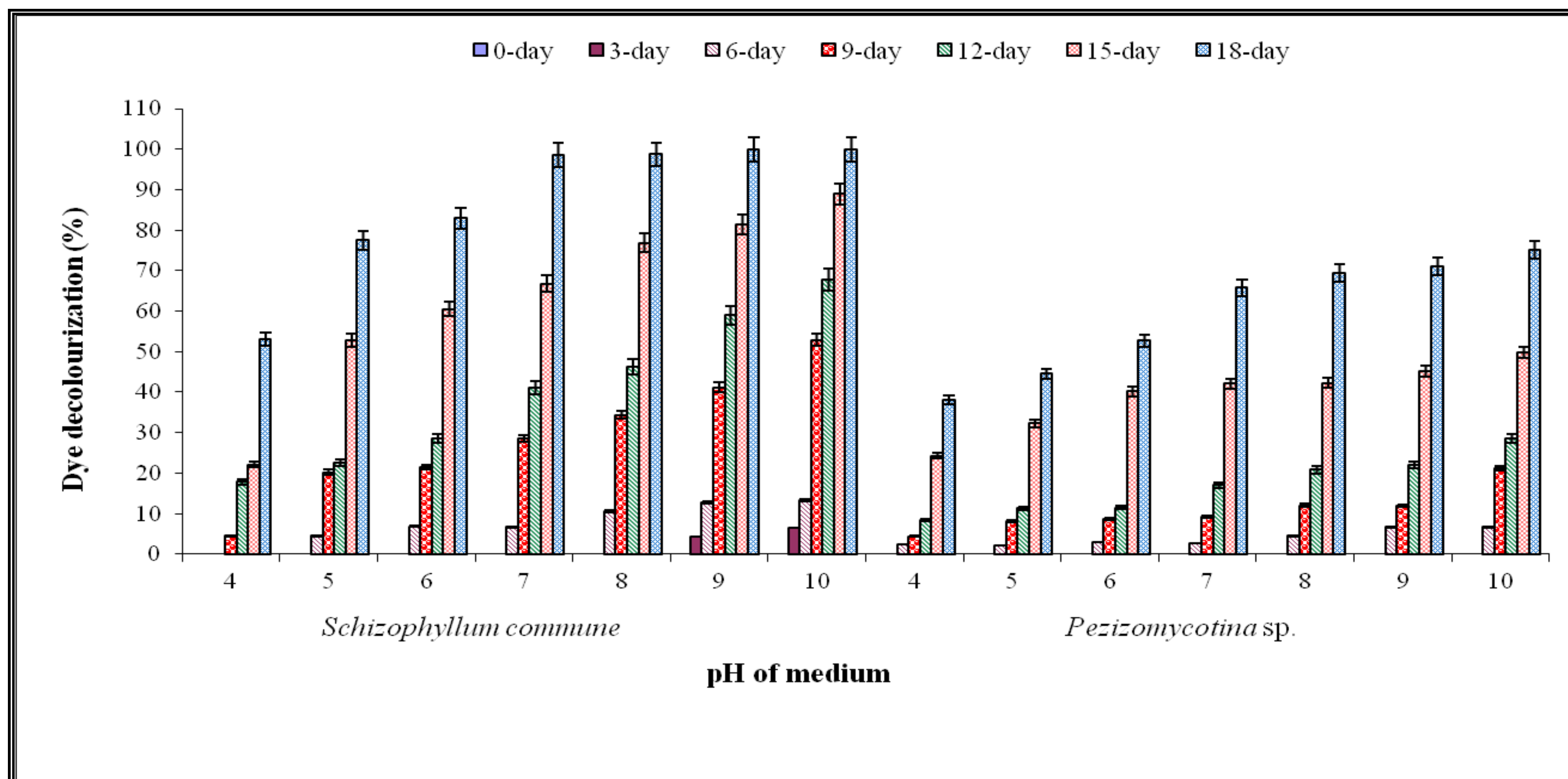


Fig. 5.9 Decolourisation of Rhodamine B using *Schizophyllum commune* and *Pezizomycotina* sp. at different pH of medium

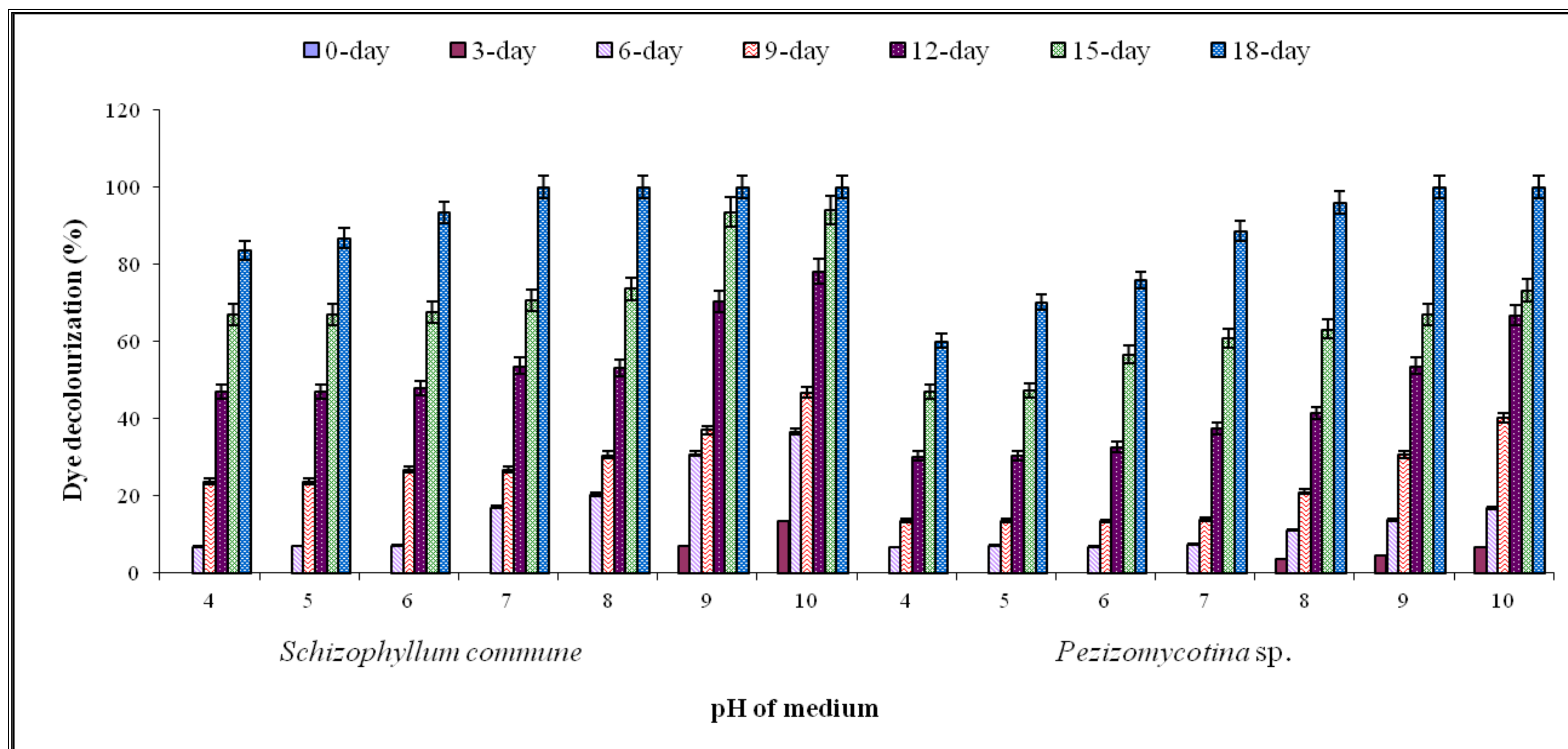


Fig. 5.10 Decolourisation of Methyl violet 2B using *Schizophyllum commune* and *Pezizomycotina* sp. at different pH of medium

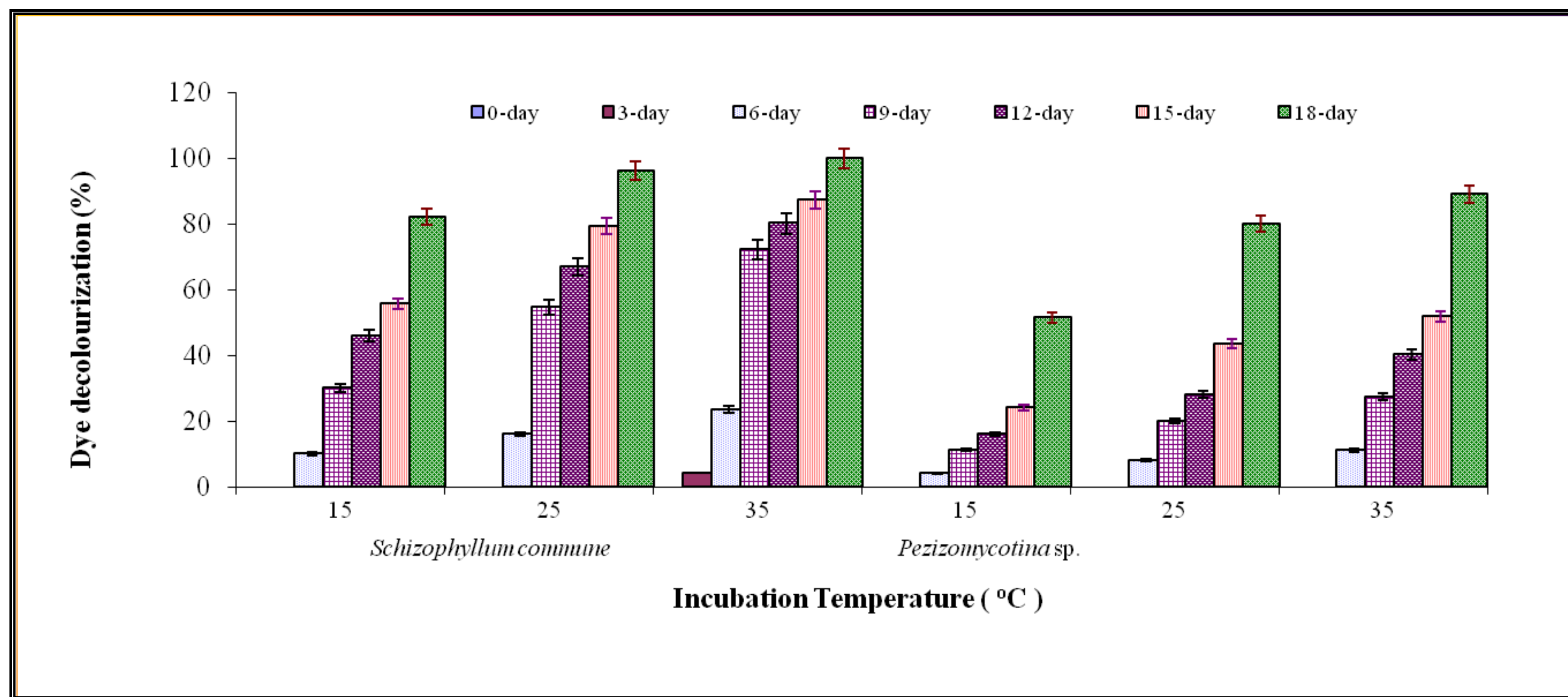


Fig. 5.11 To study the decolourisation potential of Rhodamine B using *Schizophyllum commune* and *Pezizamycotina* sp. at different temperature

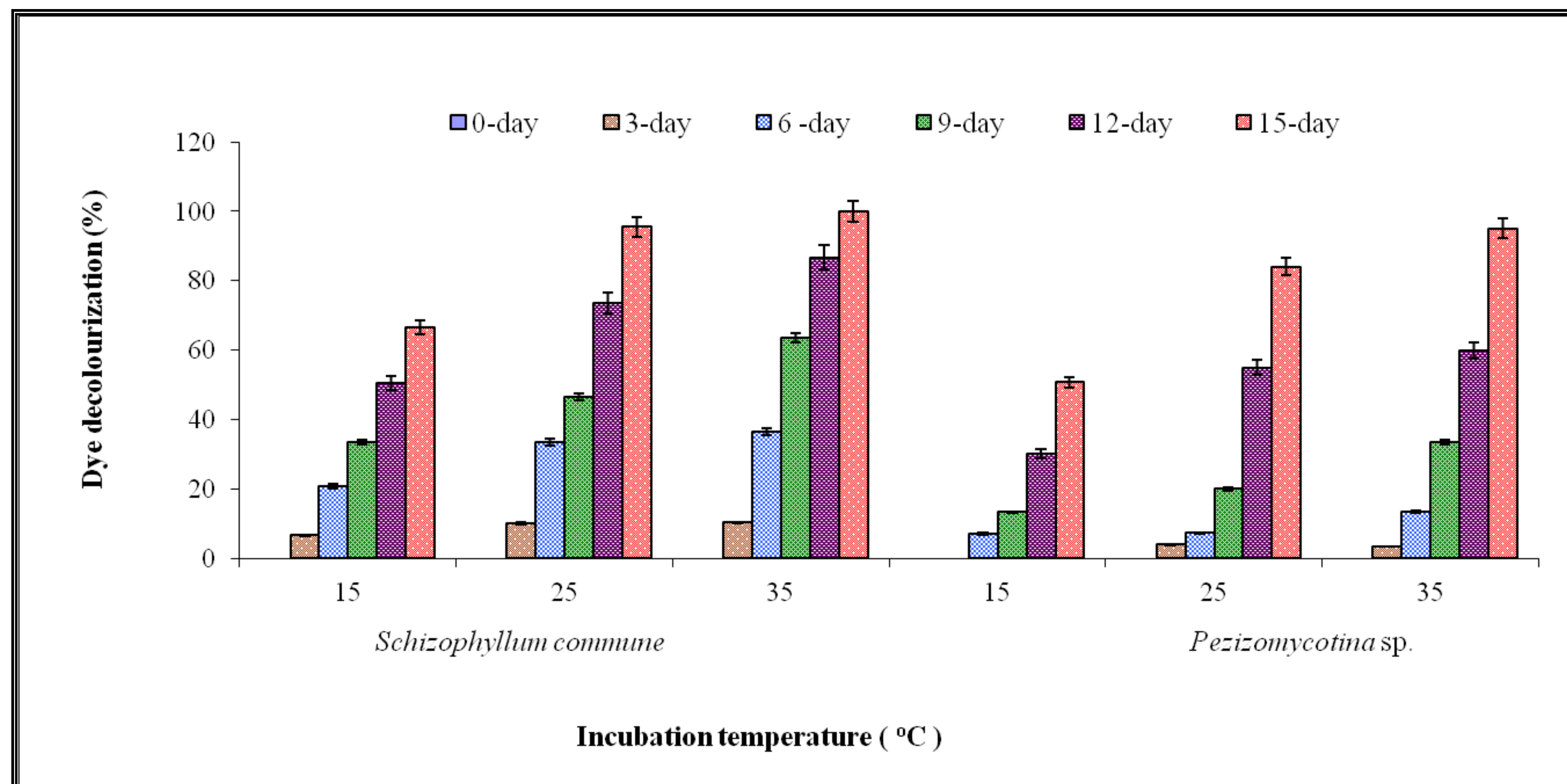


Fig. 5.12 Decolourisation of Methyl violet 2B using *Schizophyllum commune* and *Pezizomycotina* sp. at different temperature

5. 8.2 Effect of different pH values on mycelial biomass production of *Schizophyllum commune* along with dye decolourisation

Effect of varied pH values of medium on biomass production of *Pezizomycotina* sp. along with decolourisation of Rhodamine B was studied at a pH range of 4.0 to 10.0. Highest decolourisation of Rhodamine B was recorded at pH from 8.0 to 10.0, while highest biomass (6.5g/l) was recorded at pH of 9.0 (Fig. 5.13a and b). The pH from 4.0 to 6.0 was not suitable for the growth of the fungus as well as decolourisation of the dye.

5.8.3 Effect of different temperature on mycelial biomass production of *Pezizomycotina* sp. along with dyes decolourisation

Five different temperatures (15, 20, 25, 30 and 35 $^{\circ}$C) were employed for studying the effect of temperature on biomass production as well as decolourisation of Rhodamine B (Fig. 13c and d). The highest dye decolourisation of Rhodamine B was recorded at temperature ranging from 30 to 35 $^{\circ}$C, while highest biomass was recorded at temperature of 30 $^{\circ}$C.

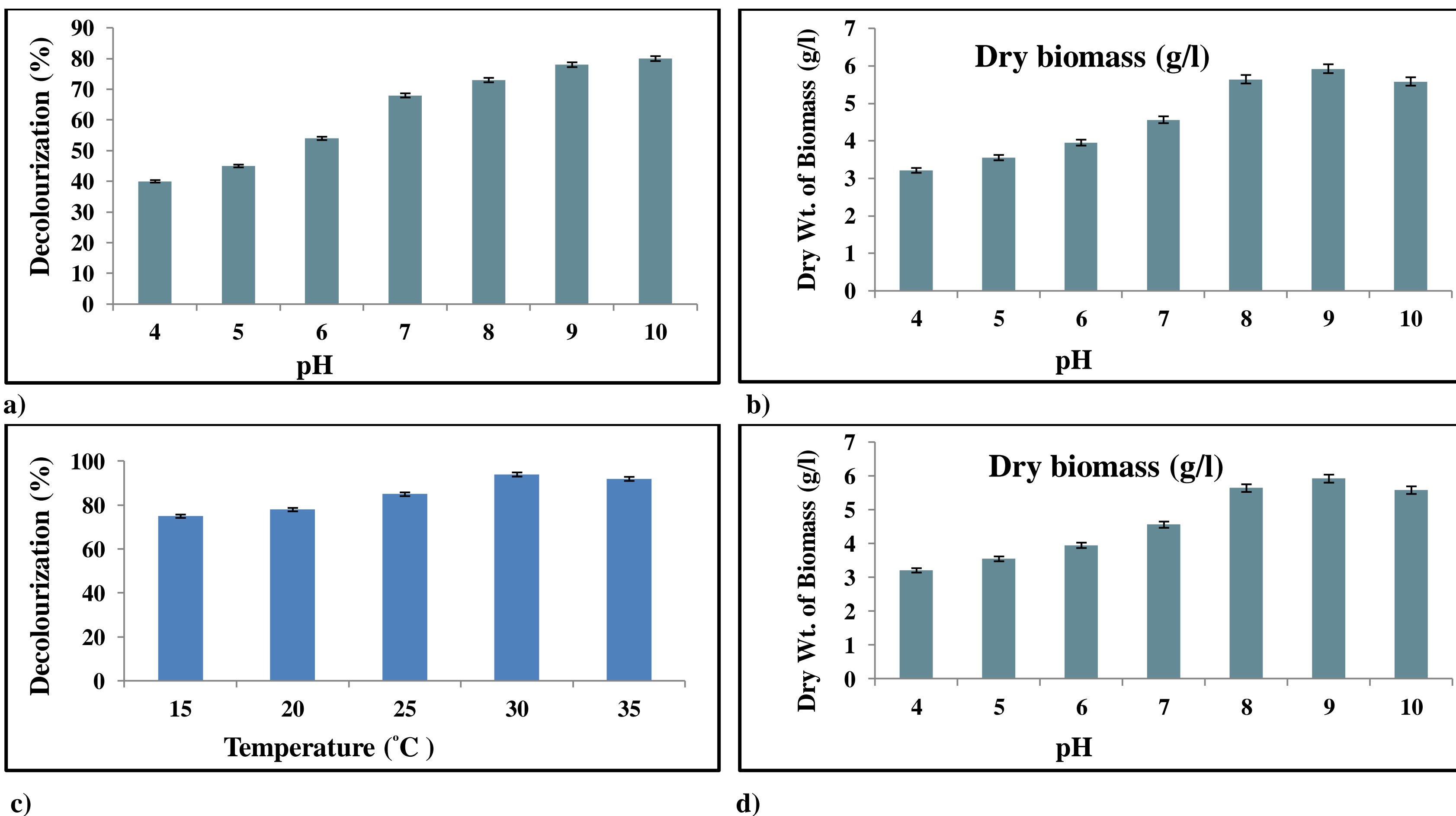


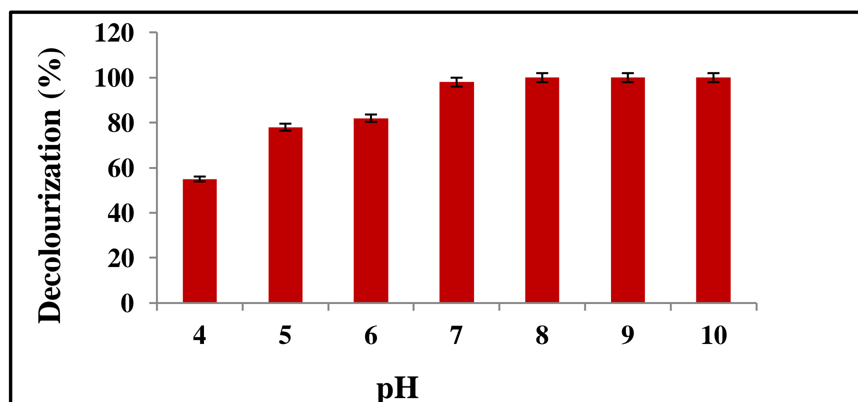
Fig. 5.13 Decolourisation of Rhodamine B and mycelial biomass production potential of *Pezizomycotina* sp. a) Rhodamine B decolourisation at different pH values; b) Dry weight of mycelial biomass at different pH values; c) Rhodamine B decolourisation at different temperature; d) Dry weight of mycelial biomass at different temperature, where the control was the amount of biomass of *Pezizomycotina* sp. growing on medium without dye; control = 4.4 gl^{-1})

5. 8.4 Effect of different pH on mycelial biomass production of *Schizophyllum commune* along with dye decolourisation

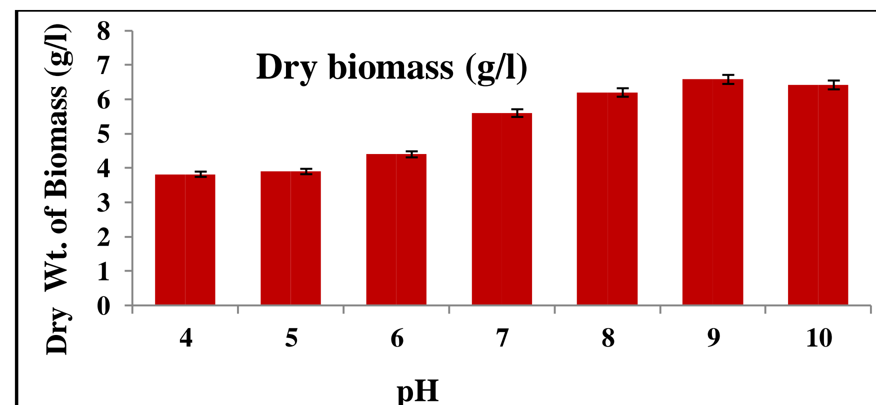
The pH optima for biomass production of *S. commune* along with Methyl violwt 2B decolourisation was studied at pH ranging from 4.0 to 10.0. The pH ranging from 7.0 to 10.0 was recorded to be optimum for achieving nearly 100% decolourisation of MV2B. However, the pH optima for biomass production was narrower and it was 9.0 to 10.0, where highest biomass (7.2 g/l) production was recorded (Fig. 5.14a and b)

5.8.5 Effect of different temperature on mycelial biomass production during dyes decolourisation by *Schizophyllum commune*

Out of 5 different temperatures (15, 20, 25, 30 and 35 °C) tested for getting the temperature optima for decolourisation of MV2B and biomass production of *S. commune*, highest decolourisation was recorded at temperature of 35 °C. However, highest mycelial biomass was obtained at temperature of 30 °C. Compared to *Pezizomycotina* sp., *S. commune* exhibited wider pH (7.0 to 10.0) and temperature (25 to 35 °C) optimas for decolourisation of dye (Fig 5.14c and d).



a)



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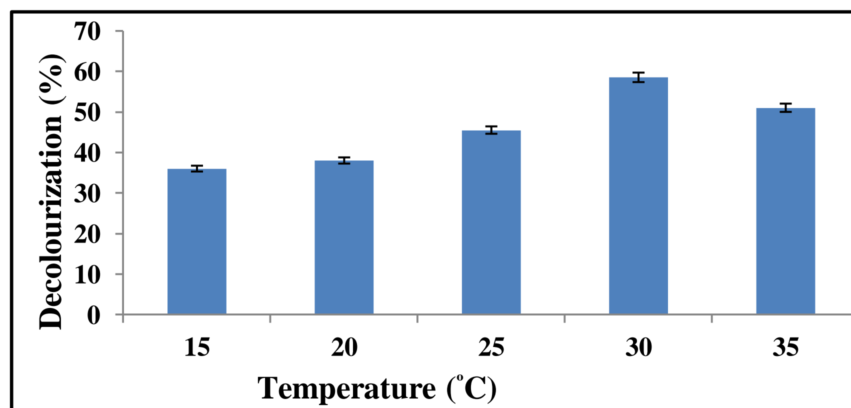
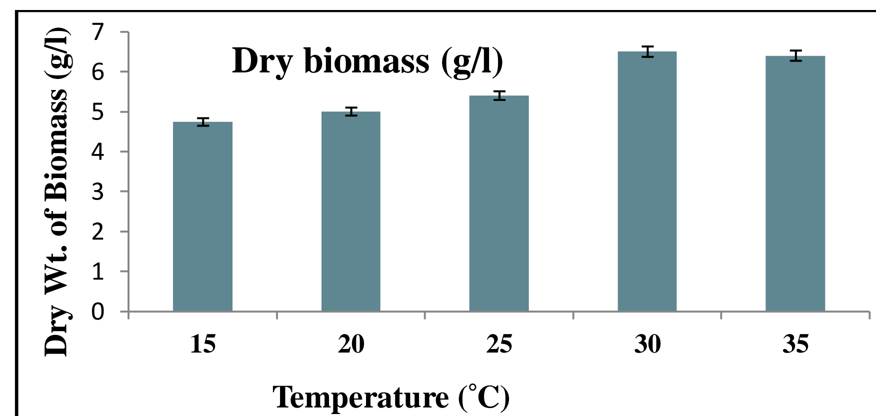


c)



d)

Fig. 5.14 Decolourisation of Methyl violet 2B and mycelial biomass production potential of *S. commune* a) MV2B decolourisation at different pH ; b) Dry weight of mycelial biomass at different pH ; c) MV2B decolourisation at different temperature ; d) Dry weight of mycelial biomass at different temperature, where the control was the amount of biomass of *S. commune* growing on medium without dye; control=6.2gl^{-1}

5.9 Fourier transform infrared spectroscopy (FTIR) analysis of Rhodamine B degradation with *Schizophyllum commune*

The FTIR spectrum of Rhodamine B dye before degradation and after degradation with *Schizophyllum commune* was outsourced from Central Instrumentation Facility at Punjab University, Chandigarh, India. The FTIR spectrum of the control showed the presence of different sharp and strong absorption peaks around 3400.97 cm^{-1} corresponding with $–NH_3$ stretches (Fig 5.15). The peaks at 2926.15 cm^{-1} indicated the stretching band of –CH. The sharp peaks observed at 1642.20 cm^{-1} could be assigned to the stretching band of C=O from amide. The peaks at 1406.66 cm^{-1} were attributed to the stretching vibration indicative of benzene ring. Other peaks observed at 1077.02 cm^{-1} indicates –OH stretching of aromatics ring and peaks at 619.72 cm^{-1} demonstrated the primary alcohols and sulphoric acid (-S=O). The FTIR spectrum after degradation of Rhodamine B with *Schizophyllum commune* displayed peaks at 3369.33, 2920 and 2851.16 cm^{-1} correspond to –C-H deformation of alkanes, -C-H symmetric stretch of the methylene group ($-CH_2$) and deformation vibration of methyl group ($-CH_3$), alkane ($-CH_2$) stretching and vibration (Fig. 5.15 b). The other peaks at 1627.92 cm^{-1} represent –C=O from amide stretching vibration, whereas 1573.31 cm^{-1} peak shows –N=N stretching of azo compound and breaking of azo bond.

5.10 Decolurization of textile effluent with microbial consortia isolated from SMS of different mushrooms

5.10.1 Effect of temperature on decolourisation of textile effluent with microbial consortia

The microbial consortia prepared from the potential fungi and bacteria isolated from SMS of different mushroom were evaluated for their potential to decolourize textile effluent at four different temperatures. Out of different temperatures of incubation, highest decolourisation of 37.10 % was recorded at 30 ºC with combined

inoculums of *S. commune* and *Pezizomycotina* sp. immobilized on wheat straw along with broth cultures of *B. pumilus*, *B. licheniformis* and *P. fluorescens* after 48 hrs of incubation (Table 5.1). It was closely followed by *Pezizomycotina* sp. alone immobilized on wheat straw (32.30 %). The same combination also exhibited highest decolourisation at 30 ºC after 96 hours (63.60 %) and 144 hrs (98.50%) of incubation, closely followed by *Pezizomycotina* sp. alone (60.00 and 91.20 %).

5.10.2 Effect of different carbon sources on decolourisation of textile effluent with different combinations of microorganisms

Out of different concentrations of five carbon sources, 0.5% of glucose and 1.0% each of rest all carbon sources were recorded to support highest level of effluent decolourisation. Out of different fungi and bacteria in isolation, highest decolourisation after 48 hours of incubation was in *P. fluorescens* in presence of 1.0 % concentration of fructose (34.60 %), sucrose (42.63 %) and starch (25.70 %), while in combined inoculum of *S. commune*, *Pezizomycotina* sp., *B. pumilus*, *B. licheniformis* and *P. fluorescens,* it was highest in presence of 0.5 % glucose (41.60 %) and 1.0 % maltose (29.30 %) (Table 5.2a). Out of different carbon sources, glucose at 0.5% and sucrose at 1.0% were significantly more effective than different concentrations of other carbon sources. After 96 hours of incubation, highest decolourisation (93.30 to 100 %) was in combined inoculum of *S. commune*, *Pezizomycotina* sp., *B. pumilus*, *B. licheniformis* and *P. fluorescens* in presence 1.0% concentration of different carbon sources (Table 5.2b). It was followed by immobilized form of *S. commune* in presence of fructose and sucrose, and *Pezizomycotina* sp. in presence of remaining carbon sources. The difference in level of decolourisation by two fungi was insignificant in presence of majority of the carbon sources. The two controls i.e. plain effluent and effluent mixed with wheat straw exhibited 0.00% decolourisation, which nullify the role of other factors including adsorption of dyes on wheat straw.

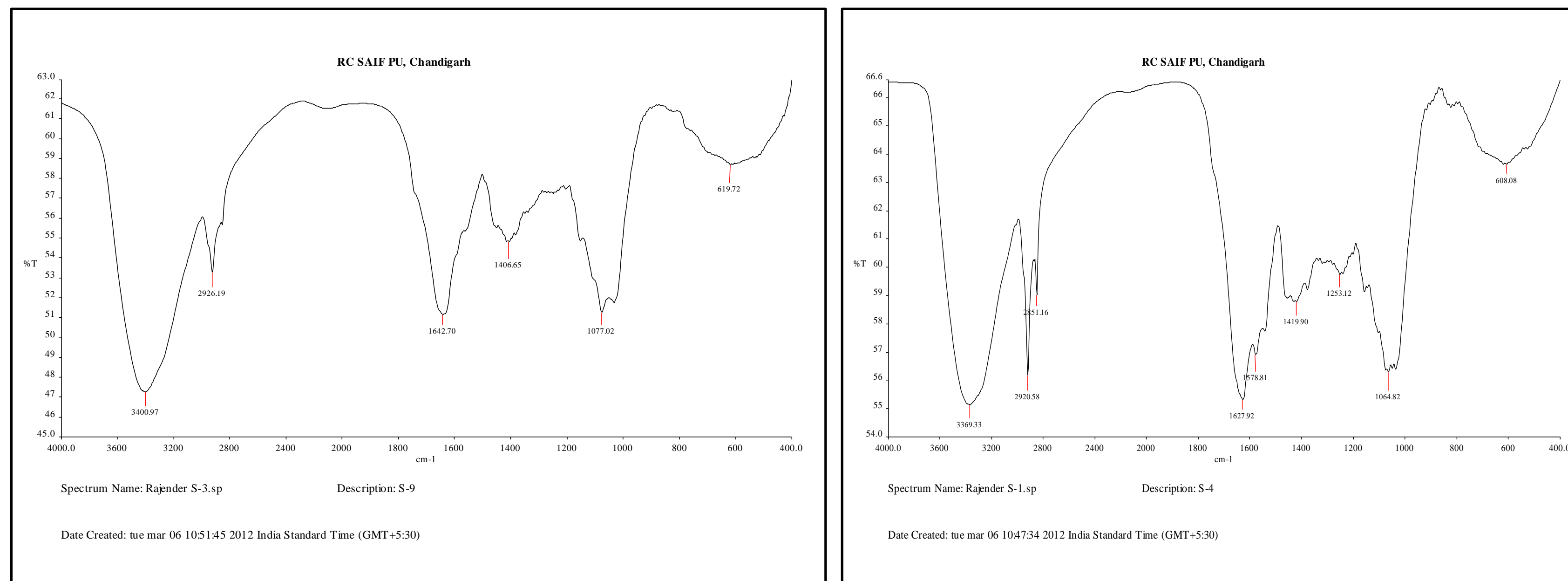


Fig. 5.15 FTIR analysis of the Rhodamine B a) Control and b) After biodegradation by *S. commune*

Table 5.1 Effect of temperature of incubation on decolourisation of textile effluent using different combinations of fungi and bacteria

Treatment	Decolourisation of textile effluent (%) at different time intervals (hrs) and at varied temperature											
	48 h				96 h				144 h			
	20 °C	25 °C	30 °C	35 °C	20 °C	25 °C	30 °C	35 °C	20 °C	25 °C	30 °C	35 °C
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	2.91	13.3	11.3	14.6	10.0	26.6	48.1	27.5	22.1	33.8	85.9	38.3
4	8.33	16.6	32.3	16.3	17.5	28.8	60.0	30.4	30.4	40.8	91.2	43.8
5	10.0	18.8	37.1	17.3	23.3	34.6	63.6	39.1	36.7	50.8	98.5	55.4
$CD_{0.05}$=	0.044	0.062	0.087	0.047	0.062	0.075	0.092	0.081	0.048	0.080	0.084	0.074

1, Plain textile effluent
2, Effluent with wheat straw
3, Wheat straw immobilized with *S. commune*
4, Wheat straw immobilized with *Pezizomycotina* sp.
5, Immobilized form of *S. commune* + Immobilized form of *Pezizomycotina* sp. + *B. pumilus* + *B. licheniformis* + *P. fluorescens*

Table 5.2a Effect of carbon sources on decolourisation of textile effluent after 48 hrs of inoculation with different combinations of fungi and bacteria

Treatment	Decolourisation of textile effluent (%) in presence of different carbon sources (48 h)														
	Glucose			Fructose			Sucrose			Maltose			Starch		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	22.10	28.80	23.80	20.00	20.80	21.04	17.80	18.10	19.30	9.30	18.10	23.22	13.80	16.60	20.71
4	23.80	31.25	28.80	20.70	21.60	25.40	18.70	19.30	20.30	14.20	19.40	26.90	16.30	18.41	23.30
5	37.50	41.60	37.50	30.80	32.91	32.10	22.08	25.50	30.00	25.30	26.70	29.30	19.20	20.00	24.91
6	23.80	24.60	29.60	25.80	26.60	28.80	20.80	25.00	26.30	14.10	16.30	16.25	24.20	21.70	24.90
7	28.30	31.70	31.30	27.90	29.60	30.00	20.40	20.70	25.80	16.30	17.50	18.30	20.00	22.10	24.94
8	29.30	32.50	32.50	30.00	31.25	34.60	31.30	35.42	42.63	16.80	19.24	23.63	21.72	25.44	25.70
$CD_{0.05}$	0.069	0.036	0.053	0.038	0.041	0.039	0.064	0.058	0.099	0.036	0.051	0.070	0.067	0.049	0.051

a, 0.25%; b, 0.50%; c, 1.0%
1, Plain textile effluent
2, Effluent with wheat straw
3, Wheat straw immobilized with *S. commune*
4, Wheat straw immobilized with *Pezizomycotina* sp.
5, Immobilized form of *S. commune* + immobilized form of *Pezizomycotina* sp. + *B. pumilus* + *B. licheniformis* + *P. fluoresences*
6, *B. pumilus*
7, *B. licheniformis*
8, *P. fluoresences*

Table 5.2b Effect of different carbon sources on decolourisation of textile effluent after 96 hrs of inoculation with different combinations of fungi and bacteria

Treatment	Decolourisation of textile effluent (%) in presence of different carbon sources (96 h)														
	Glucose			Fructose			Sucrose			Maltose			Starch		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	79.40	87.50	92.10	95.30	96.12	96.24	90.80	94.12	95.62	88.32	89.00	89.10	85.52	87.52	90.44
4	80.40	84.50	97.20	95.30	95.83	96.21	90.41	93.81	94.21	88.10	88.82	89.32	86.80	87.22	91.50
5	80.90	99.60	100.0	79.20	98.42	100.0	96.32	100.0	100.0	92.21	92.83	93.30	93.10	94.24	96.82
6	80.90	83.50	89.80	63.11	64.66	67.21	62.64	69.21	68.32	64.12	66.91	72.42	63.40	63.72	67.33
7	79.80	82.50	86.40	76.21	67.97	73.64	64.20	67.84	68.23	64.50	66.51	66.50	64.52	64.82	65.52
8	80.80	84.60	91.50	81.94	74.12	75.70	69.22	70.22	71.77	64.91	65.54	67.10	66.50	68.80	70.32
$CD_{0.05}$	0.076	0.046	0.069	0.080	0.074	0.073	0.143	0.069	0.084	0.034	0.067	0.088	0.123	0.096	0.064

a, 0.25%; b, 0.50%; c, 1.0%
1, Plain textile effluent
2, Effluent with wheat straw
3, Wheat straw immobilized with *S. commune*
4, Wheat straw immobilized with *Pezizomycotina* sp.
5, Immobilized form of *S. commune* + Immobilized form of *Pezizomycotina* sp. + *B. pumilus* + *B. licheniformis* + *P. fluorescens*
6, *B. pumilus*
7, *B. licheniformis*
8, *P. fluoresence*

5.10.3 Effect of immobilization medium on decolourisation of textile effluent with different microbes

In order to standardize the immobilization medium, wheat straw, paddy straw, peat moss, soybean straw and saw dust were used. *Schizophyllum commune* grew well over wheat straw and paddy straw, hence only these two substrates were used for *S. commune*, while all were used for *Pezizomycotina* sp. as it grew on all substrates. In case of *Pezizomycotina* sp., highest decolourisation after 144 hrs of incubation at 30 °C was on using peat moss (86.9%) as the immobilization medium, followed by soybean straw (75.5%), wheat straw (73%), paddy straw (74%) and saw dust (69.2%). In case of *S. commune*, highest decolourisation was with paddy straw (89.5 %), followed by wheat straw (86.8 %) (Fig. 5.16a). The three bacteria used for comparison exhibited very low level of decolourisation (25.80 – 38.0 %). Out of different combinations of immobilized forms of fungi and broth cultures of bacteria, the immobilized forms of *S. commune* and *Pezizomycotina* sp. separately with *B. pumilus* and *B. licheniformis* resulted in nearly 100 % decolourisation, which was at par to that of mixed inoculum of two fungi and three bacteria. The two controls again exhibited almost negligible level of decolourisation (Fig. 5.16b).

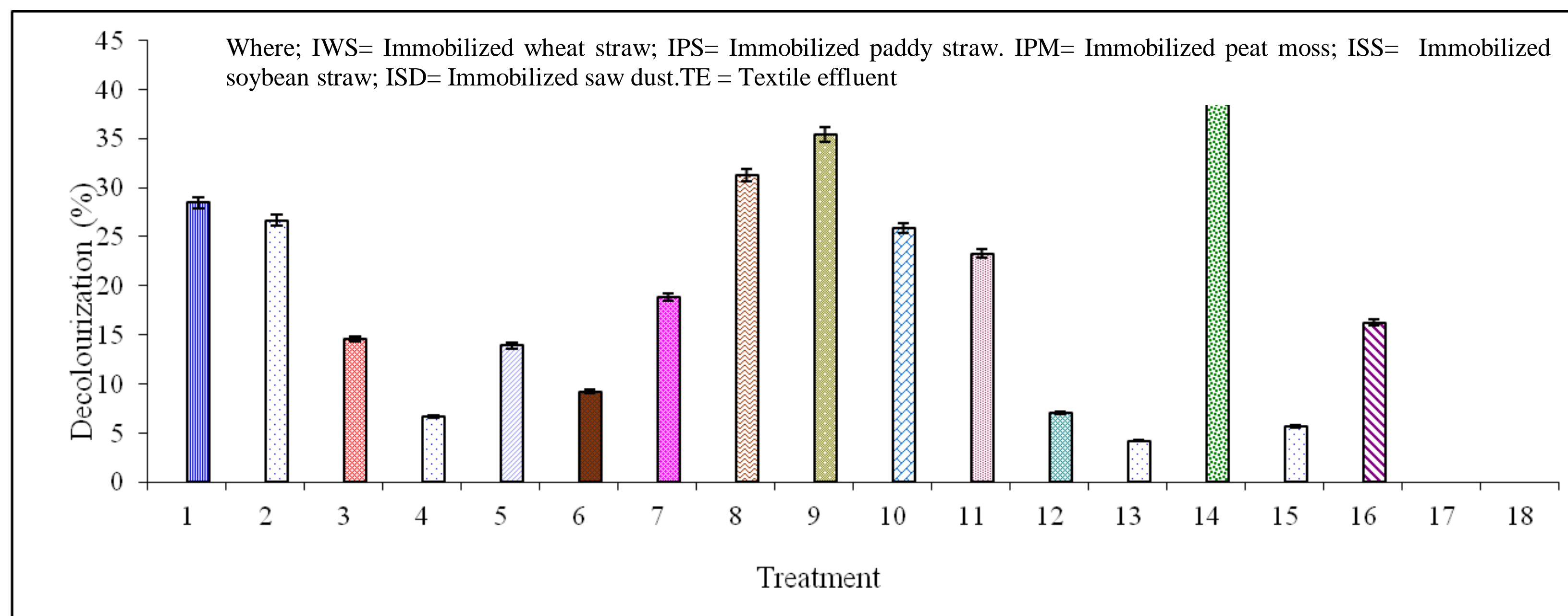


Fig. 5.16a Decolourisation of textile effluent after 48 hrs of inoculation with different combinations of fungi and bacteria

1, IWS with *S. commune* + TE; **2**, IPS with *S. commune* + TE; **3**, IWS with *Pezizomycotina* sp. + TE; **4**, IPS with *Pezizomycotina* sp. + TE; **5**, ISS with *Pezizomycotina* sp. + TE; **6**, ISD with *Pezizomycotina* sp. + TE; **7**, IPM with *Pezizomycotina* sp. + TE; **8**, IWS with *S.commune* + TE + *Bacillus pumilus*; **9**, IWS with *S.commune* + TE + *B.licheniformis*; **10**, IWS with *Pezizomycotina* sp. + TE+ *B. pumilus*; **11**, IWS with *Pezizomycotina* sp. + TE + *B. licheniformis;* **12**, *Bacillus pumilus;* **13**, *B.licheniformis;***14**, IWS with *S.commune* + IWS with *Pezizomycotina* sp. + TE + *Bacillus pumilus* + *B. licheniformis* + *P.fluorescens;* **15**, *P. fluorescens;* **16**, *P.fluorescens* + IWS with *Pezizomycotina* sp.; **17**, Control without any inoculum; **18**, Wheat straw + TE

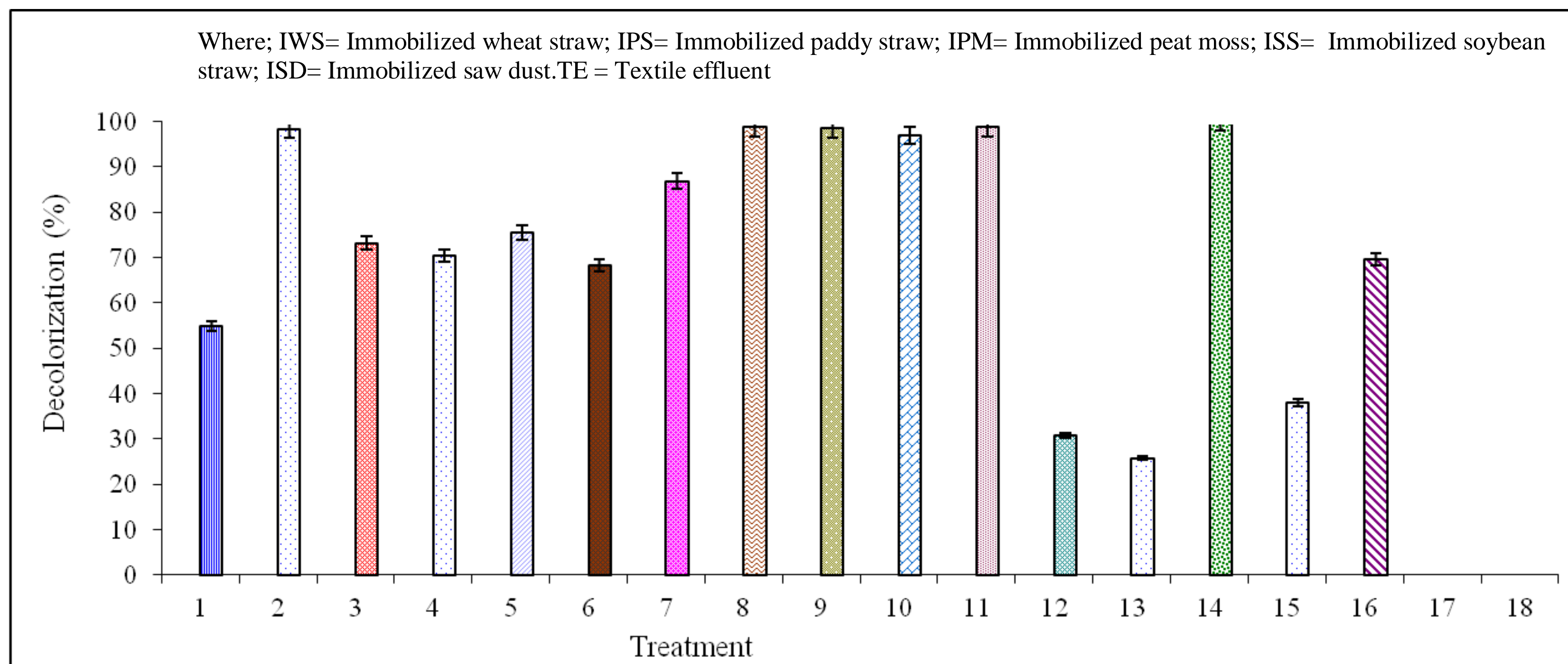
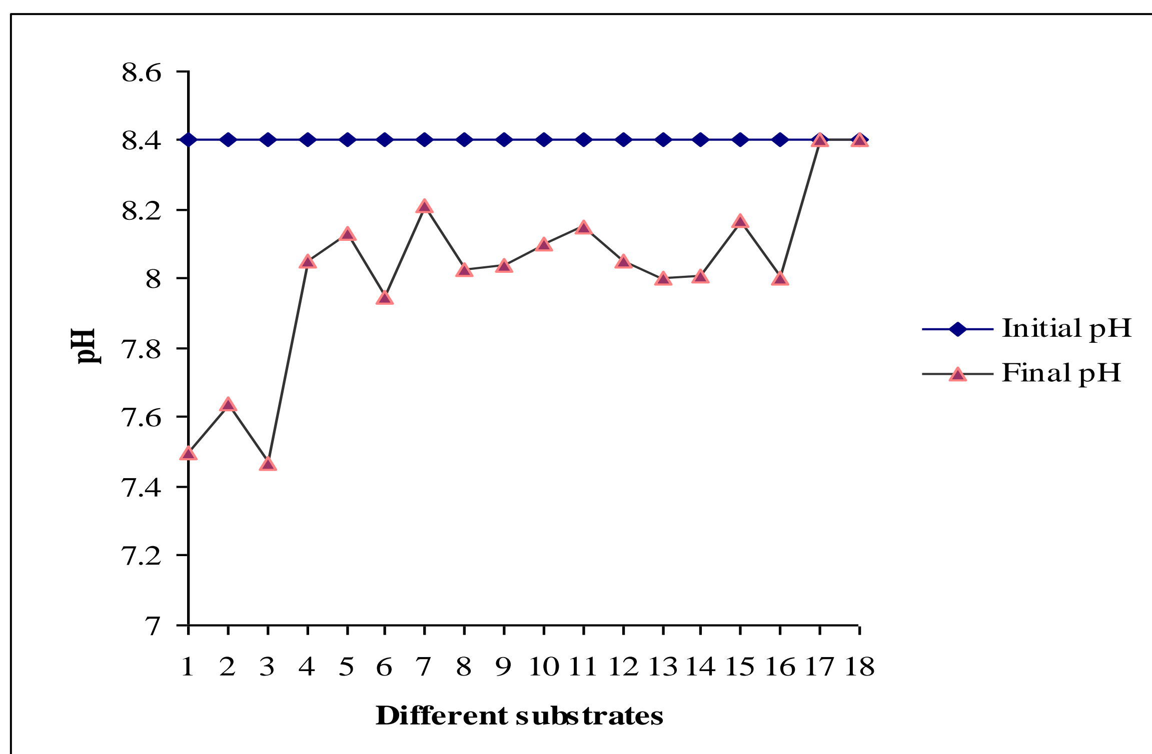


Fig. 5.16b Decolourisation of textile effluent after 144 hrs of inoculation with different combinations of fungi and bacteria

1, IWS with *S. commune* + TE; **2**, IPM with *S. commune* + TE; **3**, IWS with *Pezizomycotina* sp. + TE; **4**, IPS with *Pezizomycotina* sp. + TE; **5**, ISS with *Pezizomycotina* sp. + TE; **6**, ISD with *Pezizomycotina* sp. + TE; **7**, IPM with *Pezizomycotina* sp. + TE; **8**, IWS with *S. commune* + TE + *Bacillus pumilus*; **9**, IWS with *S.commune* + TE + *B. licheniformis*; **10**, IWS with *Pezizomycotina* sp. + TE + *B. pumilus*; **11**, IWS with *Pezizomycotina* sp. + TE+ *B. licheniformis;* **12**, *Bacillus pumilus;* **13**, *B.licheniformis;***14**, IWS with *S.commune* + IWS with *Pezizomycotina* sp. + TE + *Bacillus pumilus* + *B. licheniformis* + *P. fluorescens;* **15**, *P. fluorescens;* **16**, *P. fluorescens* + IWS with *Pezizomycotina* sp.; **17**, Control without any inoculum; **18**, Wheat straw + TE

5.10.4 pH variation during the dyes degradation

The initial pH of effluent in all treatments was 8.40 and it remained static in case of two controls incubated at 30 °C for 144 hours (6 days). However, in rest other cases, it decreased to highest of 8.20 in case of *Pezizomycotina* sp. immobilized on peat moss to lowest of just above 7.40 in case of *Pezizomycotina* sp. immobilized on wheat straw (Fig. 5.17). It was followed by *S. commune* immobilized on wheat straw (~ 7.50) and paddy straw (just above 7.60). In rest other cases, where either of the bacterium was part of inoculum, the pH varied between 8.0 to 8.1, which highlights the role of immobilization medium and microbes involved in maintaining optimum pH range for decolourisation of textile effluent.



Where;
IWS= Immobilized wheat straw
IPS= Immobilized paddy straw
IPM= Immobilized peat moss
ISS= Immobilized soybean straw
ISD= Immobilized saw dust
TE = Textile effluent

1, IWS with *S. commune* + TE; 2, IPM with *S. commune* + TE; 3, IWS with *Pezizomycotina* sp. + TE; 4, IPS with *Pezizomycotina* sp. + TE; 5, ISS with *Pezizomycotina* sp. + TE; 6, ISD with *Pezizomycotina* sp. + TE; 7, IPM with *Pezizomycotina* sp. + TE; 8, IWS with *S. commune* + TE + *Bacillus pumilus*; 9, IWS with *S.commune* + TE + *B.licheniformis*; 10, IWS with *Pezizomycotina* sp. + TE+ *B. pumilus*; 11, IWS with *Pezizomycotina* sp. + TE+ *B.licheniformis;* 12, *Bacillus pumilus;* 13, *B.licheniformis;*14, IWS with *S.commune* + IWS with *Pezizomycotina* sp. + TE + *Bacillus pumilus* + *B.licheniformis* + *P.fluorescens;* 15, *P.fluorescens;* 16, *P.fluorescens* + IWS with *Pezizomycotina* sp.; 17, Control without any inoculum; 18, Wheat straw + TE

Fig. 5.17 Change in pH of textile effluent after 144 hours of inoculation with different combinations of fungi and bacteria

Chapter-6

To Carry Out Kinetics Studies on Dyes Decolourisation by Spent Mushroom Substrate

Kinetics model offers a powerful tool to describe biological degradation processes and elucidates the quantitative degradation behaviours. In present study kinetics of dye decolourisation by spent mushroom substrate of different edible mushrooms were determined. Dye concentration at any given time was considered proportional to optical density of the reaction mixture and variation in O.D. with time was recorded in all the experimental sets and used for kinetic studies. Some of the decolourisation processes in the different treatments follow the model of first order exponential decay, represented as $A_t=A_o.e^{-kt}$. Where A_t is absorbance of the reaction mixture at any given time and A_o is initial absorbance of the reaction mixture (Mutafov *et al.,* 2006). As absorbance is directly proportional to concentration of reaction mixture at any time, therefore the exponential decay can also be represented as $C_t=C_{0.e}^{-kt}$ where C_t is concentration of the dye in the reaction mixture at any time and C_0 is the initial concentration of the dye. Potential fungi from spent substrate of *Pleurotus sajor-caju* i.e. *Schizophyllum commune* and *Pezizomycotina* sp. were also studied as a function of time, pH and temperature for achieving highest decolourisation of Methyl violet 2B.

6.1 Kinetics of Methyl violet 2B decolourisation

Decolourisation of dyes using different set of treatments was studied to determine kinetics of dye decolourisation. Obtained data was fitted for zero order, fist order and second order; by plotting dye concentration (C) versus time (t), ln C versus time and 1/C versus time according to Eq. (4), respectively.

$C = C_0 - kt$ (1)

$C = C_{0.} e^{-kt}$ (2)

$1/C = 1/C_0 - kt$ (3)

Most of the treatments in dyes decolourisation process were found to follow the first order kinetics by following equation 2 and its linear form were obtained in equation (4)

$\ln C_0/C = kt$ (4)

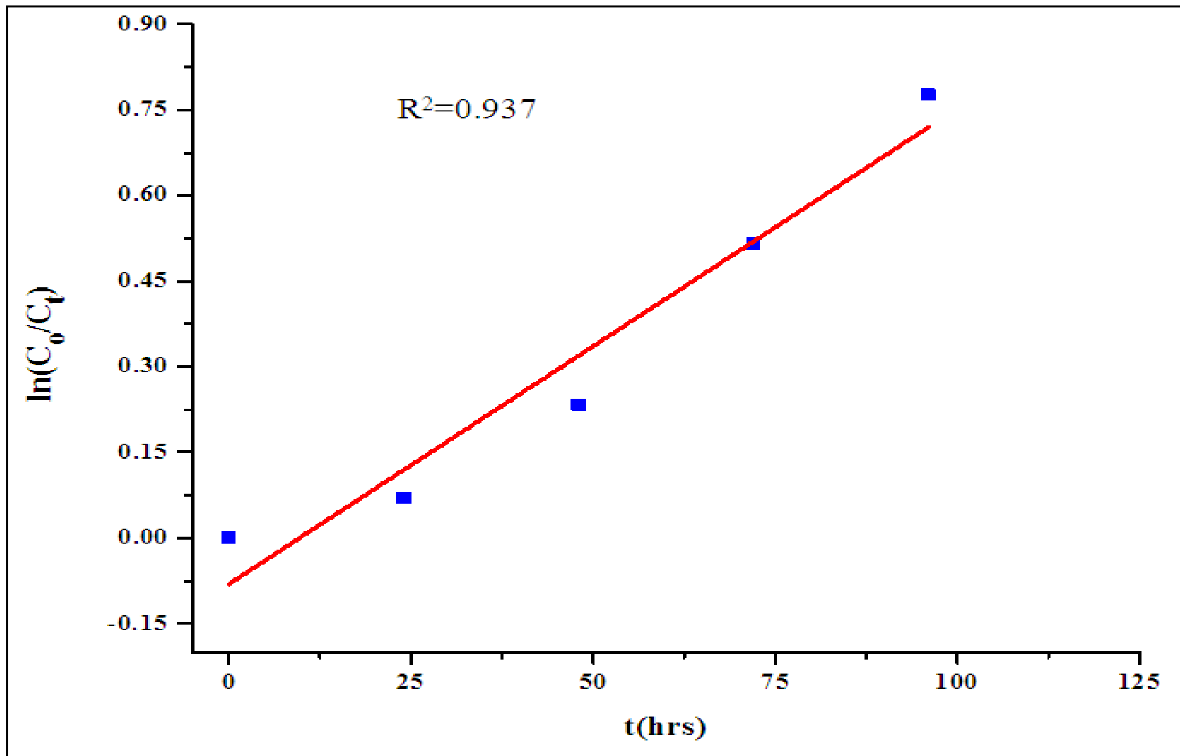
Similar kinetics for dye decolourisation have also been reported by Isik and Sponza (2004) and Karatas *et al.* (2009). The decolourisation of Methyl violet 2B using spent subsatrate of *A. bisporus* at 15 °C was observed to follow equation 2 and an almost linear plot of ln C_o/C_t versus t as shown in figure 6.1 was obtained. R^2 values were also near to unity, suggesting decolourisation to follow as a first order process. Figure 6.2 shows plot of ln C_0/C_t versus t for decolourisation of Methyl violet 2B using using spent subsatrate of *A. bisporus* at 25 °C and in this case also the plot is linear and R^2 values are also near to unity thus the decolourisation of Methyl violet 2B using using spent subsatrate of *A. bisporus* at 25 °C was also first order process.

6.1.1 Decolourisation kinetics of Methyl violet 2B using spent substrate of *Agaricus bisporus* at different temperature

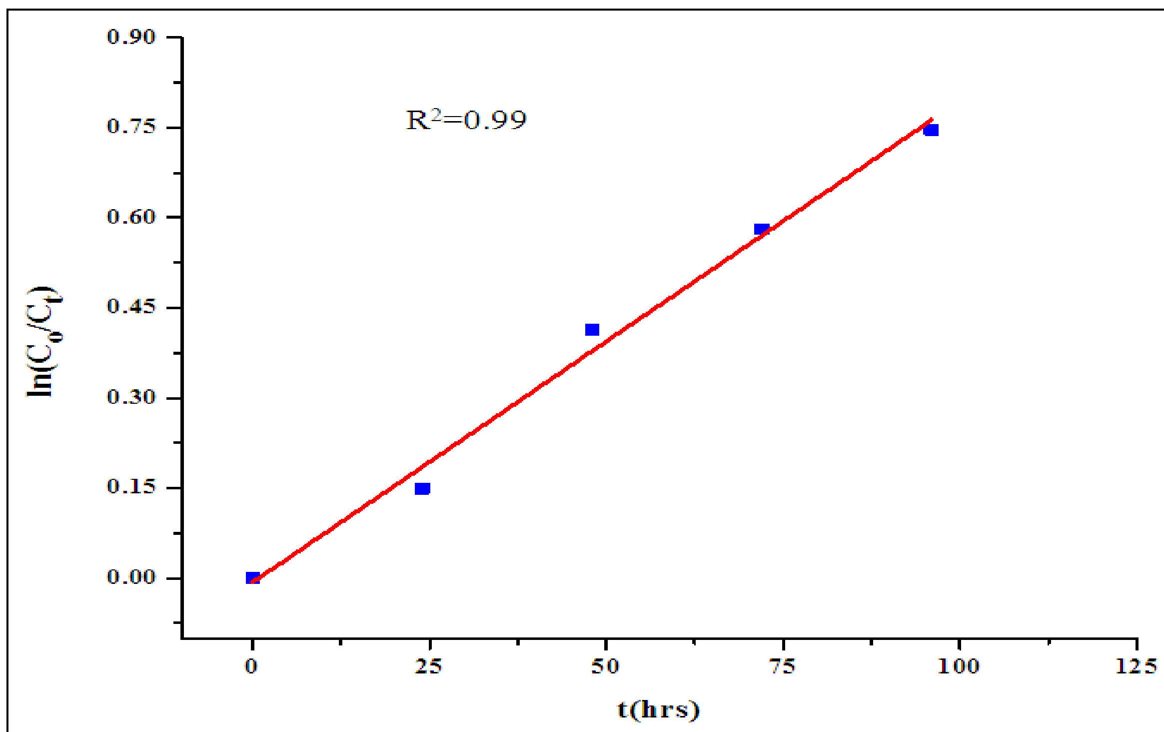
Spent substrate of *Agaricus bisporus* was used for the decolourisation of Methyl violet 2B and kinetics of decolourisation process was studied as a function of temperature. The decolourisation process was studied at temperature ranging from 15 to 35 °C for Methyl Violet 2B and highest decolourisation was recorded at 25 °C. The data obtained from the batch study with the UV-Visible double beam spectrophotometer were fitted in various orders i.e. zero, first and second order. The decolourisation process with spent substrate of *A. bisporus* was observed to follow first order kinetics as linear plots of ln (C_o/C_t) v decolourisation time were obtained and as depicted in fig 6.1. The R^2 value (Table 6.1) was also found to be good (>0.9), which is in agreement with first order kinetics for all cases

studied (Fig. 6.1). Hence, the decolourisation of dye using spent substrate of *A. bisporus* followed first order kinetics.

a)



b)



c)

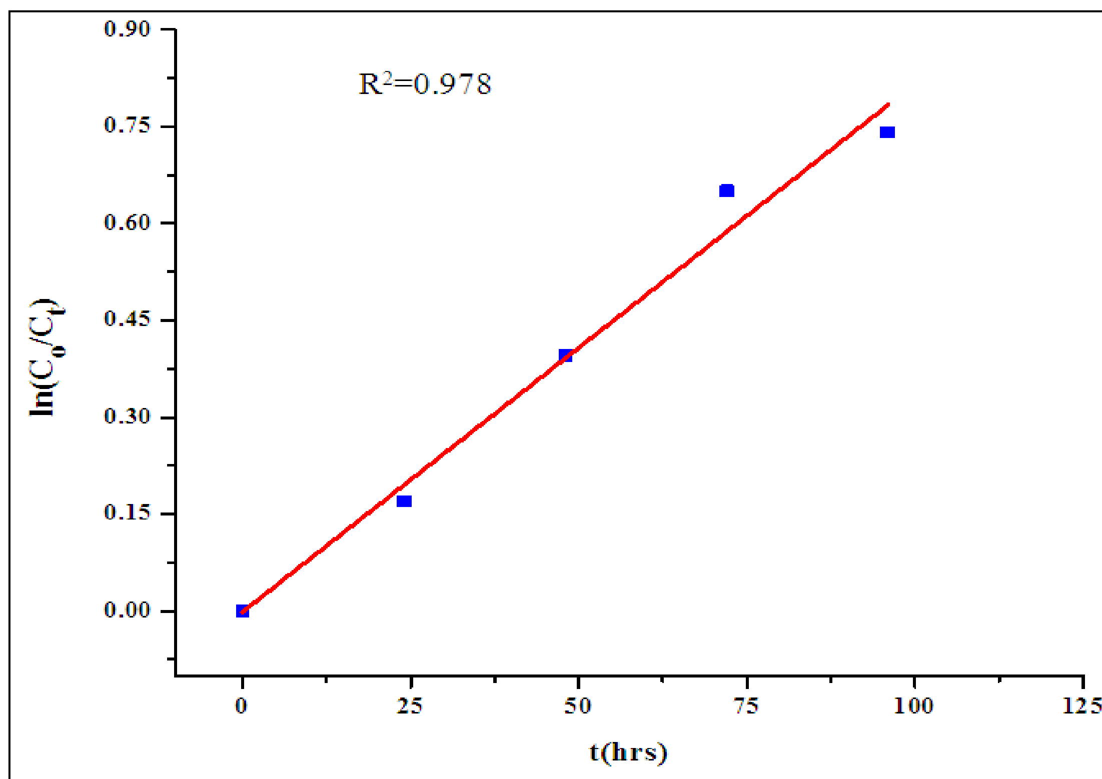
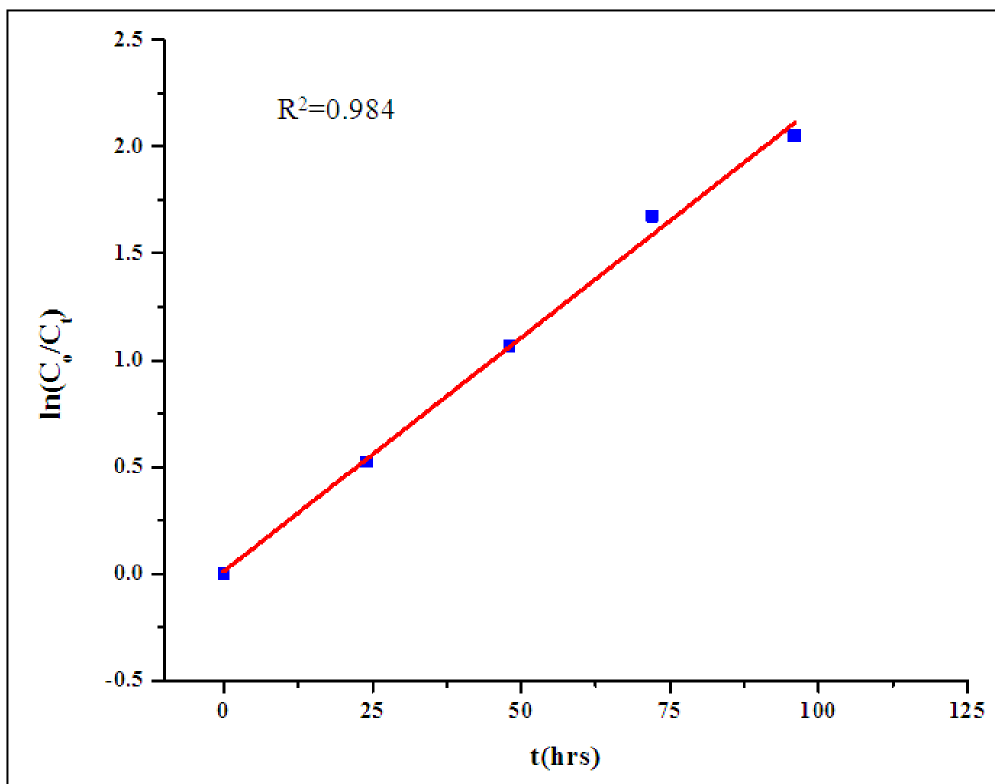


Fig. 6.1 Decolourisation kinetics of MV2B by spent substrate of *A. bisporus* at different temperatures a) 15 °C; b) 25 °C; c) 35 °C

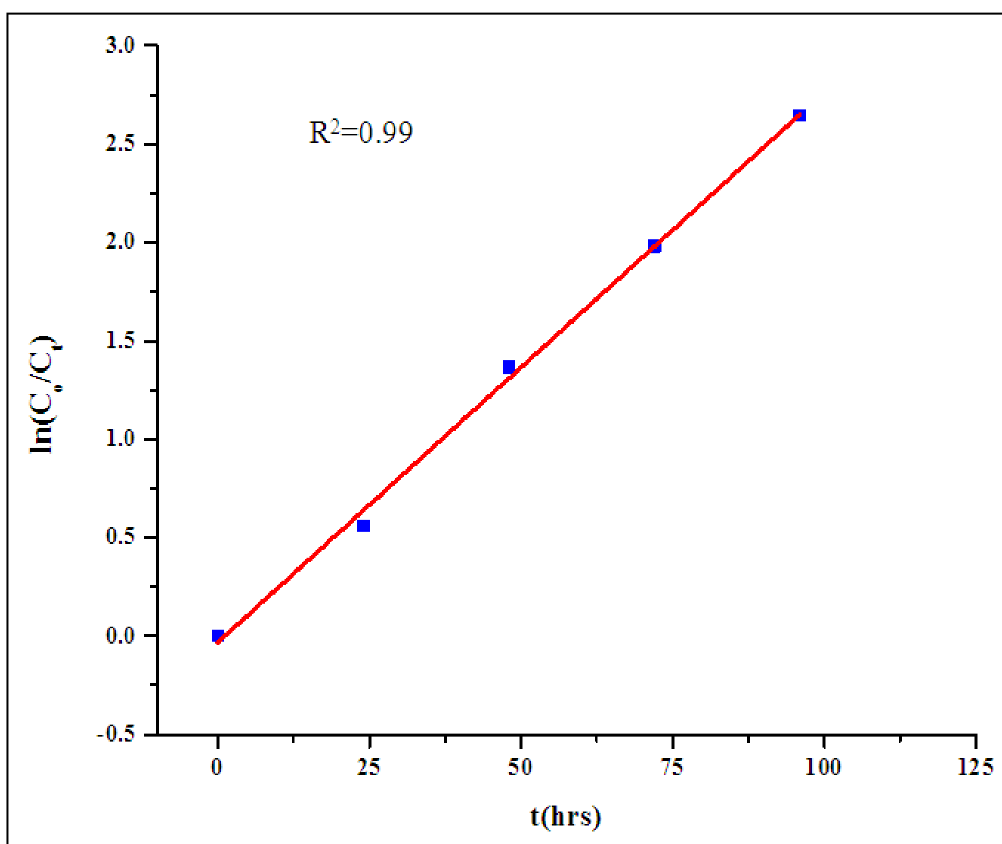
6.1.2 Decolourisation kinetics of Methyl violet 2B using spent substrate of *Agaricus bisporus* at different pH

Spent substrate of *Agaricus bisporus* was used for the decolourisation of Methyl violet 2B and kinetics of decolourisation process was studied as a function of pH values. The decolourisation process was studied at pH range of 4.0 to 10.0 and highest decolourisation was recorded at pH 10.0. The data obtained from the batch study with the UV-Visible double beam spectrophotometer were fitted in various orders i.e. zero, first and second order. The decolourisation process with spent substrate of *A. bisporus* was observed to follow first order kinetics as linear plots of ln (C_o/C_t) v decolourisation time were obtained and as depicted in figure 6.2. The R^2 value (Table 6.1) was also found to be good (>0.9), which is in agreement with first order kinetics for all cases studied (Fig. 6.2). Hence, the decolourisation of dye using spent substrate of *A. bisporus* followed first order kinetics.

a)



b)



.c)

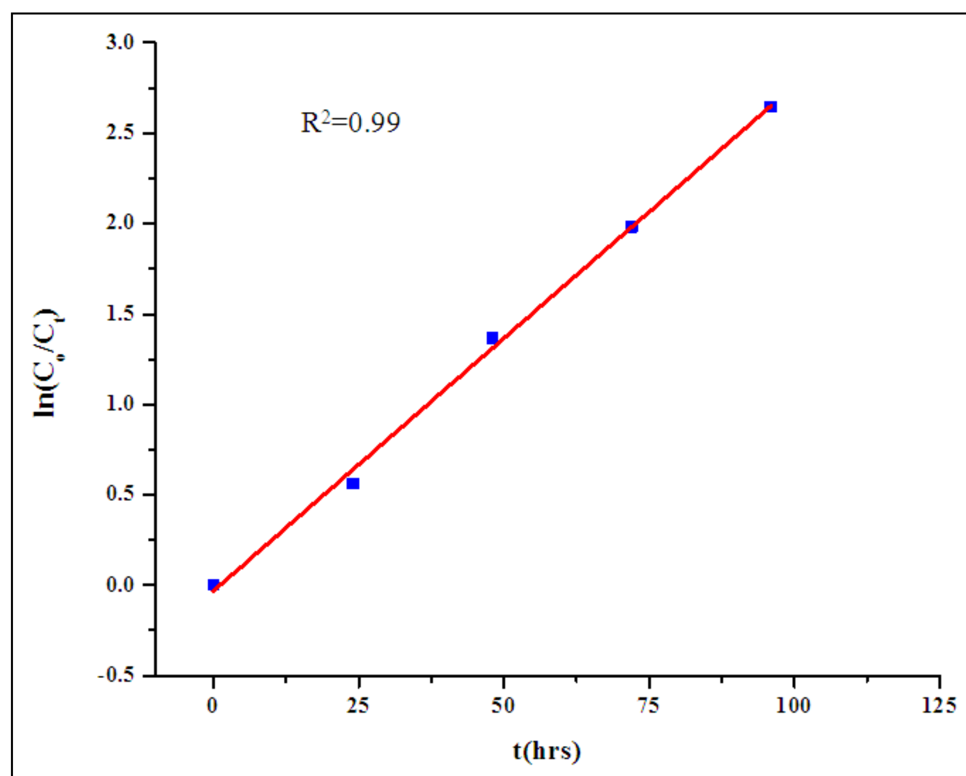


Fig. 6.2 Decolourisation kinetics of MV2B by spent substrate of *A. bisporus* at different pH a) pH 4.0; b) pH 7.0; c) pH 10.0

Table 6.1 Regression coefficient (R^2) of dyes decolourisation kinetics by SMS

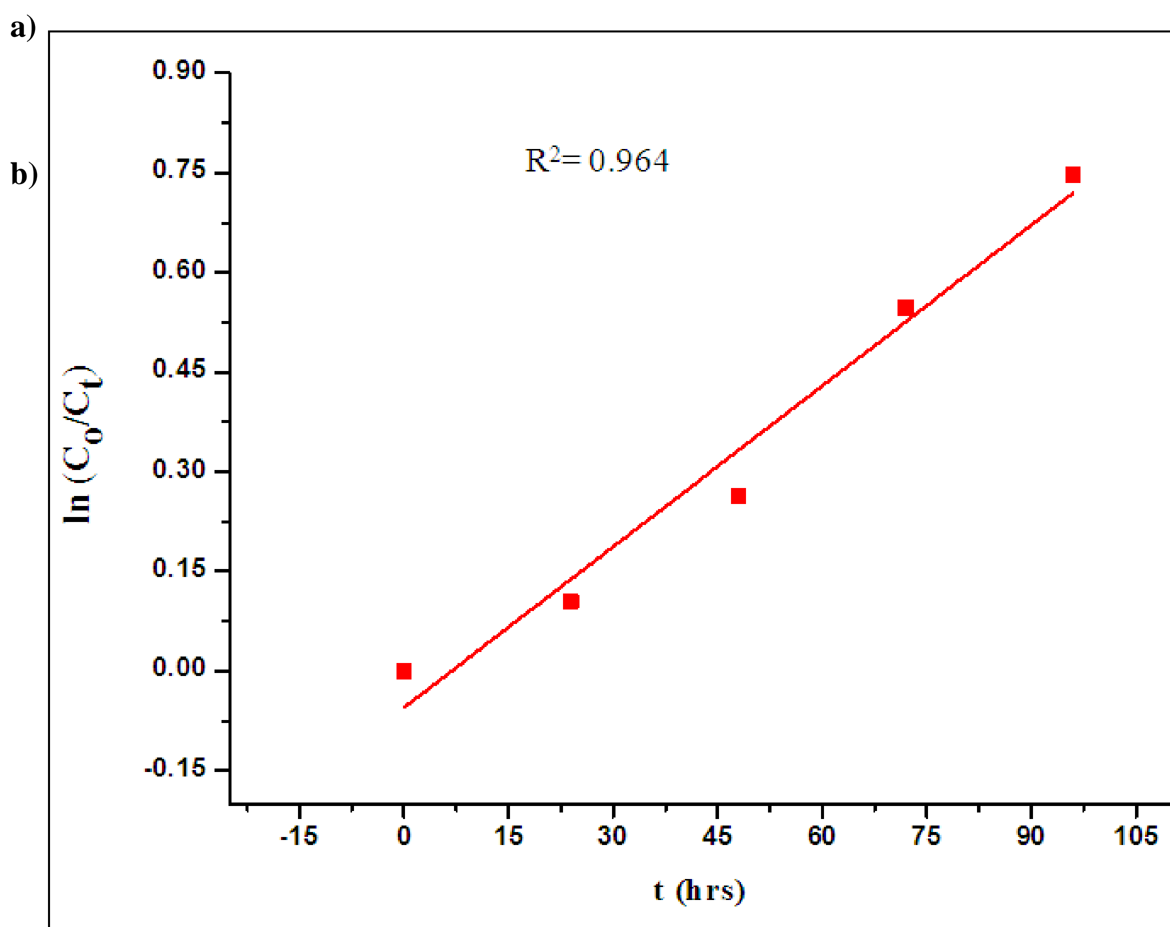
Treatment		Spent substrate of *Pleurotus sajor-caju* + Methyl violet 2B Regression coefficient (R^2)	Spent substrate of *Agaricus bisporus* + Methyl violet 2B Regression coefficient (R^2)
Temperature (°C)	15	0.964	0.937
	25	0.952	0.990
	35	0.944	0.978
pH	4.0	0.973	0.984
	7.0	0.998	0.990
	10.0	0.978	0.990

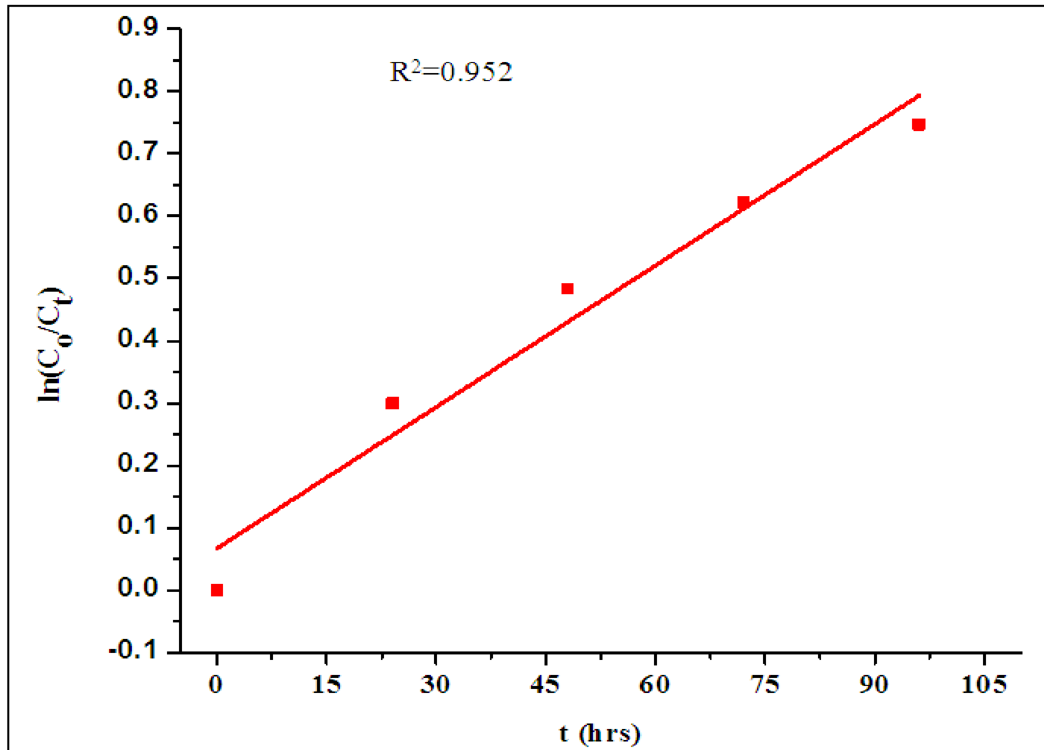
6.1.3 Decolourisation kinetics of Methyl violet 2B using spent substrate of *Pleurotus sajor-caju* at different temperatures

The first order kinetics model was applied to the experimental data of degradation of Methyl violet 2B using spent substrate of *Pleurotus sajor-caju* to verify the performance of the model in temperature ranging from 15 to 35 °C. Decolourisation time versus ln (C_o/C_t) were plotted for the data of decolourisation of dye at various temperatures (Fig. 6.3). The linear plots of ln (C_o/C_t) versus decolourisation time and regression coefficient R^2 lying close to unity, justify the use of first order kinetics. The regression coefficient (R^2) at different temperatures 15, 25 and 35 °C was 0.964, 0.952 and 0.944, respectively (Table 6.1). The highest removal of Methyl violet 2B using spent substrate of *P. sajor-caju* was obtained at 25 °C and the decolourisation process was first order.

a)

b)





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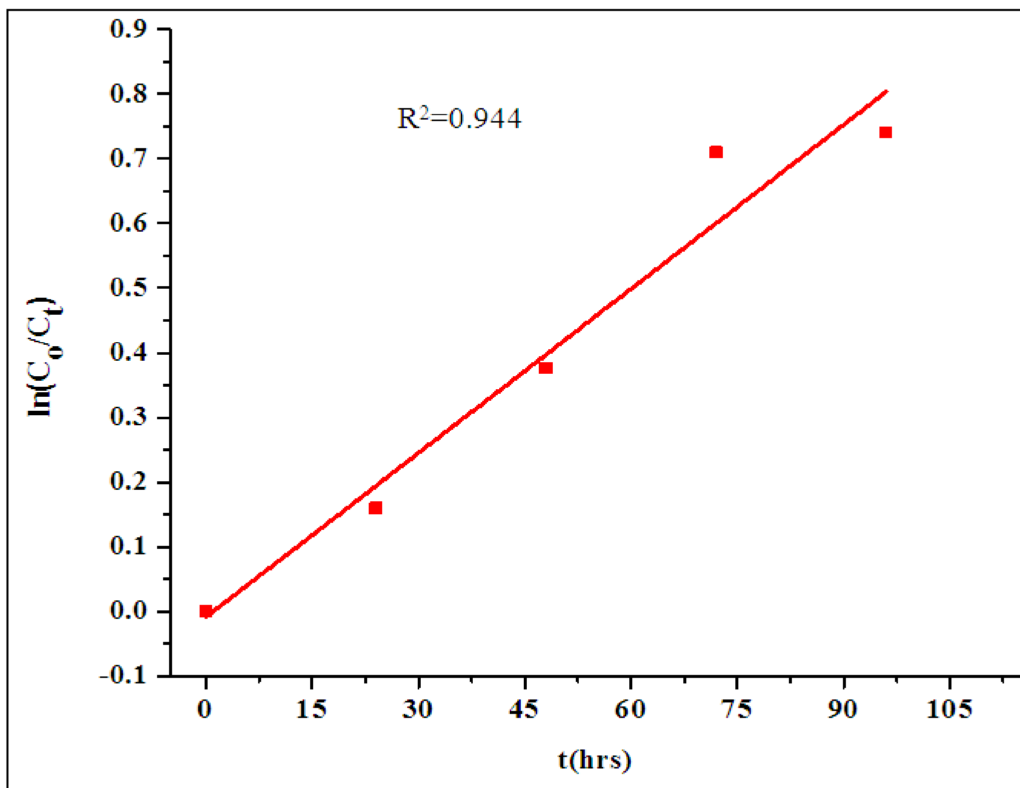
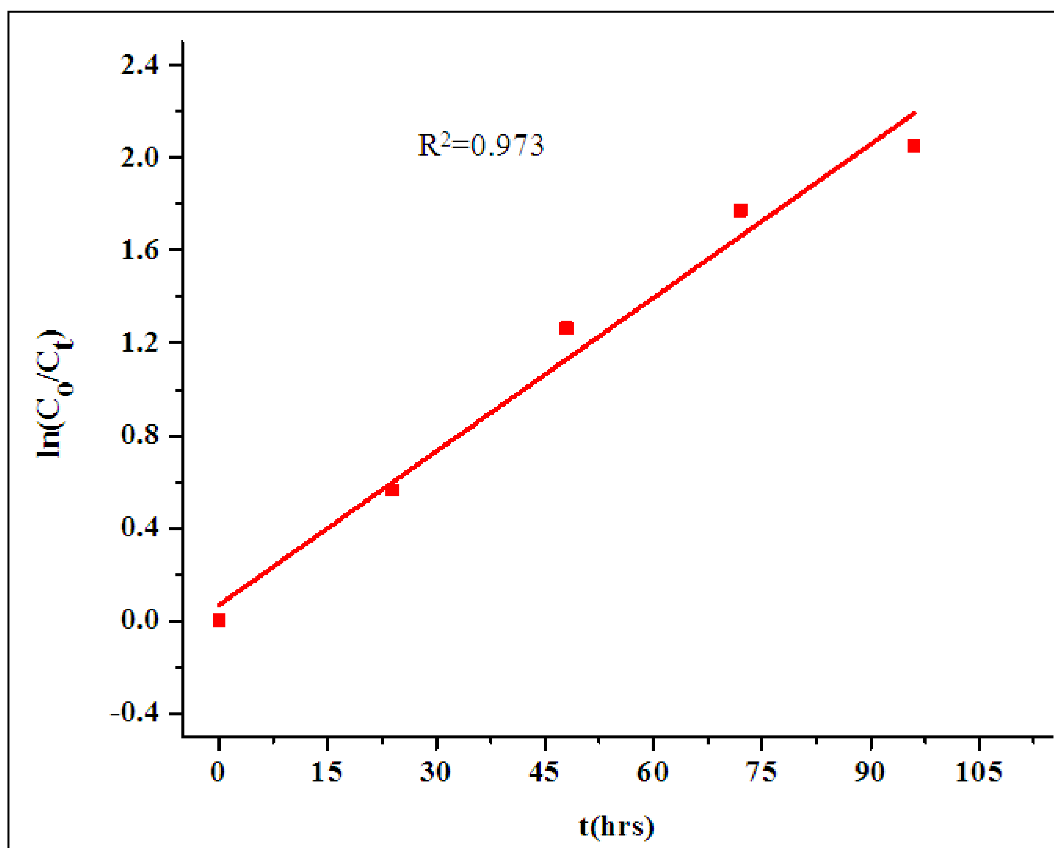


Fig. 6.3 Decolourisation kinetics of Methyl violet 2B using spent substrate of *Pleurotus sajor-caju* at different temperature: a) 15°C; b) 25°C; c) 35 °C

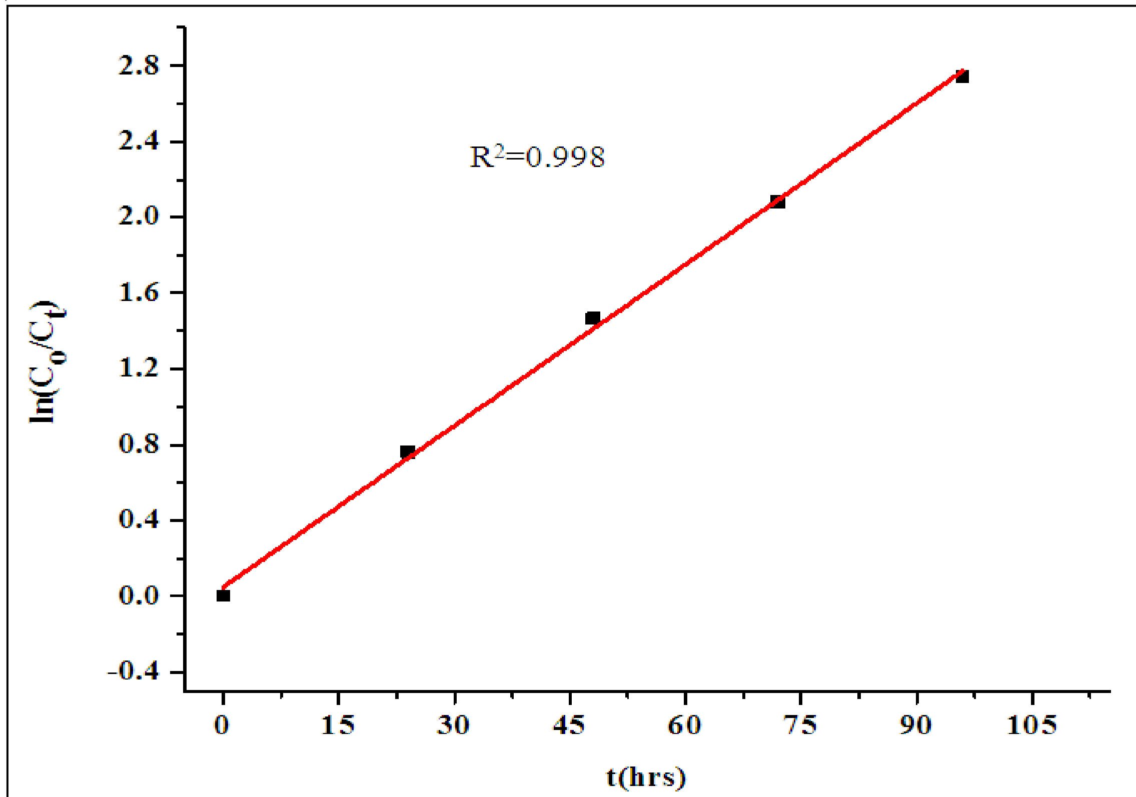
6.1.4 Decolourisation kinetics of Methyl violet 2B using spent substrate of *Pleurotus sajor-caju* at different pH

The various kinetics models were applied to the experimental data to verify the decolourisation kinetics of Methyl violet 2B with spent substrate of *P. sajor-caju* at pH values of 4.0, 7.0, and 10.0. Dye decolourisation time versus ln (C_o/C_t) was plotted for the recorded data obtained from decolourisation of dye at different time intervals at all the tested pH. Here again we obtained linear plots of ln (C_o/C_t) versus decolourisation time at all the pH values (Fig. 6.4). The Regression coefficient (R^2) was 0.973, 0.998 and 0.978 at pH 4.0, 7.0, and 10.0, respectively (Table 6.1). The values of R^2 in the proximity of unity also confirm that the decolourisation of Methyl violet 2B using spent substrate of *Pleurotus sajor-caju* followed first order kinetics.

a)



b)



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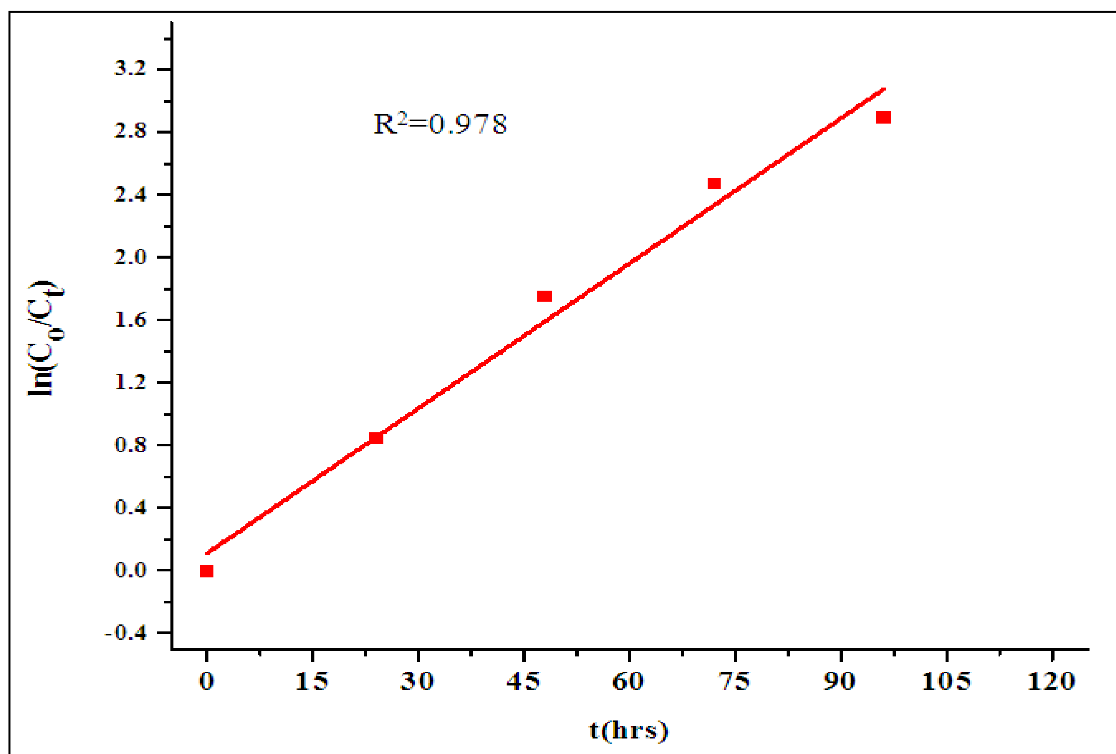
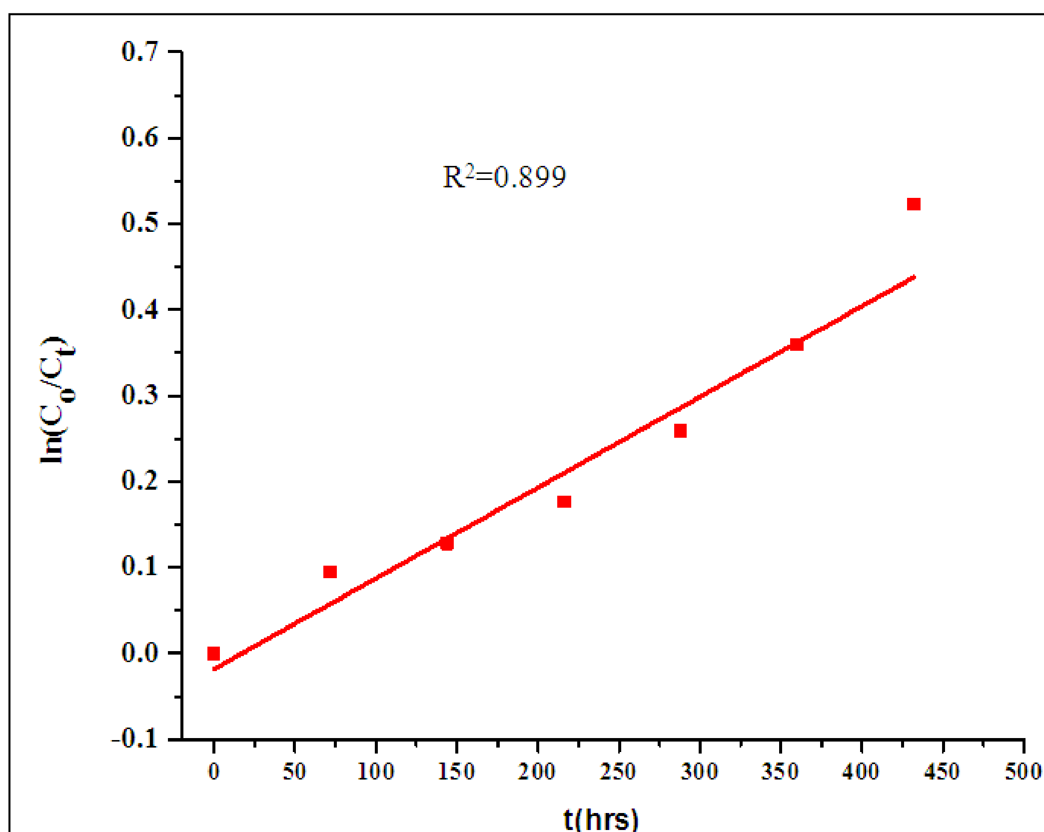


Fig. 6.4 Decolourisation kinetics of Methyl violet 2B using spent substrate of *Pleurotus sajor-caju* at different pH: a) 4.0; b) 7.0; c) 10.0

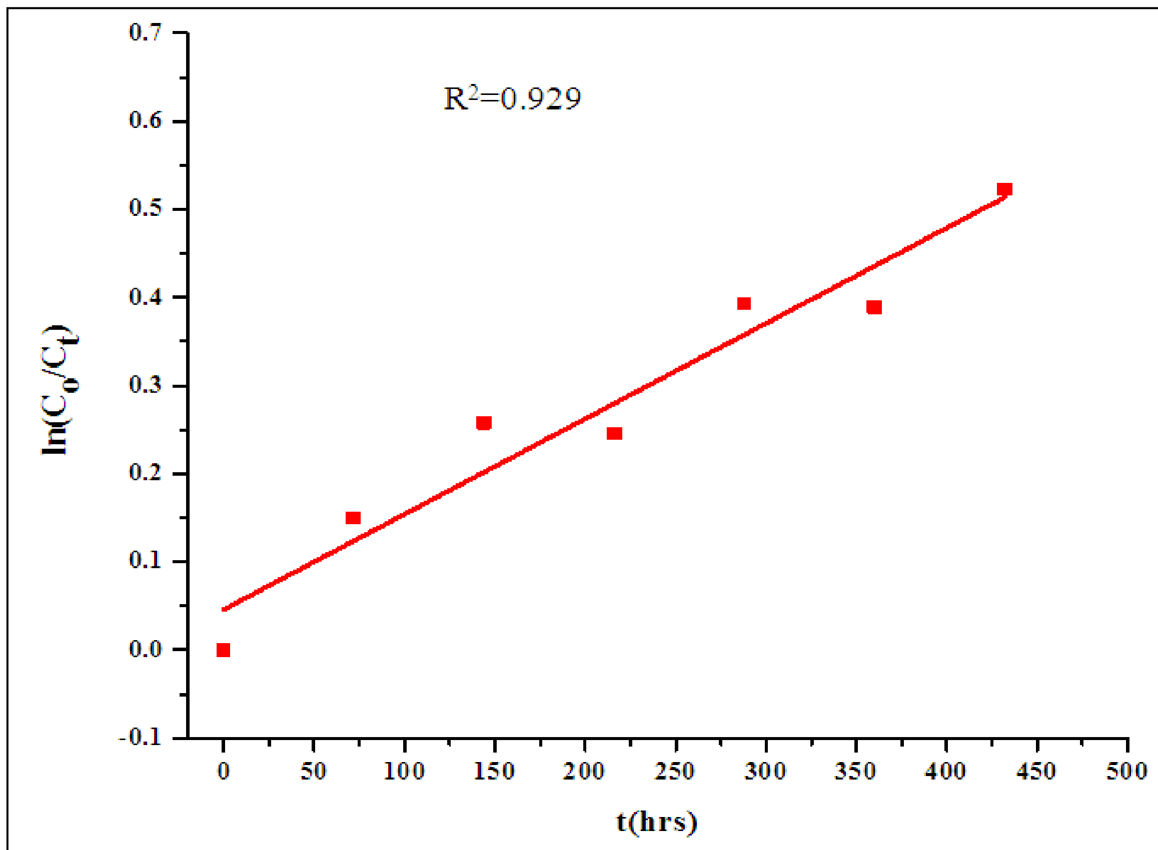
6.1.5 Decolourisation kinetics of Methyl violet 2B with *Pezizomycotina* sp. at different temperatures

The first order kinetics model was applied to the experimental data of decolourisation of Methyl violet 2B using *Pezizomycotina* sp. at temperatures of 15, 20, 25, 30 and 35 °C. Dye decolourisation time versus ln (C_o/C_t) was plotted for the data obtained from decolourisation of dye at all the tested temperatures. *Pezizomycotina* sp. showed potential for decolourisation of Methyl violet 2B at these temperatures (Fig. 6.4). Regression coefficient (R^2) value were also found to be good (>0.9) for all temperature treatments of Methyl violet 2B decolourisation. The Regression coefficient (R^2) at temperature 15, 20, 25, 30 and 35 °C was 0.899, 0.929, 0.952, 0.981 and 0.968, respectively (Table 6.2). The close to unity value of R^2 confirms that the decolourisation of Methyl violet 2B using *Pezizomycotina* sp. followed first order kinetics as in earlier cases.

a)



b)



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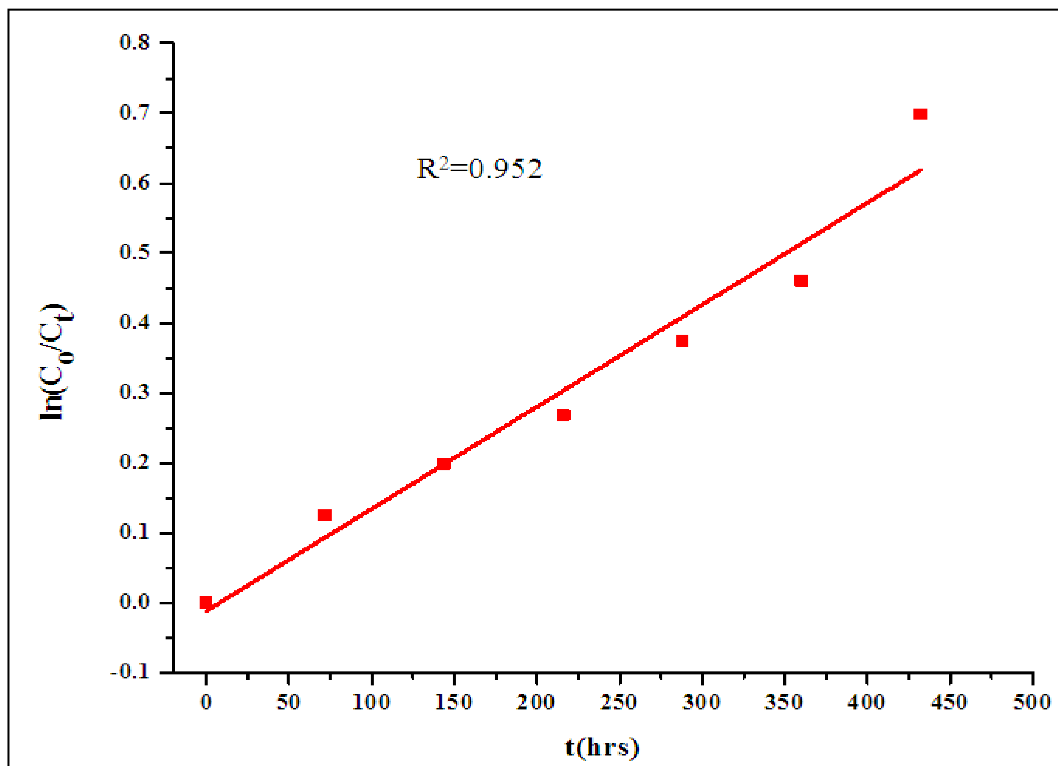

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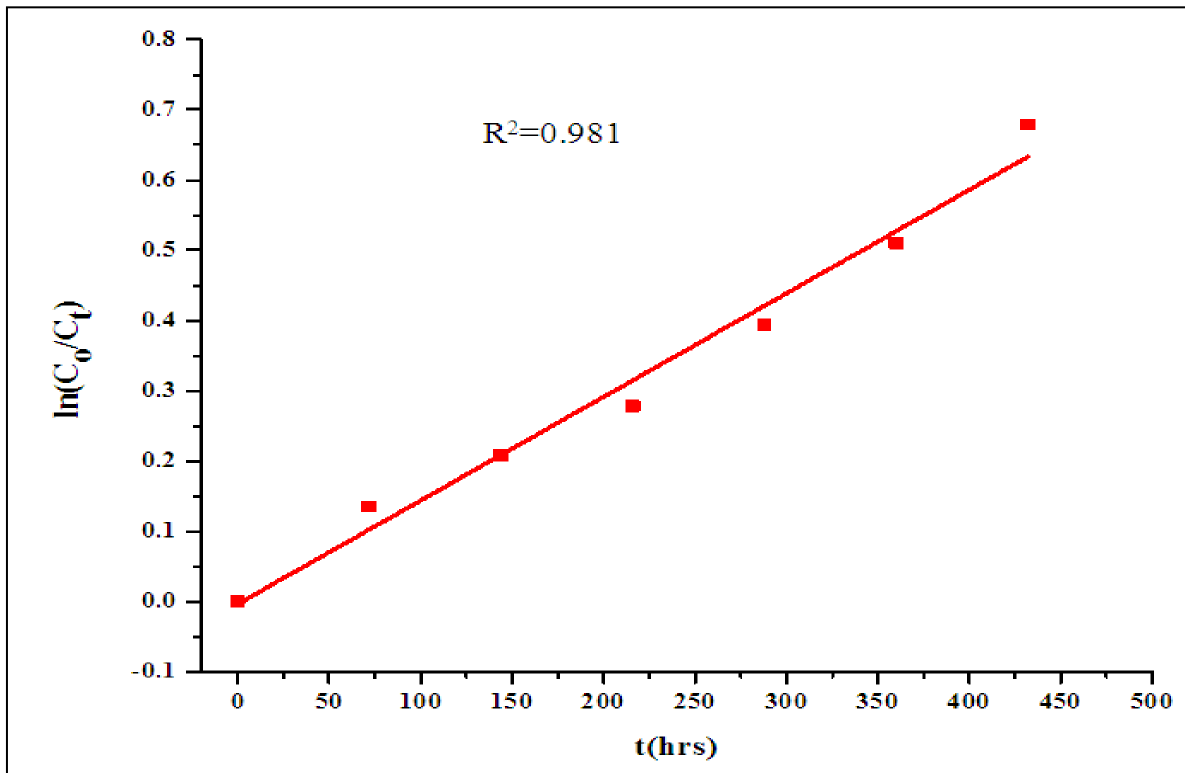

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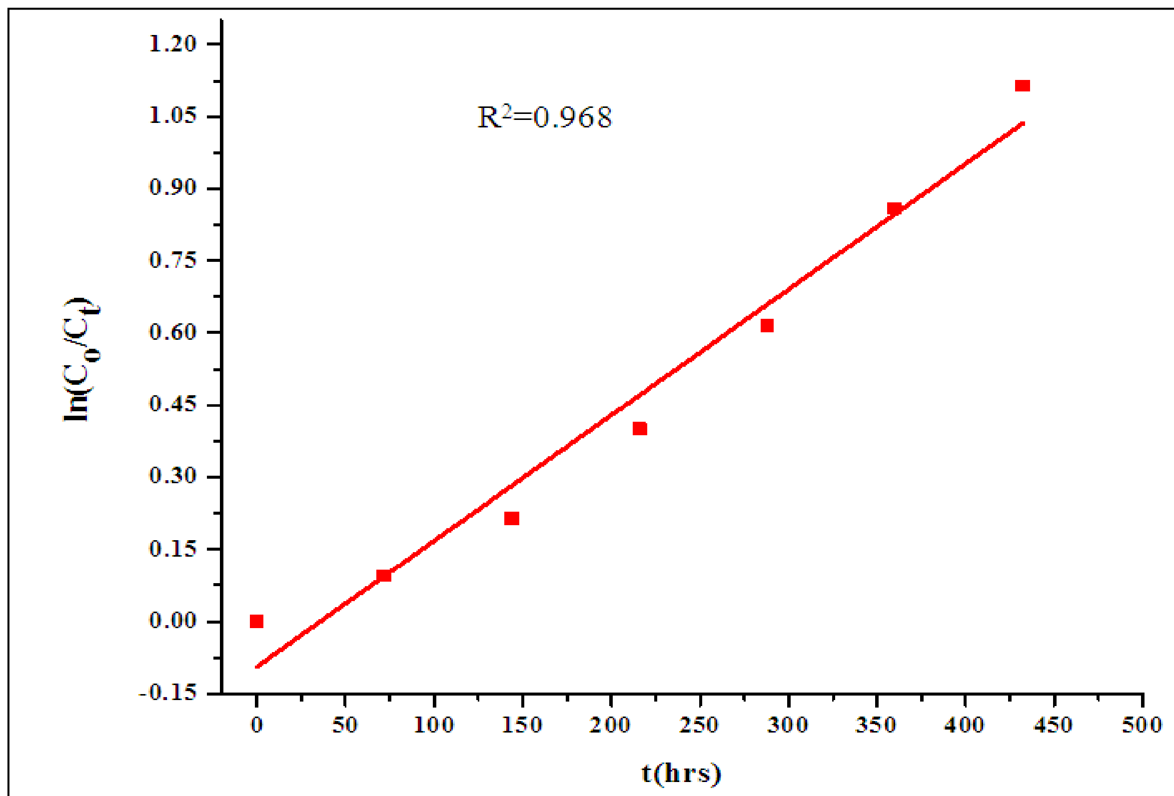
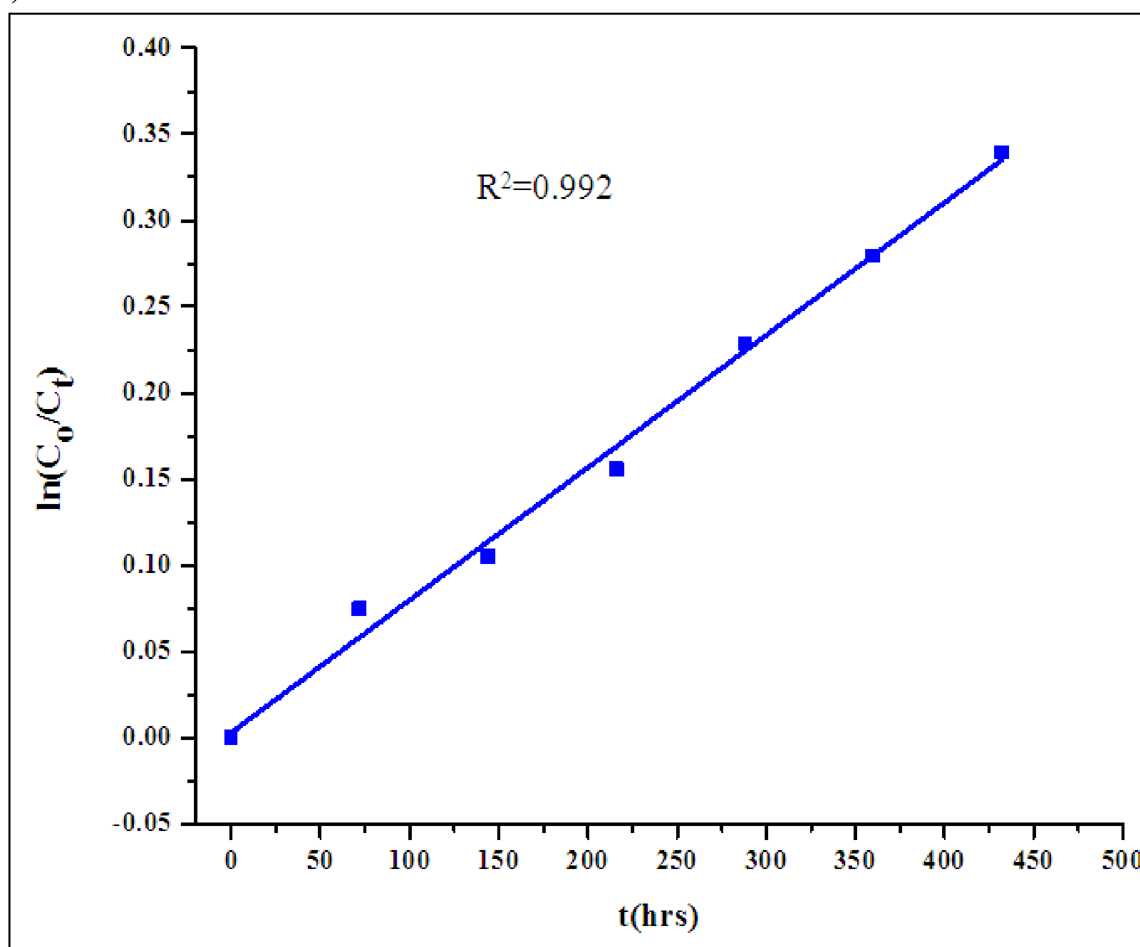


Fig. 6.5 Decolourisation kinetics of Methyl violet 2B with *Pezizomycotina* sp. at different temperatures a) 15 °C; b) 20 °C; c) 25 °C; d) 30 °C; e) 35 °C

6.1.6 Decolourisation kinetics of Methyl violet 2B with *Pezizomycotina* sp. at different pH

The first order kinetics model was applied to the experimental data of decolourisation of Methyl violet 2B with *Pezizomycotina* sp. at pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. Highest decolourisation was recorded at pH 9.0. Dye decolourisation time versus ln (C_o/C_t) was plotted for the data obtained from decolourisation of Methyl violet 2B at all the tested pH (Fig. 6.6). The Regression coefficient (R^2) at pH values 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 was 0.992, 0.952, 0.956, 0.925, 0.954, 0.966 and 0.969, respectively (Table 6.2). The R^2 values approaching to unity for the first order indicate that dye decolourisation with *Pezizomycotina* sp. also followed first order kinetics as in earlier cases.

a)



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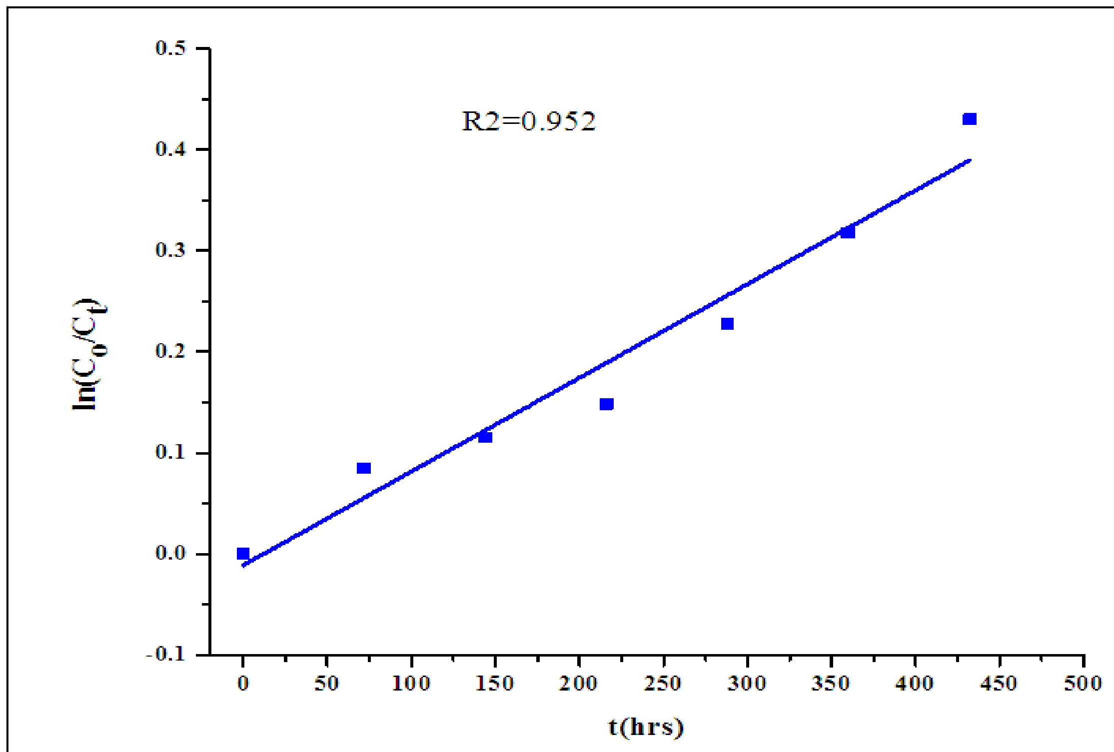

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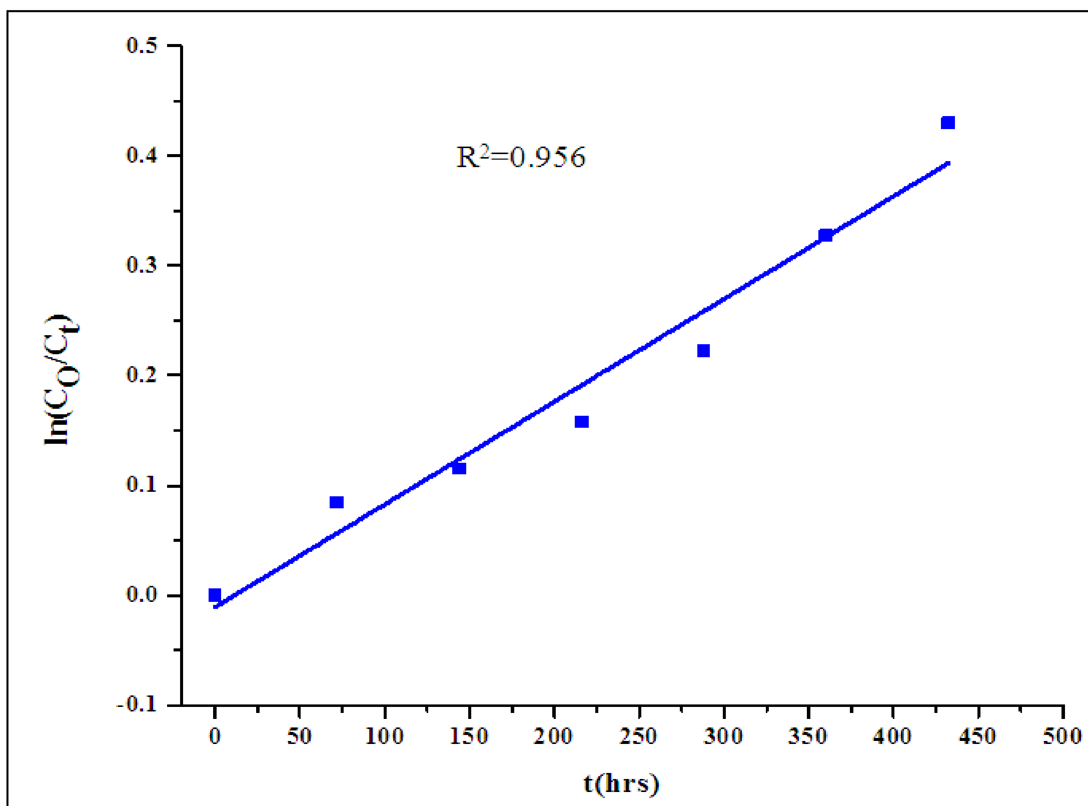

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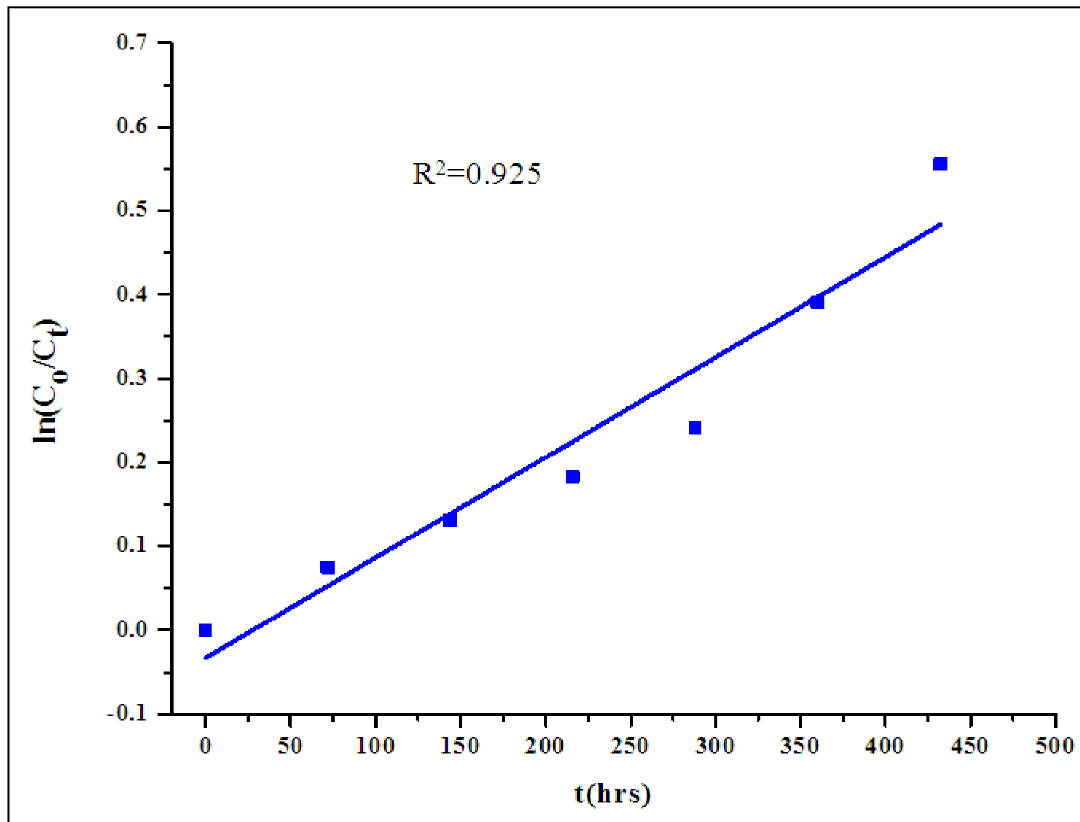

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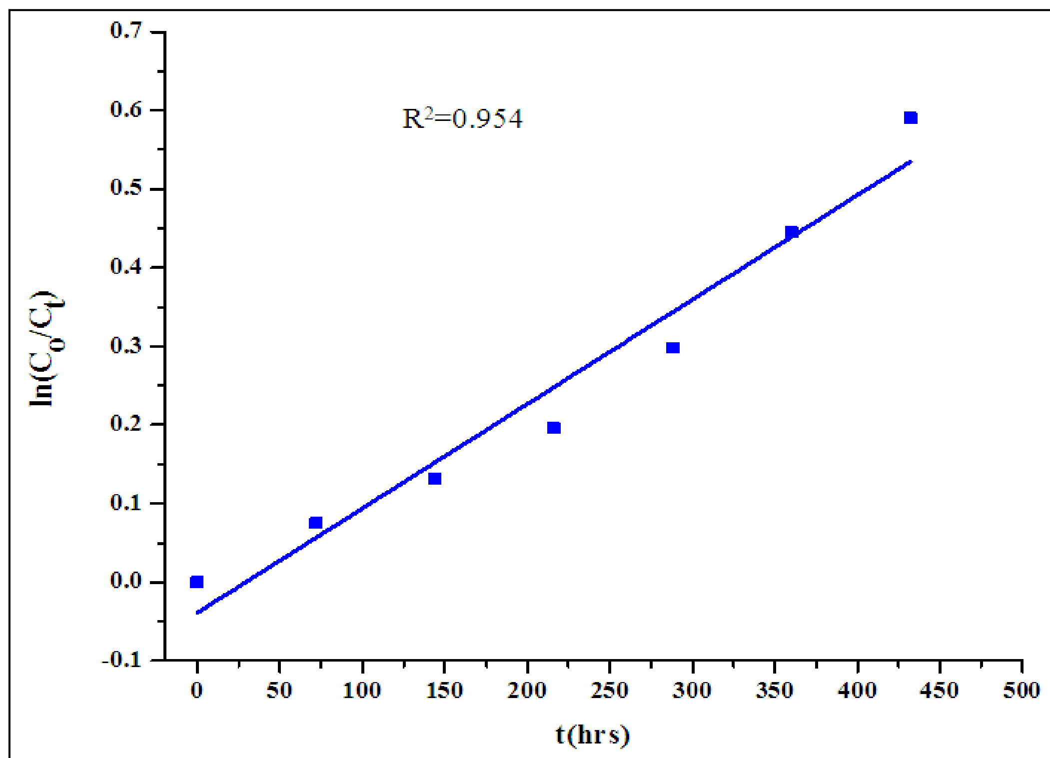

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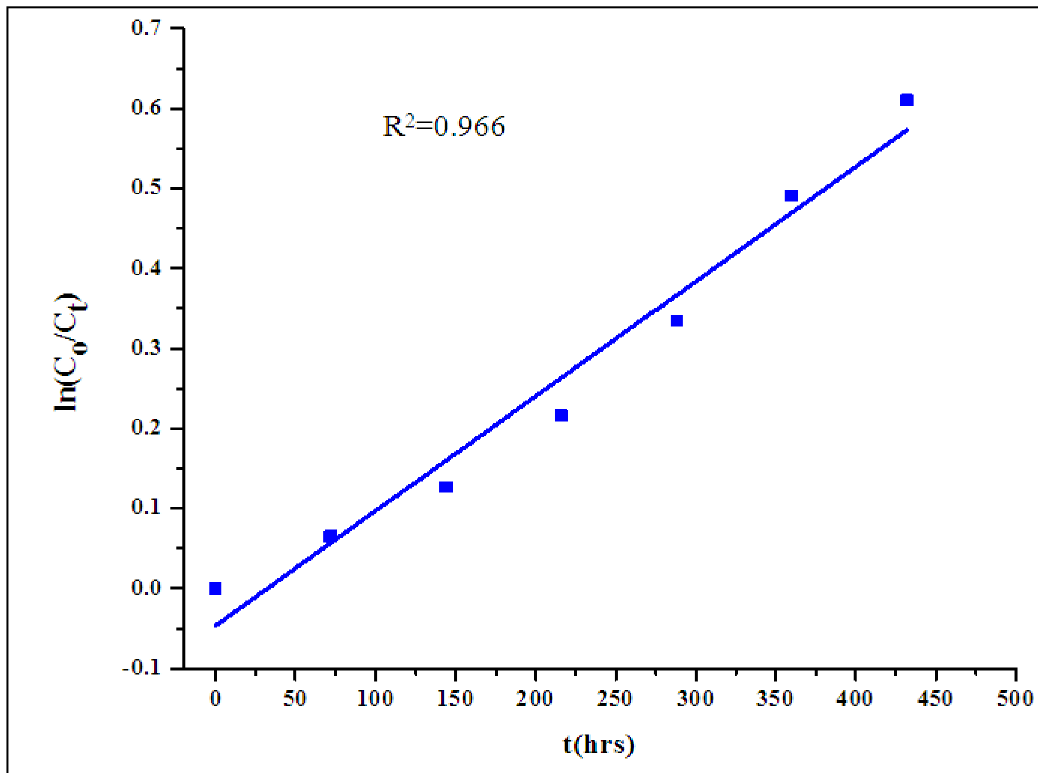


g)

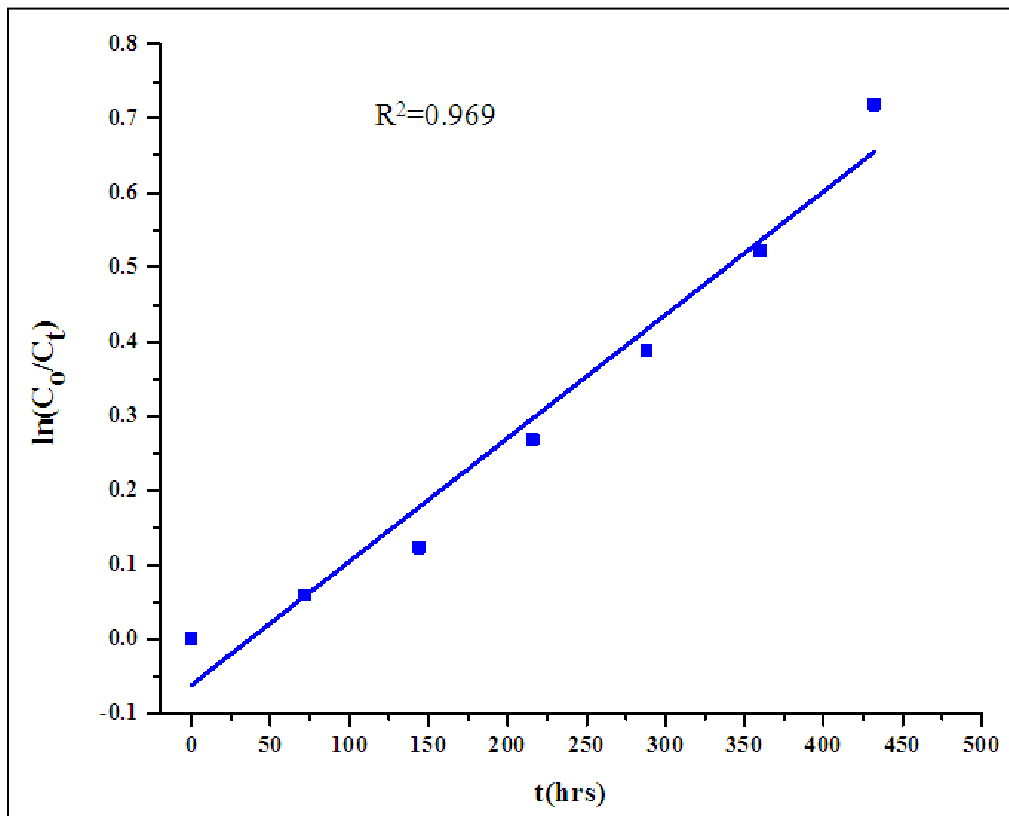


Fig. 6.6 Decolourisation kinetics of Methyl violet 2B with *Pezizomycotina* sp. at different pH : a) 4.0; b) 5.0; c) 6.0; d) 7.0; e) 8.0; f) 9.0; g) 10.0

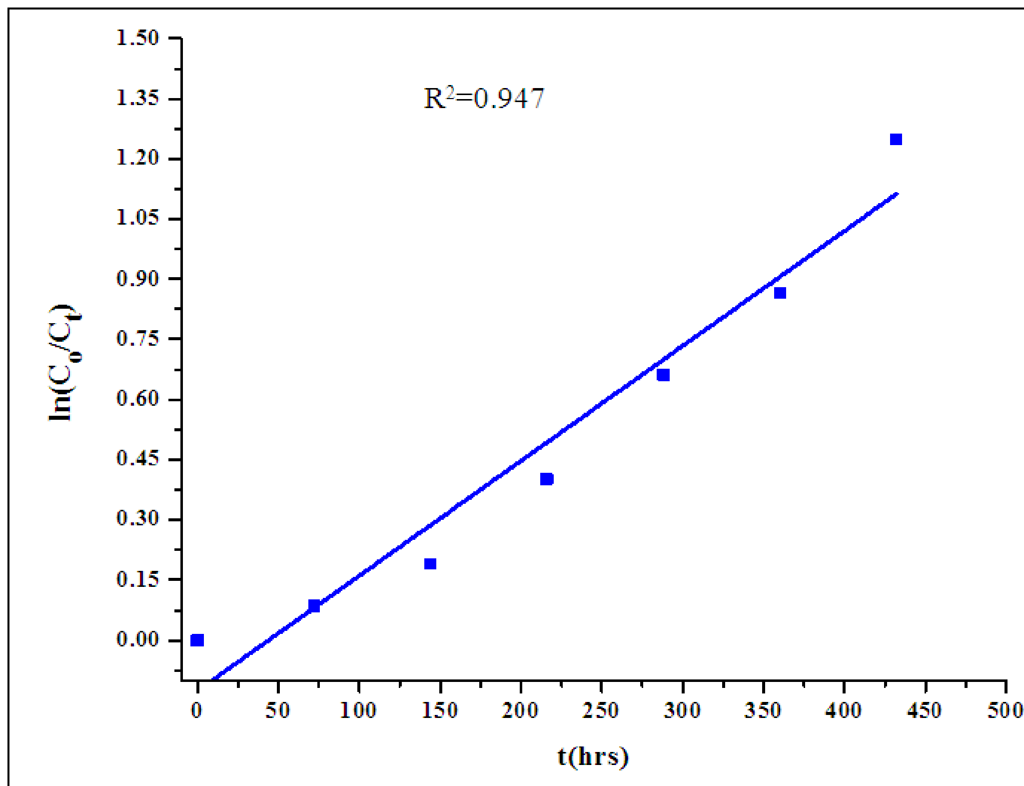
Table 6.2 Regression coefficient (R^2) of dyes decolourisation kinetics using fungi

Treatment		*Schizophyllum commune* + Methyl violet 2B Regression coefficient (R^2)	*Pezizomycotina* sp.+ Methyl violet 2B Regression coefficient (R^2)
Temperature (°C)	15	0.947	0.899
	20	0.962	0.929
	25	0.961	0.952
	30	0.974	0.981
	35	0.950	0.968
pH	4.0	0.930	0.992
	5.0	0.937	0.952
	6.0	0.958	0.956
	7.0	0.965	0.925
	8.0	0.983	0.954
	9.0	0.942	0.966
	10.0	0.956	0.969

6.1.7 Decolourisation kinetics of Methyl violet 2B with *Schizophyllum commune* at different temperatures

The first order kinetics model was applied to the experimental data of Methyl violet 2B decolourisation with *Schizophyllum commune* at different temperatures of 15, 20, 25, 30 and 35 °C. ln (C_o/C_t) versus decolourisation time was plotted for the data obtained from decolourisation of dye at all the tested temperatures (Fig. 6.7). The Regression coefficient (R^2) for first order at temperature 15, 20, 25, 30 and 35 °C was 0.947, 0.962, 0.961, 0.974 and 0.950, respectively (Table 6.2). The R^2 value of decolourisation of Methyl violet 2B with *Schizophyllum commune* indicates the fitness of the first order model. Hence, dye decolourisation with *Schizophyllum commune* also followed first order kinetics.

a)



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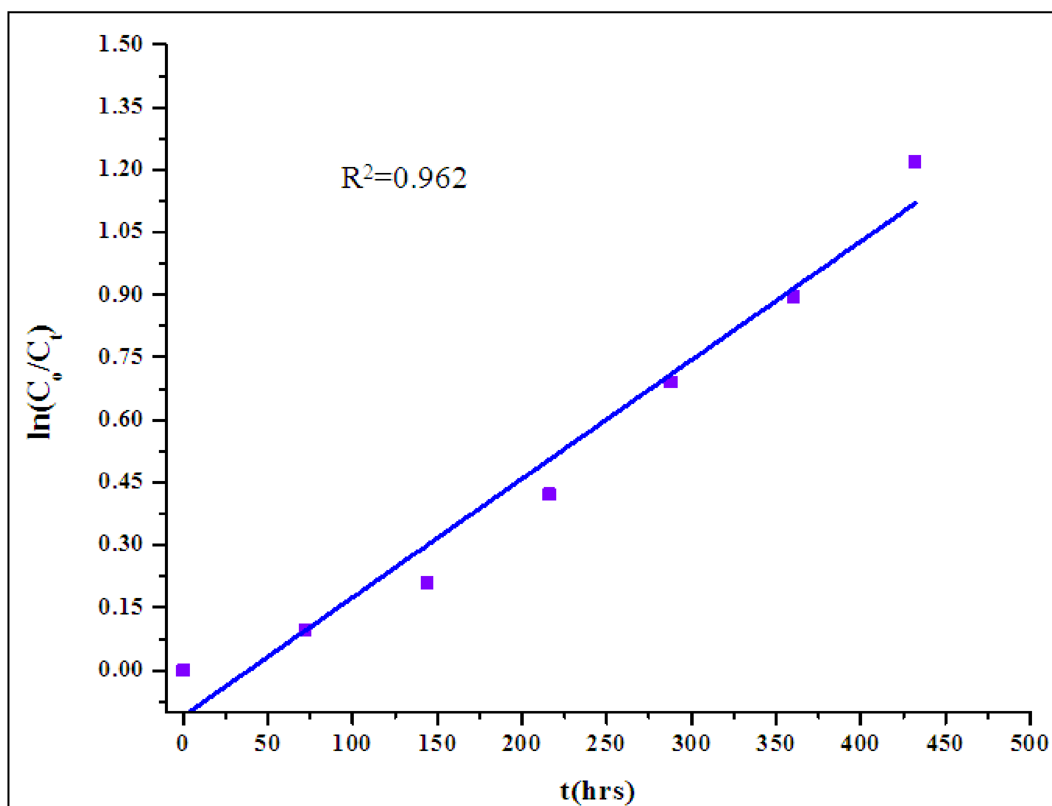

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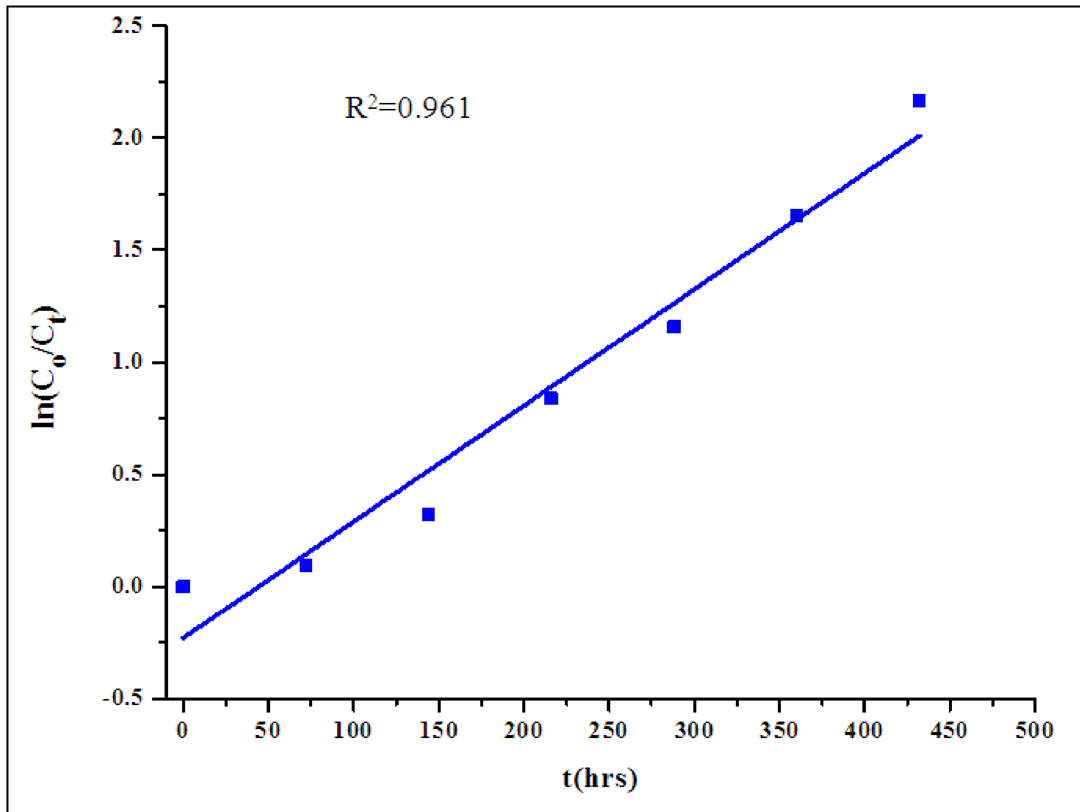

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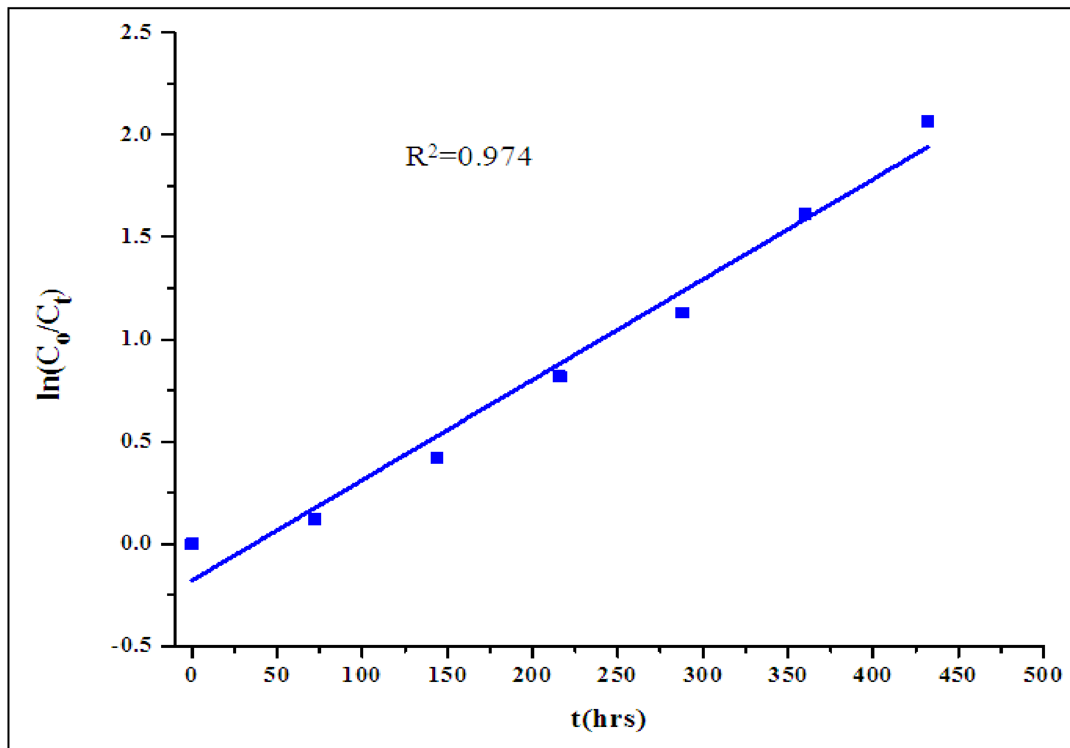

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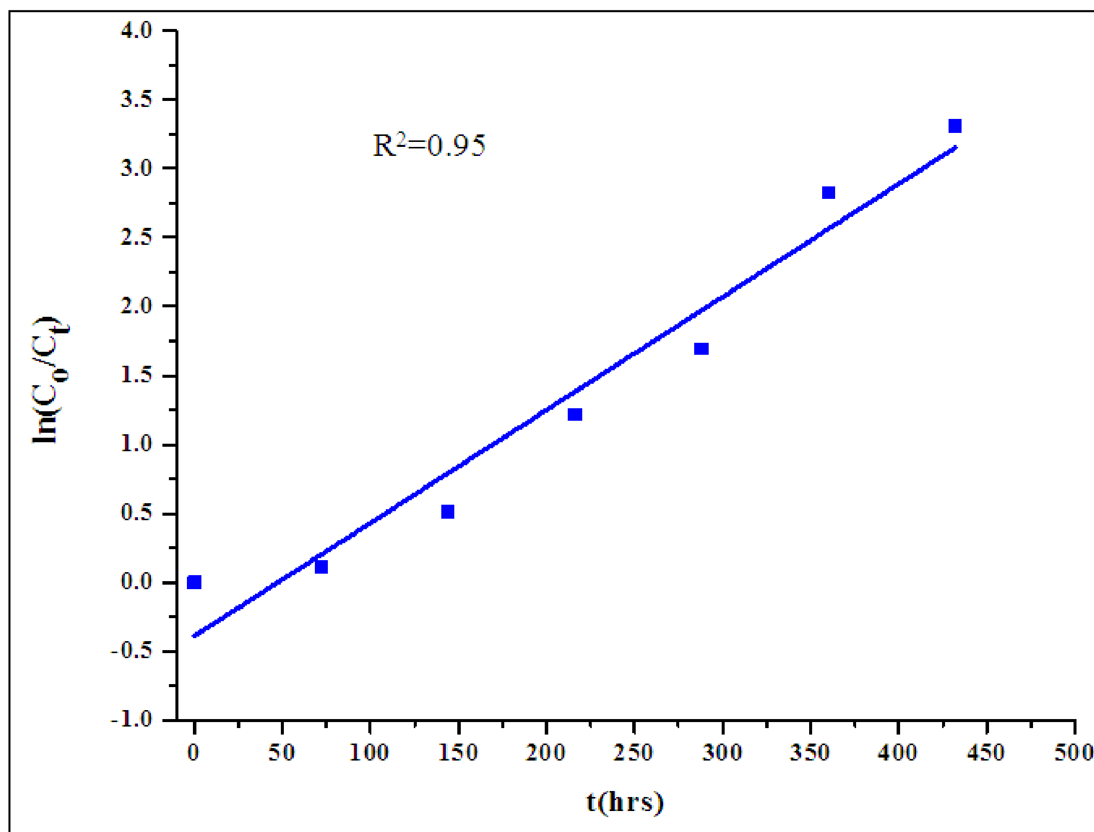
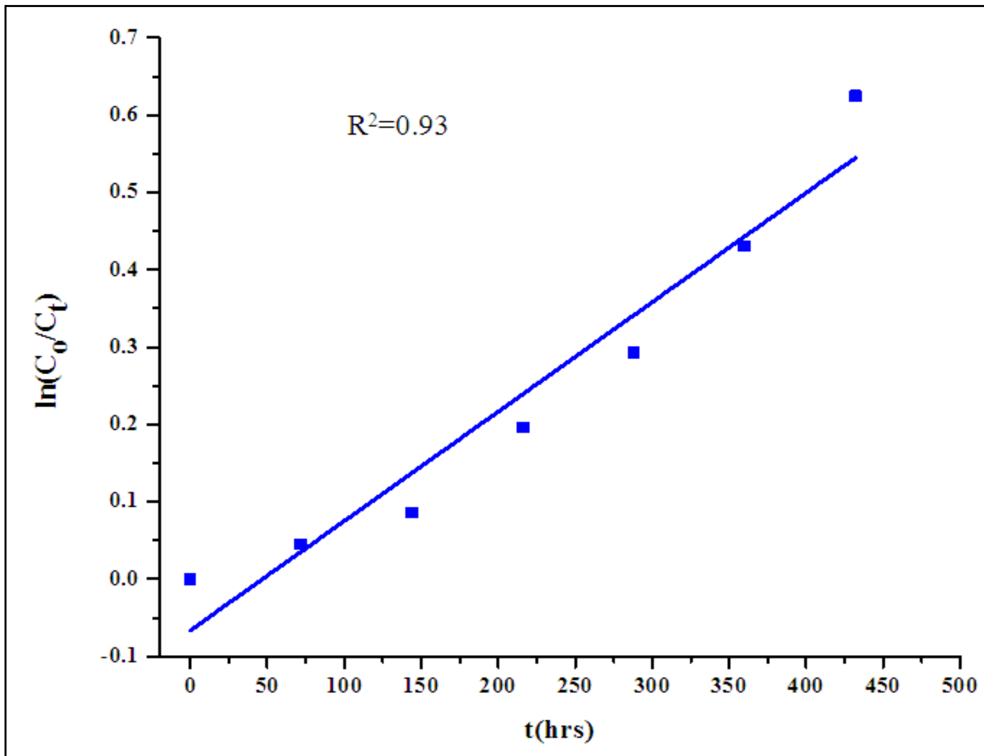


Fig. 6.7 Decolourisation kinetics of Methyl violet 2B by *Schizophyllum commune* at temperatures a) 15 °C; b) 20 °C; c) 25 °C ; d) 30 °C; e) 35 °C

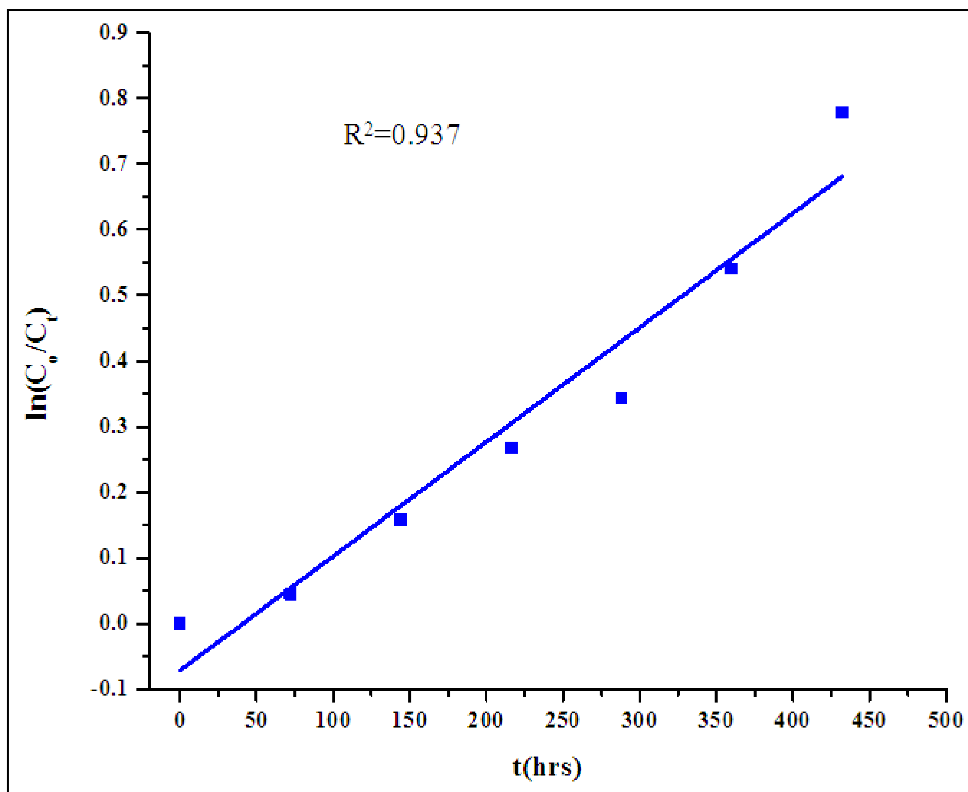
6.1.8 Decolourisation kinetics of Methyl violet 2B with *Schizophyllum commune* at different pH

The first order kinetics model was applied to the experimental data obtained from decolurization of Methyl violet 2B with *Schizophyllum commune* to verify the performance of the model at pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. ln (C_o/C_t) versus decolourisation time was plotted for the data obtained from decolourisation of dye at all the tested pH values (Fig. 6.8). The Regression coefficient (R^2) for first order at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 was 0.930, 0.937, 0.958, 0.965, 0.983, 0.942 and 0.956, respectively (Table 6.2). The R^2 value close to unity indicates the fitness of the first order kinetics model for the decolourisation Methyl violet 2B with *Schizophyllum commune*. Hence, dye decolourisation with *S. commune* also followed first order kinetics as in earlier cases.

a)



b)



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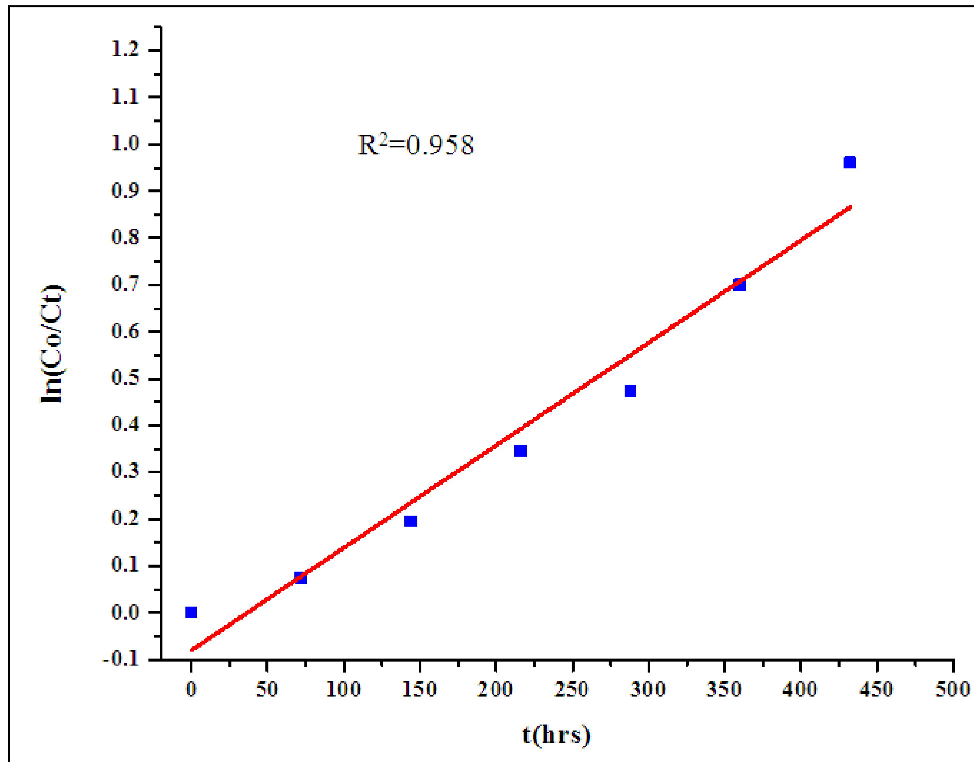

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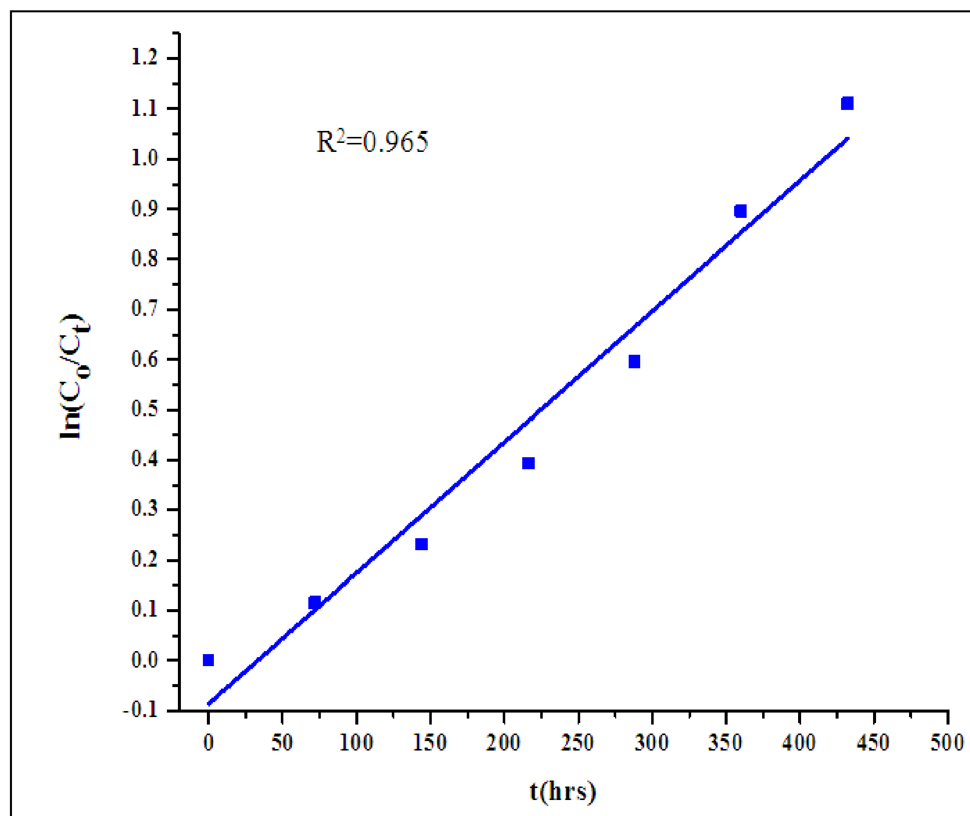
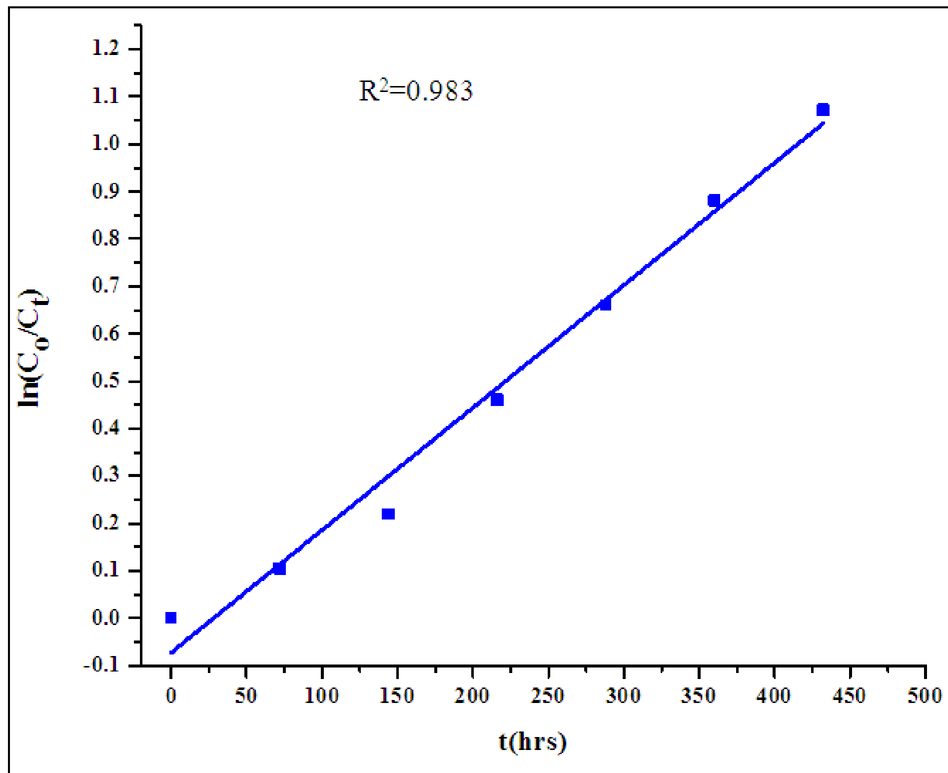
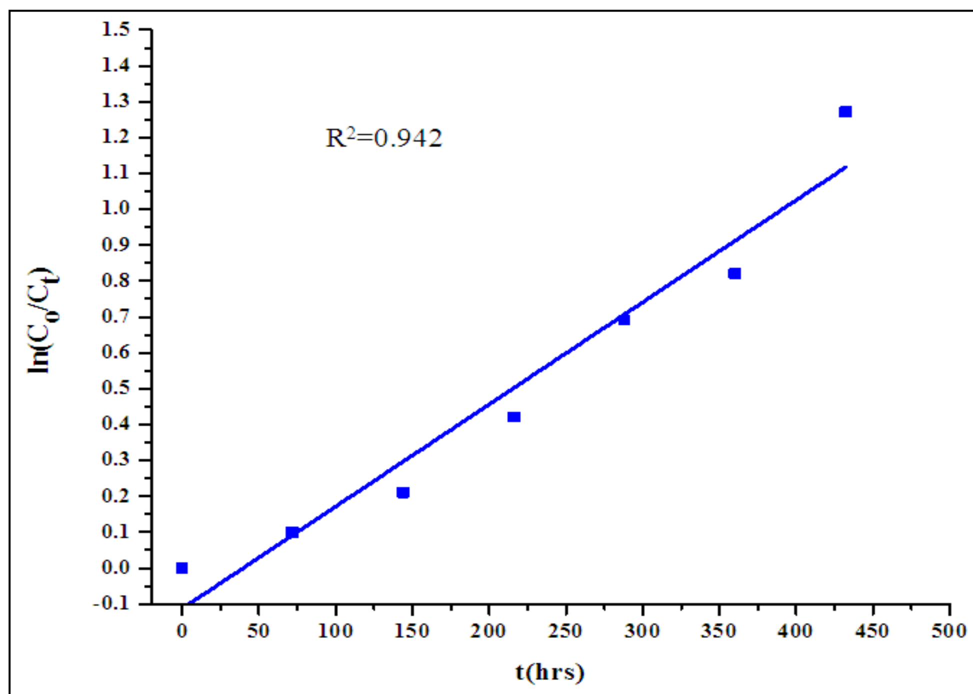


e)



f)



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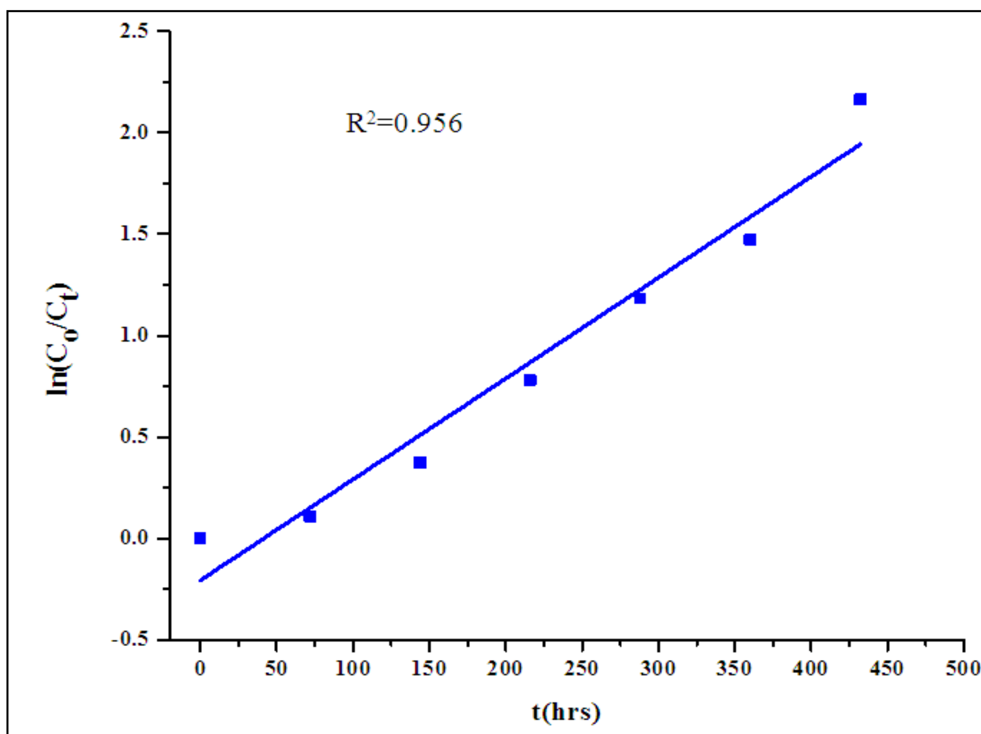

Fig. 6.8 Decolourisation kinetics of Methyl violet 2B with *Schizophyllum commune* at different pH a) 4.0; b) 5.0; c) 6.0; d) 7.0; e) 8.0; f) 9.0; g) 10.0

Chapter-7

Discussion

7.1 To evaluate the ligninolytic enzymes activity and dyes decolourisation potential of spent mushroom substrate of different mushrooms

Spent mushroom substrate (SMS), a by-product of mushroom industry is generated in large quantity every year. Different edible mushrooms namely *Agaricus bisporus*, *Pleurotus* spp. *Volvariella volvacea*, *Calocybe indica*, etc. are cultivated in different regions of the country, which generate large amount of spent substrate after crop harvest. The remaining mycelia in SMS along with the abundant quantity of extracellular ligninolytic enzymes (Laccase, Lignin peroxidase, Manganese peroxidase) in it play crucial role in complete biodegradation of the lignin present in mushroom substrate, mechanism of which is quite similar to that of dyes degradation. Due to its nutrient status and physico-chemical properties SMS works as a good source of nutrients for the growing mushroom mycelia and other associated bacteria and fungi. The SMS also harbours very high population of heterotrophic bacteria and fungi. These bacteria and fungi play an important role in dyes decolourisation with the involvement of their extracellular ligninolytic enzymes. In general perception, SMS is considered as waste after harvesting of mushroom crop, and further extension of the crop becomes unremunerative. Moreover, disposal of SMS is a costly affair due to its bulky nature. In the present study, spent substrate of *P. sajor-caju* and *A.bisporus* has been studied for their potential in dyes decolourisation.

7.1.1 Dye decolourisation potential of different edible mushroom species

A simple agar-plate test was performed for determining the decolourisation capability of different edible mushrooms species against structurally different dyes. The study proved that different dyes supported different levels of mycelial growth (radial growth dia. in mm) and the mushrooms species were also recorded to vary in their mycelial growth as well as

their dyes decolourisation potentials against a specific dye. Two *Pleurotus* spp. i.e. *Pleurotus sajor-caju* and *P. flabellatus* were recorded to exhibit higher radial mycelial growth in presence of different dyes as well as their decolourisation in agar plate. *P. sajor-caju* showed highest mycelial growth (90.00 mm) in presence of Reactive blue, followed by Chicago sky blue (88.30 mm) after 15 days of incubation at 25 °C. Similarly, out of the tested strains of *Agaricus* spp. *Agaricus bisporus* strain U3 showed higher mycelial growth compared to strain S-11 against majority of the tested dyes.

In earlier studies several microorganisms have been studied for their dye decolourisation potential and in one such study, Eichlerova *et al.,* (2007) recorded highest decolourisation capacity in *Bjerkandera adusta*, *Phanerochaete chrysosporium* and *Pleurotus ostreatus* against Orange G, Amaranth, Remazol brilliant blue R, Cu-phthalocyanine and Poly R-478. Novotny *et al.,* (2001) have also reported decolourisation of structurally different dyes (azo and anthraquinone dyes) in agar plates by *Pleurotus ostreatus* and *Irpex lacteus*.

7.1.2 Fungal and bacterial microflora in SMS of different mushrooms

SMS from three mushrooms was recorded to vary in both population as well as diversity of fungi. SMS from *Pleurotus sajor-caju* was found to harbour highest fungal population, dominated by *Aspergillus fumigatus*, followed by *Schizophyllum commune* and *Pezizomycotina* sp. Spent substrate of *Agaricus bisporus*, harbored next highest population exclusively dominated by *A. fumigatus*. Spent substrate from *Volvariella volvacea* although harbored three dominating fungi like spent substrate of *P. sajor-caju*, but it exhibited two very distinct fungi (*Paecilomyces variotii* and *Pichia guilliermondii*) along with *A. fumigatus*. Information on microbial population in SMS from different mushrooms is scanty. However, SMS from *A. bisporus* has been reported to be dominated by *Aspergillus* sp., *Trichoderma* sp., *Mucor* sp. and *Glycodium* sp. (Ahlawat *et al.,* 2010). Present study has also reported highest population of *A. fumigatus* in spent substrates of *A. bisporus* and *P. sajor-caju*.

Spent substrate of *P. sajor-caju* was found to harbour highest population as well as diversity of bacteria comprised of four different species (*Bacillus licheniformis*, *Bacillus subtilis*, *Rummeliibacillus stabekisii* and *Pseudomonas fluorescens*) compared with spent substrate of *A. bisporus* and *V. volvacea*, which supported three and one species, respectively. Spent substrate of *Volvariella volvacea*, harbored second highest population, dominated by only one species (*Bacillus pumilus*). Although information on microbial population of SMS is again scanty, however, in one study, Ntougias *et al.* (2004) observed 14 different operational taxonomic units based on 16S rDNA sequencing technique in *A. bisporus* SMS. Out of these 14 different operational taxonomic units, 12 were Gram-positive bacteria, while rest two had link to *Comamonas* and *Sphingobacterium*. Among Gram-positive bacteria, the important were *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Brevibacterium*, *Arthrobacter*, *Microbacterium*, etc. (Ntougias *et al.,* 2004). The present study has also proved the dominance of *Bacillus* sp. in SMS of *A. bisporus* and *V. volvacea*, while *R. stabekisii* and *P. fluorescens* in *P. sajor-caju* SMS. The microbial variability has the relevance in the light of SMS as source of potential microorganisms with their role in dyes decolourisation.

7.1.3 Extracellular ligninolytic enzymes activity in spent mushroom substrate and associated microorganisms

Fungal ligninolytic enzymes are commercially in use in textile industry and have potential for more industrial applications. One of the possible alternatives for treatment of coloured effluents, the use of white-rot fungi is considered as cost effective, efficient and environment friendly. The decolourisation by white rot fungi is mediated by their extracellular ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase, which can oxidize a wide spectrum of organic pollutants including synthetic dyes (Kokol *et al.,* 2007; Levin *et al.,* 2004; Pointing, 2001). In present study the spent substrate of *P. sajor-caju* exhibited highest activity of MnP (55 UmL^{-1}), followed by

laccase (47 UmL^{-1}). Lower activity of LiP was recorded as compared to other two enzymes in both the spent substrates. The present study envisages that out of three enzymes, activity of MnP was higher in two SMSs.

Out of different fungi and bacteria isolated from SMS of different mushrooms, highest activity of laccase was in *S. commune*, followed by *Pezizomycotina* sp. in the natural medium containing wheat straw as the main substrate. The activity of laccase in three bacteria was nearly 3 to 4 folds lesser than the two fungi. Contrary to this, the activity of Manganese peroxidase (MnP) was highest in two bacteria (*P. fluorescens* and *B. licheniformis*), followed by *Pezizomycotina* sp. Lignin peroxidase (LiP) activity was highest in *S. commune*, followed by *Pezizomycotina* sp. and *P. fluorescens*. Activity of LiP was lowest in *B. pumilus*. Under sub-merged conditions, the activities of three extracellular ligninolytic enzymes (LiP, MnP and Laccase) were higher in *Schizophyllum commune* compared with *Pezizomycotina* sp. The activity of MnP in *S. commune* was almost double to that in *Pezizomycotina* sp. The difference in activities of laccase and LiP was not that much between two fungi. Compared to other two enzymes, the activity of LiP was lowest in two fungi. The study proved the higher utility of *S. commune* in dye decolourisation studies compared with *Pezizomycotina* sp.

The present study envisages that out of three enzymes, activity of laccase and LiP was higher in fungi, while that of MnP was higher in bacteria. The role of ligninolytic enzymes produced by fungi and bacteria in dye decolourisation is well documented (Bhatti *et al.,* 2008). In one study, the SMS of *Pleurotus sajor-caju* has been reported to be the main potential source of ligninolytic enzymes, including lignin peroxidase (LiP) (Singh *et al.*, 2011).

7.1.4 Effect of medium pH on dyes decolourisation using spent mushroom substrate

The pH of the growing medium influences the growth of the microbial population and activity of extracellular ligninolytic enzymes having direct role in dye decolourisation process, hence whole decolourisation process has been found to be affected by pH of the cultural medium. In present study, decolourisation of Rhodamine B and Methyl violet 2B dyes with spent substrate of *A. bisporus* and *P. sajor-caju*, was more effective at pH 4.0 and 7.0. Highest decolourisation of Rhodamine B (93-95%) and Methyl violet 2B (91-100%) was with spent substrate of *A. bisporus* after 3 days of incubation. In case of Chicago sky blue and Quinaldine red, decolourisation was almost similar at pH 4.0 and 7.0 on using spent substrate of two mushrooms and it was higher in comparison to decolourisation at pH 10.0. In present study, dyes have been recorded to have different pH optima for decolourisation with spent substrate of different mushrooms, which vary in their physico-chemical properties; hence also in inhabiting microbial population.

Although several previous studies have highlighted the role of white rot fungi including *Pleurotus* spp. in dyes decolourisation (Singh *et al.,* 2011; Zilly *et al.,* 2002) but none has highlighted the role of spent substrate of different mushrooms in decolourisation of different dyes at different pH, excepting some preliminary studies, wherein spent substrate of *A. bisporus* has been reported to perform better than spent substrate of *Pleurotus* spp. (Ahlawat *et al.,* 2006). For the role of pH in decolourisation of dyes with individual microorganisms, Jin *et al.* (2007) has reported pH 8.0 for efficient decolourisation of textile effluents with *Aspergillus fumigatus*.

7.1.5 Effect of incubation temperature on dyes decolourisation using spent mushroom substrate

Like pH, temperature of incubation has direct role is sustaining enzymatic activities and growth of different microorganisms in a cultural medium, which ultimately influences dye decolourisation process. In present study, decolourisation of Rhodamine B and Methyl

violet 2B was higher at 35 °C than at other two temperatures with SMS of *P. sajor-caju* and *A. bisporus*. However, after 3 days of incubation, there was marginal difference in levels of decolourisation at 25 and 35 °C. In spent substrate of *P. sajor-caju*, decolourisation recorded at 15 °C was comparatively lesser than that at other temperatures. In case of Chicago sky blue and Quinaldine red, almost similar trend was recorded at initial stage of decolourisation. However, at later stage, incubation temperature of 25 and 35 °C were equally effective in decolourisation of both dyes, particularly with spent substrate of *P. sajor-caju,* whereas with *Agaricus bisporus,* highest decolourisation for Quinaldine red was at 35 °C. Out of three temperature levels, 25 °C and 35 °C were the more preferred temperatures compared to 15 °C.

In several earlier studies, specific temperature has been indicated for decolourisation of specific dye through a specific microorganism. In these studies, optimum decolourisation of Reactive red has been reported through *Citrobacter* sp. CK3 at 32 °C (Wang *et al.,* 2009), while that of Acid red 151 through *Aspergillus niger* SA1 at mesophilic temperature (25 to 45 °C) and Remazol brilliant blue R through *Streptomyces psammoticus* at 32 °C (Ali *et al.,* 2008). However, in none of the cases, where mushroom species have been used as test organism, temperature optima studies have been concluded. In present study, variation in temperature optima for decolourisation of different dyes through spent mushroom substrate of two mushrooms is mainly because of variation in microbial population harboured by these spent mushroom substrates. Excepting few cases, decolourisation was higher at 25 and 35 °C, because of 25 °C is the optimum temperature for vegetative mycelial growth of these two mushroom species and 25 to 35 °C as the mesophilic temperature range suitable for the growth and dye decolourisation through mesophilic microorganisms like *Aspergillus niger* (Ali *et al.,* 2008), which also dominates microbial population thriving in spent mushroom substrate. In few cases, decolourisation was at par at 15, 25 and 35 °C, and it is attributed to 15 °C as an optimum temperature for fructification in *A. bisporus*. Mushroom species are

grown on different substrates. Although, mushrooms have pH and temperature optima of 7.0 to 8.0 and 15 to 28 °C, respectively for vegetative growth and fruiting, but their spent substrates also harbour other microorganisms of mesophilic nature, which have significant ligninolytic enzymes activity and contribute towards dyes decolourisation at same temperature and pH.

7.1.6 Dye decolourisation by fungi isolated from SMS of *P. sajor-caju*

Out of fungi isolated from spent mushroom substrate of three mushrooms, *Schizophyllum commune* exhibited whole plate decolourisation against Chicago sky blue, Quinaldine red, Rhodamine B and Methyl violet 2B in agar plate assay. Similarly, under broth culture conditions, highest decolourisation of Chicago sky blue after 12 days of incubation was with *Schizophyllum commune* isolated from spent substrate of *P. sajor-caju*, followed by *Pezizomycotina* sp. again from the same SMS. Rhodamine B and Methyl violet 2B were also decolourized to the highest extent by these two fungi (*Schizophyllum commune* and *Pezizomycotina* sp.), followed by three isolates of *A. fumigatus* isolated from three different spent mushroom substrates. Decolourisation was completely absent in *Pichia guilliermondii* isolated from spent substrate of *V. volvacea*.

Reports on dyes decolourisation with fungi isolated from spent mushroom substrates are quite scanty. However, work carried out with other fungi has proved the synthetic dye decolourisation capability of white rot fungus, *Phanerochaete chrysosporium* (Cripps *et al.,* 1990; Spadaro *et al.,* 1992). It has also been stressed upon that different fungi have varied decolourisation potential against chemically different dyes; hence the screening of fungi for ligninolytic enzymes activity and dye decolourisation must be conducted under similar conditions (Dos-Santo *et al.,* 2004; Swamy *et al.,* 1999; Kapdan *et al.,* 2000). In one study conducted with *Lentinula edodes*, it has been noted that the fungal mycelial growth became visible after 24 hrs of inoculation, and the medium became completely decolorized within 6

days (Boer *et al.,* 2004). Similarly the role of temperature on dye decolourisation potential of a fungus has also been studied and temperature optima of 30 to 37 °C has been recorded for highest decolourisation of chemically diverse dyestuffs by using white-rot fungi (Boer *et al.,* (2004), while 25 to 35 °C specifically for decolourisation of Cibacron Red FN- 2BL by *Schizophyllum commune* (Bhatti *et al.,* 2008).

7.1.7 Dye decolourisation by bacteria isolated from SMS of different mushrooms

Out of different bacteria isolated from SMS of three mushrooms, *Bacillus licheniformis* isolated from *P. sajor-caju* SMS, exhibited highest decolourisation of Orange II sodium salt (66.1%), followed by *B. pumilus* (58.0%) isolated from *V. volvacea* SMS. Lowest decolourisation was with *B. subtilis* and *Rummelibacillus stabekisii* both isolated from *P. sajor-caju* SMS. Even the three isolates of *B. pumilus* isolated from SMS of three different mushrooms varied in their dye decolourisation potential. In majority of the dyes, highest decolourisation was achieved up to 6 days of incubation. Out of the two potential bacteria (*B. licheniformis* and *B. pumilus*) isolated from *P. sajor caju* SMS, *B. pumilus* was more effective against Azure B and Methylene blue (44.6 and 91.3%) compared to *B. licheniformis*. In several earlier studies role of various bacteria for azo dyes decolourisation i.e. *Pseudomonas fluorescens* for Acid yellow (Pandey *et al.,* 2007), *Bacillus fusiformis* for Disperse blue 79 acid orange (Kolekar *et al.,* 2008), *Pseudomonas aeruginosa* for Navitan fast blue (Nachiyar *et al.,* 2003) and *Bacillus* sp. for Disperse dye (Maier *et al.,* 2004) have been reported.

The temperature optima for decolourisation of Orange II sodium salt through *B. licheniformis* was in the range of 20 to 25 °C. Similarly, highest decolourisation of Azure B (44.6%) and Methylene blue (91.3%) with *B. pumilus* was also at 25 °C. This range of temperature optima is attributed to the same range of temperature (20 to 25 °C) requirement for mycelial growth and fruiting in *P. sajor-caju*. Accordingly, the spent substrate of *P. sajor-*

caju also supports the growth of microorganisms with their temperature optima in this temperature range. In few earlier studies, the decolourisation has also been reported at par at 15, 25 and 35 °C, which can be justified in light with varied temperature requirement for vegetative growth and fruiting in different mushrooms (Rigas *et al.,* 2006). In order to further standardize the dyes decolourisation using bacteria isolated from SMS, the pH optima for decolourisation of Orange II sodium salt was studied at different pH values. With most efficient dye decolourizing bacterium, *B. licheniformis*, highest decolourisation of Orange II sodium salt was recorded at pH levels of 6.0 and 7.0, followed by 8.0.

Prior to this, several researchers have studied the pH and temperature optima of the microorganisms of their interest and have reported wide range of pH (Banat *et al.,* 1996; O'Neill *et al.,* 1999; Joe *et al.,* 2008; Pointing, 2001; Ahlawat *et al.,* 2010; Ahlawat *et al.,* 2006; Neelamegam *et al.,* 2004) and temperature (20 to 40 °C) for optimum decolourisation by those microorganisms (Joe *et al.,* 2008). In literature, a vide variation in pH requirement for decolourisation of different dyes through different microorganisms has been cited and it varied from 10.0 in case of *Clostridium bifermentans* SL186 for Reactive dyes (Joe *et al.,* 2008), 7.0 in *Citrobacter* sp. CK3 for Reactive red (Wang *et al.* 2009) and 5.0 in *Aspergillus niger* SA1 for Acid red 151 (Ali *et al.,* 2008). Again for temperature, a specific temperature has been indicated for decolourisation of specific dye through a specific microorganism (Ali *et al.,* 2008; Wang *et al.,* 2009). However, unlike present study, the optimum decolourisation of Reactive red has been cited through *Citrobacter* sp. CK3 at 32 °C, while that of Acid red 151 through *Aspergillus niger* SA1 at mesophilic temperature (25-45 °C) and Remazol brilliant blue R through *Streptomyces psammoticus* at 32 °C (Ali *et al.,* 2008). However, in none of the cases, the temperature optima of SMS inhabiting bacteria have been studied.

7.2 Role of cultural conditions on dyes decolourisation

The prevailing cultural conditions not only determine the level of growth of the target microorganism (s) used for dyes decolourisation but these also determine the production as well the activities of the extracellular ligninolytic enzymes with their role in dyes decolourisation process. With this concept in mind, the role of cultural conditions *viz*., effect of initial concentration of dye, decolourisation medium, carbon sources, heavy metals, enzymes inducers/mediators, static/agitated growth conditions and intact/pellet forms of mycelia were studied for their role in dyes decolourisation.

7.2.1 Effect of initial concentration of dyes on decolourisation

Our results have shown that initial concentration of Rhodamine B, Azure B and Methyl violet 2B influences the decolourisation process with spent substrate of *Pleurotus sajor-caju*. On completion of the experiment, it was significantly higher in the lowest initial dye concentration than the highest initial dye concentration (150 ppm). The results have also shown that with lowest initial concentration of dye (25 ppm), the decolourisation reached up to 100%. In several earlier studies, the response of initial concentrations of dyes towards their decolourisation has been reported to vary from dye to dye (Neelamegam *et al.,* 2004). However, in a good number of cases, where fungi have been used as decolourizing agents, decolourisation has been recorded higher at lower initial dye concentration (Ali *et al.,* 2008; Wang *et al.,* 2008) and like present study this was more evident in studies with *Pleurotus florida* (Sathiya *et al.,* 2007) and *Pleurotus ostreatus* (Neelamegam *et al.,* 2004). The lesser decolourisation at higher initial dye concentration in case of *Schizophyllum commune* has been explained in light of growth inhibitory activity of azo dyes (Renganathan *et al.,* 2006).

7.2.2 Effect of media on dyes decolourisation

The present study has revealed that highest decolourisation of Quinaldine red, Methyl violet 2B, Chicago sky blue and Rhodamine B was achieved in potato dextrose broth compared to malt extract broth and plain distilled water with spent substrate of *P. sajor-caju*.

The roles of composition of cultural media and presence of dyes in media have also been studied earlier in *Pleurotus* species (Eichlerova *et al*. 2006) and both of these have been reported to influence the decolourisation process. The activities of laccase and manganese peroxidase were recorded higher in malt extract broth compared to kirk medium. Literature supports our conviction that the composition of the media substantially influences the decolourisation process in fungi; this opinion has also been shared by other authors (Hatvani *et al.,* 2002; Swamy *et al.,* 1999; Kim *et al.,* 1996).

7.2.3 Role of carbon sources on dyes decolourisation

In the present study, at the end of experiment, sucrose and starch added media exhibited highest decolourisation of Methyl violet 2B compared with other carbon sources. Highest decolourisation was recorded with sucrose (87.75%), followed by starch (87.48%), while only 80.29% in control and 5 to 7% in second control inoculated with wheat straw alone. In subsequent study, out of three different concentrations (0.25%, 0.50% and 1.0%) of five carbon sources (glucose, fructose, sucrose, maltose and starch), 0.5% of glucose and 1.0% of rest all carbon sources supported higher decolourisation of textile effluent in different microbial combination treatments. Out of different concentrations of carbon sources, glucose at 0.5% and sucrose at 1.0% were more effective than different concentrations of other carbon sources.

Addition of glucose to the dye decolourisation medium provides easily metabolizable energy source to the fungus and creates an environment to enhance decolourisation rate of dyes. The carbon sources are the primary source of energy for the growth of different microorganisms, however, their requirement vary from microbe to microbe and their growth conditions. The textile effluent may be rich in several kinds of carbon sources but they may not be in sufficient quantity or suiting to the requirements for the SMS-borne microorganisms. The earlier studies have also proved that adding of additional amount of

glucose at 0.2–2.0% enhances dye decolourisation (Joe *et al.,* 2008; Ali *et al.,* 2008). The present study has also showed higher decolourisation in presence of different carbon sources, out of which glucose has been found to be effective at a lower concentration (0.50%) than other carbon sources (1.0%). This enhancement in dye decolourisation or effluent decolourisation is attributed to easy utilization of glucose by growing microorganisms, leading to shortening of their lag phase of growth (Leung and Pointing, 2002).

7.2.4 Effect of veratryl alcohol and manganese sulfate on dye decolourisation

In present study, the lower concentration of veratryl alcohol (0.025%) supported highest dye decolourisation at the initial stage, while at later stages, highest concentration (0.1%) supported highest decolourisation using spent substrate of *Pleurotus sajor-caju*. Same was the situation with Manganese sulphate, wherein the lower concentration of Manganese ions supported higher decolourisation at the initial stages, however, the trend get reversed at the later stages.

The earlier studies have also highlighted the role of veratryl alcohol and Mn^{2+} in dye decolourisation of different dyes and have reported that the addition of 0.4 mM veratryl alcohol (VA) into the medium considerably increased the decolourisation rate in sunflower-seed shell cultivation (Enayatizamir *et al.* 2011). It has been reported that the addition of veratryl alcohol into fungal cultures leads to higher lignin peroxidase activities and dye decolourisation but has no significant effect on manganese peroxidase production (Bibi *et al.,* 2010). Further, Couto *et al.* (2002) have reported that veratryl alcohol stimulates and is necessary for lignin peroxidase secretion by white rot fungi. In another study, the optimal concentrations of Mn^{2+} and dosage of H_2O_2 (the cofactors involved in the catalytic cycle of the manganese peroxidase enzyme and responsible for maximizing the efficiency of the degrading and/or decolourizing processes *in vitro*), have been reported to depend on the nature and concentration of the dyes to be used (Contreras *et al.* 2012).

7.2.5 Effect of heavy metal on dyes decolourisation

Normally the presence of heavy metal ions in growing medium is considered as detrimental for the growth of microorganisms including fungi, however in present study, presence of lead, cadmium, cobalt and nickel ions has supported higher decolourisation compared to control. The present phenomenon can be explained in light with the justification given by Younes *et al.,* (2007), wherein they suggested that the presence of metal ions provide more stability to laccase, which is having role in dye decolourizatiom by the white rot fungi. Presence of heavy metals in coloured industrial wastewater has also been reported to affect succession of microbial community and hence decolourisation (Tan *et al.,* 2009). Regulation of laccase expression by metals has been widely reported in fungi. In line with our findings, Soden *et al.* (2001) and Baldrian *et al.* (2002) have also reported increased laccase activity in *P. ostreatus* in presence of cadmium, copper and manganese ions.

7.2.6 Role of agitated, stationary, intact and pellet form of mycelia

Amongst the cultural conditions studied i.e. agitated vs stationary growth conditions and intact vs pellet form of mycelia, higher decolourisation of Methyl violet 2B was recorded under agitated growth conditions and with inoculation of pellet form of mushroom mycelia. Difference in decolourisation at initial stage was less significant mainly because of the lag phase of growth of mushroom mycelia, as it took few days to attain appreciable mycelial growth and to show its effect on dye decolourisation. Nearly 100% decolourisation of Methyl violet 2B was recorded in pellet form of mycelia treatment after 15 days of inoculation compared with 96% in intact form of mycelia.

In an earlier study conducted under static and shaking conditions, Bibi *et al.* (2012) reported highest decolourisation (83.75%) of Remazol brilliant blue R dye under static conditions as compared to shaking by *Trametes hirsuta*, a white rot fungus. However contrary to our findings, Wesenberg *et al.,* (2003) have reported higher decolourisation

efficiency under stationary (71.3%) conditions than with agitated cultures (57.2%) and the best decolourisation efficiency (78%) was when the two conditions were combined. In favour of higher decolourisation in presence of pellet form of mushroom mycelia, Kaushik *et al.,* (2009) have attributed it to the uniform suspension of these spherical pallets in growing medium contributing to higher level of decolourisation under such conditions.

7.3 Dye decolourisation along with biomass production

The two potential fungi *Schizophyllum commune* and *Pezizomycotina* sp. were studied for pH and temperature optima for decolourisation of Rhodamine B and Methyl violet 2B along with their mycelial biomass production. Highest decolourisation of Rhodamine B was recorded at pH from 8.0 to 10.0, while highest biomass was recorded at pH of 9.0 by *Pezizomycotina* sp. On the other hand the pH ranging from 7.0 to 10.0 was recorded to be optimum for achieving nearly 100% decolourisation of Methyl violet 2B and pH 9.0 to 10.0 for highest biomass production by *Schizophyllum commune* at 30 °C. In present study, highest fungal biomass was recorded almost in the same pH range where highest decolourisation of two dyes was recorded with the two fungi. It proves the adaptability of the two fungi to grow and decolourize the dyes simultaneously at same pH and temperature optima, which is crucial for their industrial scale application.

While working on similar lines, Balu, (2009) reported that the rate of enzyme utilization and dye decolourisation increase linearly with increasing initial biomass concentration. In batch scale experiment it was recorded that decolourisation of Acid orange 10 was a function of both the substrate (dye) and biomass concentrations. Highest dye decolourisation (99.65%) was obtained with 200 beads where there was high biomass concentration, while optimum biomass concentrations (above 50 beads) were generally recorded to be advantageous resulting in shorter time period and higher percentage of decolourisation.

7.4 Effect of immobilization medium on decolourisation through different microbes isolated from SMS of different mushrooms

Out of five different immobilization media *viz*., wheat straw, paddy straw, peat moss, soybean straw and saw dust used for immobilization of two potential fungi. Wheat straw and paddy straw were found suitable for *Schizophyllum commune*, while all were used for *Pezizomycotina* sp. In case of *Pezizomycotina* sp., highest decolourisation at 30 °C was with peat moss (86.9%), followed by soybean straw (75.5%), wheat straw (73%), paddy straw (74%) and saw dust (69.2%). However in case of *S. commune*, highest decolourisation was with paddy straw (89.5 %), followed by wheat straw (86.8 %). The three bacteria exhibited very low level of decolourisation (25.80 to 38.0 %). Out of different combinations of immobilized forms of fungi and broth cultures of bacteria, the immobilized forms of *S. commune* and *Pezizomycotina* sp. separately with *B. pumilus* and *B. licheniformis* resulted in nearly 100 % decolourisation, which was at par to that of mixed inoculum of two fungi and three bacteria. The two controls again exhibited almost negligible level of decolourisation.

The effect of growing medium on growth and activity of microorganisms is well known and fungi are not the exception (Snajdr and Baldrian, 2007). The immobilization of fungi on suitable solid supports has been studied by several workers and they have reported that it helps them to grow well ensuring higher enzyme production, dye decolourisation and pollutant degradation (Pandey, 2003). Subsequently, Tavcart *et al.* (2006) have also reported many more advantages of using immobilized forms of fungi in dye decolourisation like it helps in their long time use, handling large volume, possibility of refreshing cultures between different cycles and allowing the persistence in competition with faster growing species. The higher suitability of certain immobilizing media over others is attributed to their ability to support the growth of these two fungi and their ability to do so continuously.

7.5 Kinetics of dye decolourisation by spent substrate of *Pleurotus sajor-caju* and *A. bisporus*

Spent substrate of *Pleurotus sajor-caju* and *Agaricus bisporus* were used for the decolourisation of Methyl violet 2B and kinetics of decolourisation process was studied as a function of temperature and pH. The decolourisation process was studied at temperature range 15 to 35 °C and highest decolourisation was recorded at 25 °C. The decolourisation process was observed to follow the nearly first order kinetics showing exponential decrease in dye concentration with time. The decolourisation process was also studied at pH range of 4.0 to 10.0 and Methyl Violet 2B was decolourized to maximum extent at pH 4.0 by SMS of two mushrooms and the dye decolourisation process was found to follow the first order kinetics. The study also showed that at identical temperatures and pH, the absolute decolourisation rate with spent mushroom substrate of *P. sajor-caju* was considerably higher than that observed with SMS of *A. bisporus*, and the decolourisation were found to dependent upon the nature of particular dye (Ahlawat and Singh, 2009).

7.6 Kinetics of dye decolourisation by *Schizophyllum commune* and *Pezizomycotina* sp.

Schizophyllum commune and *Pezizomycotina* sp. were used for decolourisation of Methyl violet 2B and kinetics of decolourisation process was studied as a function of temperature and pH. The decolourisation process was studied at temperature range of 15 to 35 °C and highest decolourisation was recorded at 30 to 35 °C. The decolourisation process was recorded to follow the nearly first order kinetics showing exponential decrease in dye concentration with time. The decolourisation process was also studied at pH range of 4.0 to 10.0 and highest decolourisation was recorded at pH range of 8.0 to 9.0 with both fungi (*Schizophyllum commune* and *Pezizomycotina* sp.). The decolourisation process was again found to follow the nearly first order kinetics. While studying the degradation of

environmental pollutants especially petroleum contaminated site with bacteria, Abbassi and Shquirat (2007) have reported first order kinetics for the degradation process. The using of first order kinetics has been reported to have many advantages like easy of presenting and analyzing the data, the simplicity of plotting logarithm of the dye remaining versus decolourisation time as a straight line and the case of predicting concentrations (Boonchan *et al.,* 2000; Bonaventura and Johnson, 1996; Greene *et al.,* 2000). While working with Reishi mushroom (*Ganoderma lucidum*) for decolourisation of textile dye wastewater, Selvakumar *et al.* (2013) have also reported first order kinetics.

Proper disposal of SMS has always remained a challenging task for mushroom growers' as till now mushrooms are considered as prime product of mushroom cultivation and left over residual substrate known as spent mushroom substrate, which contains a variety of nutrients and microbes is discarded unsystematically. Present study highlights both quantitative and qualitative variation in microflora of spent substrate from three different mushrooms and same has been proved earlier, as spent substrate from different mushrooms vary in their physico-chemical properties; hence also in inhabiting microbial population. The study has also revealed higher population and diversity of both fungi and bacteria in SMS from *P. sajor-caju*, which has again been proved in some earlier studies (Ahlawat *et al.,* 2004). Amongst the inhabiting microflora from spent substrate of different mushrooms, the fungus (*Schizophyllum commune)* and bacterium (*B. licheniformis)* have been found to have higher decolourisation potential than others and likewise in earlier several reports, the SMS of oyster mushroom or the *Pleurotus* mycelium has been reported to have higher decolourisation ability than SMS of other mushrooms or microbes from SMS of other mushroom (Ahlawat and Singh, 2009). *P. sajor-caju* is grown on pasteurized wheat straw/paddy straw substrate. Although, this mushroom has pH and temperature optima of 7.0 to 8.0 and 20 to 28 °C, respectively for vegetative growth and fruiting, but its spent substrate

also harbor other microorganisms of mesophilic nature, which have significant ligninolytic enzymes activity (Ahlawat *et al.,* 2006; Ahlawat *et al.,* 2004) and contribute towards dyes decolourisation (Ahlawat *et al.,* 2009). The present study has also proved higher dye decolourisation ability and ligninolytic enzymes activity in SMS of *P. sajor- caju* and in fungi as well as bacteria isolated from SMS of *P. sajor-caju.*

Like in present study, the microorganisms from different ecosystems have also been tested earlier for their dye decolourisation potential (Forss *et al.,* 2009; Machado *et al.,* 2006), and one aerobic non-filamentous bacterium from composting environment (Lopez *et al.,* 2006), *Clostridium bifermentans* from contaminated soil (Joe *et al.,* 2008), *B. cereus* from dye house effluent (Modi *et al.,* 2010) and *Micrococcus* sp. from refuse dump soil (Olukanni *et al.,* 2009) have been reported to have significant dye decolourisation potential. In most of the earlier studies, the dye decolourisation potential of specific bacterium have been studied against several dyes and bacteria belonging to *Bacillus* spp. have been reported to have good decolourisation potential (Lopez *et al.,* 2006; Modi *et al.,* 2010) like we got in the present study.

The present study has also shown that the SMS of *P. sajor-caju* could be a cheap source of ligninolytic enzymes for decolourisation of dyes in textile industry wastewaters. The combined inocula of two fungi and three bacteria isolated from SMS of different mushrooms proved as the best microbial combination for textile effluent decolourisation. Alike our study, wherein *S. commune* isolated from *P. sajor-caju* SMS, has been found as superior dye decolourizing microbe, Bhatti *et al.* (2008) had also reported the role of *Schizophyllum commune* IBL-6 for the biodegradation of reactive dye Cinacorn red FN-2BL. Among different enzymes involved in dye decolourisation process, manganese peroxidase has been reported as the major peroxidase with minor role of lignin peroxidase and laccase. The role of ligninolytic enzymes and the influencing factors like temperature and pH in

enzymatic activities in *Phanerochaete chrysosporium* have been studied by several workers and have reported higher decolourisation of dyes by optimizing the conditions (Couto *et al.,* 2006; Shah *et al.,* 2003; Wesenberg *et al.,* 2003). Similarly the role of other factors like of carbon source in dye decolourisation process have been studied and have detected high ligninolytic enzyme (laccase and MnP) at all the tested glucose concentrations (Verma and Madamwar, 2005). This indicates that the fungus exhibits the enzyme activity even in primary growth phase. In present study also, the spent substrate of *P. sajor-caju* used for decolourisation of Methyl violet 2B showed higher decolourisation under carbon rich conditions. Consortium of different potential bacteria and fungi was also observed to exhibit higher textile effluent decolourisation with the addition of carbon sources. The crude enzyme extract from SMS of *P. sajor-caju* was also found to have quite high dye decolourisation ability. Among different dyes, Starch azure exhibited higher decolourisation in wide range of ratios (1:4 to 1:9) between crude enzymes extract and dye supplemented broth, and nearly 100% decolourisation was recorded after 2 days of incubation.

Chapter-8

Conclusions

8.1 Dye decolourisation by spent mushroom substrate

i. Highest decolourisation of Methyl violet 2B (96%) and Rhodamine B (74%) using spent substrate of *P. sajor-caju* was recorded at pH range of 7.0 to 10.0. However, highest decolourisation of Chicago sky blue (96%) was recorded at pH 4.0 with same spent mushroom substrate.

ii. Highest decolourisation of Rhodamine B (58%), Methyl violet 2B (98%), Chicago sky blue (95%), Quinaldine red (91%) and Reactive blue (100%) was recorded at 25 °C after 72 hrs of incubation using spent substrate of *P. sajor-caju*.

iii. Spent substrate of *Pleurotus sajor-caju* did harbour highest population as well as diversity of bacteria comprised of four different species (*Bacillus licheniformis*, *Bacillus subtilis*, *Rummeliibacillus stabekisii* and *Pseudomonas fluorescens*) compared with spent substrate of *Agaricus bisporus* and *Volvariella volvacea*, which supported three and one species, respectively. Spent substrate of *Volvariella volvacea* harboured second highest population, dominated by *Bacillus pumilus*.

iv. Spent substrate of *P. sajor-caju* harboured highest population of fungi, dominated by *Aspergillus fumigatus*, followed by *Schizophyllum commune* and *Pezizomycotina* sp.

v. Out of the isolated bacteria and fungi from SMS of different edible mushrooms, the fungus (*Schizophyllum commune*) and bacterium (*Bacillus licheniformis*) isolated from *P. sajor-caju* SMS, exhibited highest decolourisation against different dyes.

vi. The results revealed that the spent substrate of *P. sajor-caju* was capable of decolourizing different dyes over a wide range of pH from 4.0 to 10.0.

vii. Out of different spent mushroom substrates, the SMS from *P. sajor-caju* harboured fungi (*Schizophyllum commune* and *Pezizomycotina* sp.) and bacteria (*Bacillus licheniformis*, *Pseudomonas fluorescens* and *Bacillus pumilus*) with appreciable level of ligninolytic enzymes activities and decolourisation potential against different textile dyes.

viii. Highest activity of laccase (11.8 U mL^{-1}) was recorded in *Schizophyllum commune*, followed by *Pezizomycotina* sp. (8.32 U mL^{-1}).

ix. Spent substrate of *P. sajor caju* exhibited highest activity of Manganese peroxidase (55 U mL^{-1}), followed by laccase (47 U mL^{-1}) whereas, spent substrate of *A. bisporus* also exhibited highest activity of Manganese peroxidase (33 U mL^{-1}), followed by laccase (22 U mL^{-1}).

x. Ratios of 1:4, 1:5 and 1:6 of crude enzyme extract of spent mushroom substrate and dye supplemented growth medium were recorded for highest decolourisation of Methyl violet 2B, Rhodamine B, Chicago sky blue and Azure B.

8.2 Role of cultural conditions on dyes decolourisation

i. Lower concentration (25 mg/L) of Methyl violet 2B, Rhodamine B and Azure B showed higher decolourisation (100%) with spent substrate of *P. sajor-caju* compared with higher initial concentrations of different dyes.

ii. The results concluded that the lower concentration of manganese ions, veratryl alcohol and addition of sucrose and starch enhances the decolourisation of Methyl violet 2B compared with controls without any additional source of these.

iii. The presence of heavy metal ions (lead, cadmium, cobalt and nickel) enhanced the decolourisation of Methyl violet 2B compared with presence of ions of other heavy metals and the control.

iv. The inoculation of dye supplemented medium with pellet form of *P. sajor-caju* mycelia (100%) and maintaining of agitated conditions (70%) supported highest decolourisation of Methyl violet 2B compared with inoculation with intact form of mycelia and maintaining of stationary conditions.

v. Presence of carbon sources and immobilization of fungi on wheat straw enhanced the decolourisation and reduced the time required for complete decolourisation of textile effluent. The pH and temperature optima for decolourisation were in the same range as for the growth of heterotrophic mesophilic fungi, bacteria and *P. sajor-caju*.

vi. After 48 h of incubation at four different temperatures, highest decolourisation of 37.10% was recorded at 30 °C with combined inoculums of *S. commune* and *Pezizomycotina* sp. immobilized on wheat straw along with broth cultures of *B. pumilus*, *B. licheniformis* and *P. fluorescens.* The same combination also exhibited highest decolourisation at 30 °C after 96 (63.60%) and 144 hrs (98.50%) of incubation, closely followed by *Pezizomycotina* sp. alone (60.00% and 91.20%). *S. commune* was third best at all stages of textile effluent decolourisation.

vii. Out of three different concentrations (0.25%, 0.50% and 1.0%) of five carbon sources (glucose, fructose, sucrose, maltose and starch), 0.5% of glucose and 1.0% of rest all carbon sources supported higher effluent decolourisation in different microbial combination treatments.

viii. After 96 hrs of incubation, highest decolourisation (93.30–100%) was in combined inocula of *S. commune*, *Pezizomycotina* sp., *B. pumilus*, *B. licheniformis* and *P. fluorescens* in presence of 1.0% concentrations of different carbon sources. With this microbial combination, the 0.50% and 0.25% concentrations performed almost at par with 1.0% concentration in case of sucrose, maltose and starch, while only 0.50% concentration in case of glucose and fructose.

ix. In case of *Schizophyllum commune*, highest decolourisation was on its immobilization at paddy straw (99.5%), followed by wheat straw (57.8%). The three bacteria (*B. pumilus*, *B. licheniformis* and *P. fluorescens*) used separately for comparison exhibited very low level of decolourisation (25.80–38.0%).

x. In case of *Pezizomycotina* sp., highest decolourisation after 144 h of incubation at 30 °C was on immobilization at peat moss (86.9%), followed by soybean straw (75.5%), wheat straw (74%), paddy straw (73%) and saw dust (69.2%).

xi. Out of different microbial combinations, the immobilized forms of *S. commune* and *Pezizomycotina* sp. used separately with *B. pumilus, B. licheniformis* and *P. fluorescens* and resulted in nearly 100% decolourisation, which was at par to that of consortium comprised of both two fungi and all three bacteria.

xii. The study has the potential to go a long way and can provide better alternatives for SMS disposal as well as textile effluent decolourisation.

8.3 Kinetic of dyes decolourisation with spent mushroom substrate

i. The regression coefficient (R^2) at different temperatures 15, 25 and 35 °C were 0.964, 0.952 and 0.944, respectively for decolourisation kinetics of Methyl violet 2B using spent substrate of *P. sajor-caju.*

ii. The Regression coefficient (R^2) was 0.973, 0.998 and 0.978 at pH 4.0, 7.0, and 10.0, respectively for decolourisation kinetics of Methyl violet 2B using spent substrate of *P. sajor-caju.*

iii. The decolourisation process with spent substrate of *A. bisporus* was observed to follow first order kinetics as linear plots of ln (C_0/C_t) v decolourisation time were obtained. The R^2 value was also found to be good (>0.9), which was in agreement with first order kinetics for all cases studied.

iv. First order kinetics was recorded for the dye decolourisation process by using spent substrate of *P. sajor-caju* and *A. bisporus*, and fungi i.e. *Schizophyllum commune* and *Pezizomycotina* sp. isolated from SMS of *P. sajor-caju*.

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