TEXTILE DYEING WASTEWATER TREATMENT POTENTIAL OF *PHANEROCHAETE CHRYSOSPORIUM*: EXPERIMENTS AND SIMULATION

Thesis submitted by

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Dedicated
to
My Family

INDIAN INSTITUTE OF TECHNOLOGYGUWAHATI

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STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology and Department of Physics, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Kannan Pakshirajan and Dr. Sitangshu Bikas Santra.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work described in this thesis entitled "Textile dyeing wastewater treatment potential of *Phanerochaete chrysosporium*: experiments and simulation" by KausikSen for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Department of Biotechnology and Department of Physics, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

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Contents

Chapter-1

Introduction

Environmental pollution due to discharge of wastes from industries remains a major threat to the ecosystem. Essential components of an effective strategy for maintaining the environmental quality includes reduced generation, efficient treatment and utilization of wastes, besides conservation and better utilization of resources.A major concern with the growth of industrial sectors is the discharge of a huge volume of wastewater contaminated with toxic organic substances like dyes. The major industries that discharge dye wastewater include textile and dye manufacturing, which employ a large variety of dyes and chemicals additives.The major units in these industries that utilize dyes are the dyeing and finishing operations, which require the input of a wide range of chemicals and dyestuffs that are majorly organic compounds of complex structure. Among the dyes used by these industries, azo dyes and their pigments are versatile and most common synthetic colorants. They are also typically amenable to structural modification and representative azo dyes can be made to bind most synthetic and natural textile fibers. The removal of colour from textile industry and dyestuff manufacturing industry wastewaters thus represents a major environmental concern due to their high persistence levels (only 47% of 87% of dyestuff are biodegradable) [Pagga and Brown, 1986]. Interest in pollution prevention from textile dyeing industries has been of primary concern owing to their possible toxicity and carcinogenicity. This is mainly due to the fact that many dyes are made from known carcinogens, such as benzidine and other aromatic compounds, all of which might be reformed as a result of microbial metabolism [Clarke and Anliker, 1980]. It has been shown that azo- and nitro-compounds are reduced in sediments and in the intestinal environment [Chung et al., 1978], resulting in regeneration of parent toxic amines. Therefore, removal of these dyes to sufficiently low levels in

wastewater is mandatory and appropriate strategies of wastewater treatment have to be employed in order to counterbalance these growing environmental problems. For the last two decades, rigorous pollution control and legislation in many countries have resulted in an intensive search for new and more efficient effluent treatment technologies.

1.1. Dye Decolourization Techniques

In the past, municipal treatment systems were mainly used for purification of textile mill wastewaters. These systems depended mainly on biological activity and were mostly found inefficient for the removal of more resistant synthetic dyes; less sensitive, yet more effective methods, therefore, were developed and tested for dye removal. These methods were primarily physical or chemicaltreatment processes, occasionally in conjunction with biological treatment [Groff, 1993]. The physical and chemical techniques were numerous and included physico-chemical flocculation combined with flotation, electroflotation, flocculation with Fe(II)/$Ca(OH)_2$, membrane-filtration, electrokinetics coagulation, electrochemical destruction, ion-exchange, irradiation, precipitation, ozonation, adsorption and the Katox treatment method involving the use of activated carbon and air mixtures [Lin and Lin, 1993]. Although some of these techniques have shown to be effective, shortcomings such as excess amount of chemical usage, unavoidable sludge disposal problems, costly plant requirements or operating expenses, inefficient treatment of colour, particularly with sulfonated azo dyes etc. have been observed. Other techniques involve chemical oxidation using sodium hypochlorite to remove the colour. They, however, release a lot of aromatic amines that are carcinogenic, or otherwise toxic compounds which subsequently aggravate the problem [Anliker, 1979]. Several authors have also

suggested their treatment by adsorption, which does not involve biodegradation and release of intermediate products. This technique rather utilizes the recalcitrance of dyes and affinity to adhere to surfaces[De Angelis and Rodrigues, 1987]. The above-mentioned technologies have been the subject of several reviews, yetno one specific treatment process seems to be able to handle decolourization of textile wastewaters and, generally, a customized process, probably involving a combination of methods, could be more applicable. The treatment of textile wastewater by purely biological processes may be possible even without the inclusion of other carbon sources, e.g. municipal wastewater. Several microbial cultures have been tested or are implicated in textile dye decolourization.

1.2. Azo Dyes

Dye manufacturing is one of the sectors in chemical industry dealing with the greatest variety of products and intermediates. Azo colorants can enter the environment from their own manufacture processes but the most significant routes include their use in subsequent industrial sectors, such as textile, paper, plastics, food and drugs colouring or the production of paints and lacquers. Roughly two thirds of the dyestuff market is directed to the textile sector, and it is estimated that around 12% of the used colorants are lost in wastewaters [Riu et al., 1997]. Due to the general association of dye toxicity with hydrophobic character [Benigniand Passerini, 2002] low toxicity has often been achieved through the introduction of polar moieties in the dye structure, also resulting in higher aqueous solubility, sulfonation being a widespread character particularly in the reactive dye group. However, this feature can hinder their removal in wastewater treatment works. Increased hydrophilicity has been described as unfavourable for dye bio-elimination in activated sludge systems, through absorption

onto the biomass. Azo dyes have also been long known to resist effective biodegradation in aerobic conditions with the exception of a few simple structured dyes [Pagga and Brown, 1986]. The recalcitrance of azo dyes has been attributed to the presence of sulfonated groups and azo bonds, two features generally considered as xenobiotic. The azo bond in azo colorant molecules is however vulnerable to reductive cleavage. Due to its potential for eliminating colour impact in azo dye containing effluents, the biologically- mediated decolourization of azo dyes through azo bond reduction has been extensively investigated in the past 20 years. Several reviews have been published on or including this subject, with emphasis on wastewater purification [Slokar and Marechal, 1998; Pearce et al., 2003]. However, only a few of the studies on microbial azo dye reduction included a clear demonstration of total or partial, subsequent biodegradation of the resulting metabolites, aromatic amines. Although the possibility of total elimination of azo dye reduction metabolites through biotransformation has been demonstrated, the process appears clearly less straightforward than the primary reduction step. Basic limitations are the need for specific, adapted microbial strains and, in some cases, co-cultures of several strains [Rieger et al., 2002], the tendency of some metabolites to undergo chemical oxidation to more recalcitrant products, and the lack of knowledge on the bio-reactivity of the majority of the aromatic amines which can be formed in wastewater treatment plants or water courses as a result of the reduction of the discharged azo dyes presently in use. The identified carcinogenic amines have been found to pose a significant risk of bioaccumulation in the environment [Ollgaard et al., 1998], though, again, little is known of the more hydrophilic amines which could result from azo bond cleavage in water soluble dyes. Thus, the likely persistence and largely unknown effect of azo dye derived aromatic amines in the environment makes

them a desirable target for discharge monitoring and environmental distribution studies, in accordance with the precautionary principle basis.

Azo compounds are compounds bearing the functional group R-N=N-R`, in which R and R` can be either aryl or alkyl and the N=N group is called an azo group. The more stable derivatives contain two aryl groups. As a consequence of π-delocalization, aryl azo compounds have vivid colours, especially reds, oranges and yellows. Therefore they are used as dyes and are commonly referred to as azo dyes. Direct Red 80 is a very important dye usedwidely in industries, which is however carcinogenic and hence need to be removed from constituent wastewaters.

Direct Red 80 has molecular weight 1373.07 and absorbance λ_{max} at 523 nm. Its molecular formula is $C_{45}H_{26}N_{10}Na_6O_{21}S_6$ and its structure is given in Fig 1.

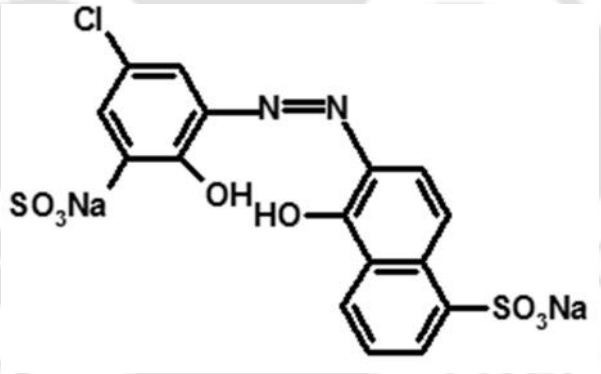

Figure 1. Molecular Structure of DR – 80 dye used in this study

1.3. *Phanerochaete chrysosporium*

Among the different microorganisms capable of degrading recalcitrant organics, white-rot fungi are those organisms that are able to degrade lignin, the structural polymer found in woody plants [Barr and Aust, 1994]. The most widely studied white-rot fungus, in regards to xenobiotic degradation, is *Phanerochaete*

chrysosporium. This fungus is capable of degrading dioxins, polychlorinated biphenyls (PCBs) and other chloro-organics [Chao and Lee, 1994]. Kirby [1999]showed that *P.chrysosporium* had the ability to decolorize artificial textile effluent by up to 99% within 7 days. White-rot fungi are able to degrade dyes using enzymes, such as lignin peroxidases (LiP), manganese dependent peroxidases (MnP). Other enzymes used for this purpose include H_2O_2-producing enzymes, such as, glucose-1-oxidase and glucose-2-oxidase, along with laccase, and a phenoloxidase enzyme [Archibald and Roy, 1992]. These are the same enzymes used for the lignin degradation. Azo dyes, the largest class of commercially produced dyes, are not readily degraded by micro-organisms but these can be degraded by *P. Chrysosporium* [Paszczynski and Crawford, 1991].

1.4. Lignin Peroxidase

An important extracellular enzyme secreted by *P. chrysosponium* is lignin peroxidase (LiP) [Glennand Gold, 1983]. This enzyme has been demonstrated to be a major component of the lignin degradation system in this fungus. The catalytic cycle of LiP is similar to Horse Radish Peroxidase (HRP) [Gold et al., 1989]. The primary reaction product of LiP with H_2O_2 is the two-electron oxidized state compound I, LiPI. As with HRP, LiPI is reduced back to the native enzyme via two single-electron steps with compound II, LiPII as an intermediate (scheme 1) In the process, the aromatic reducing substrate is oxidized to an aryl cation radical (Ar^+) as shown in the following scheme:

$$LiP + H_2O_2 \rightarrow LiPI + H_2O$$

$$LiPI + Ar \rightarrow LiPII + Ar^+$$

$$LiPII + Ar \rightarrow LiP + Ar^+ + H_2O$$

LiP catalyzes the oxidation of nonphenolic lignin model compounds such as veratryl alcohol to veratryl aldehyde. Therefore the unique feature of this enzyme is that it is able to oxidize aromatic compounds with redox potentials beyond the reach of HRP and many other peroxidases.

1.5. Aim and Scope of this Study

Wastewater from textile dyeing industries poses a serious threat to the receiving environment due to the toxic chemicals present, particularly due to the dyes. Therefore, its treatment is necessary prior to its discharge. *P. chrysosporium* is a potential organism that can degrade the dyes mainly by establishing its biomass growth and secreting necessary enzymes in presence of such toxic compounds. However, these two aspects have not been sufficiently addressed in the literature. Therefore, this work was aimed at investigating the biomass growth and enzyme secretion by *P. chrysosporium* in absence and in presence of DR-80, a commonly used azo dye in textile dyeing industries, and to develop a suitable model to explain these two biological processes. This aim was achieved by fulfilling the following objectives:

- Effect of physico-chemical parameters on biomass growth and LiP activity of the fungus in absence of DR-80.
- Development of a suitable model to describe biomass growth and LiP secretion by the fungus.
- Effect of physico-chemical parameters on biomass growth and LiP secretion by the fungus in presence of DR-80.

1.6 Organization of this Thesis

This thesis contains six chapters. The present chapter (Chapter 1) gives the general introduction about textile dyeing wastewater, azo dyes, the potential of P. chrysosporium in wastewater treatment and the objectives of this research. Literature review on several aspects including textile dyeing wastewater treatment has been elaborated in Chapter 2. Chapter 3 provides the details of investigations on biomass growth and enzyme secretion by *P. chrysosporium*, modeling of these two phenomena and discussion. Chapter 4 describes biomass growth and enzyme secretion by *P. chrysosporium* inpresence of DR-80 as a toxic pollutant and its modeling. Whereas Chapter 5 describes effect of physico-chemical factors such as nitrogen source, initial pH, temperature, and agitation, both in absence and in presence of DR-80, on biomass growth and enzyme secretion by *P. chrysospoirum*, Chapter 6 gives the summary and conclusions of the work as well as some the recommendations for future work.

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Chapter-2

Literature Review

The treatment of industrial wastewaters can be performed by many processes. Among which many physical and chemical processes are used industrially. This chapter presents a review on these various treatment processes and also elaborates the importance of biological treatment over the other processes used in the industry.

2.1. Environmental Impact of Textile Dyeing Industry Wastewaters

Textile industries consume large volumes of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products [Mishra and Tripathy, 1993]. The presence of very low concentrations of dyes in effluent is highly visible and undesirable [Nigam et al., 2000]. There are more than 100,000 commercially available dyes with over 7×10^5 ton of dye-stuff produced annually [Zollinger, 1987]. Due to their recalcitrant nature, dyes are resistant to fading on exposure to light, water and many chemicals [Poots and McKay, 1976a]. Many dyes are difficult to decolourize due to their complex structure and synthetic origin. There are many structural varieties, such as, acidic, basic, disperse, azo, diazo, anthroquinone based and metal complex dyes. Decolourisation of textile dye effluent does not occur when treated aerobically by municipal sewerage systems [Willmott et al., 1998].

Many dyes are made from known carcinogens, such as benzidine and other aromatic compounds, all of which might be transformed as a result of microbial metabolism [Clarke and Anliker, 1980]. It has been shown that azo- and nitro-compounds are reduced in sediments and in the intestinal environment [Chung et al., 1978], resulting

in the regeneration of the parent toxic amines. Anthraquinone based dyes are the most resistant to degradation due to their fused aromatic structures, which remain coloured for long periods of time. Basic dyes have high brilliance and therefore higher colour intensity, making them more difficult to decolourize, while metal-based complex dyes, such as chromium-based dyes, can lead to the release of carcinogenic chromium, into water supplies.

Anliker et al. [1981] and Baughman and Perenich [1988] reported that some disperse dyes tend to bio-accumulate and heavy-metal ions from textile effluents have also been reported at high concentrations in both algae and higher plants exposed to such effluents.

2.2.Major Treatment Techniques for Textile Dyeing Industry Wastewater

Various methods to treat textile dyeing effluents are presented under three categories: physical, chemical and biological.

2.2.1. Physical treatment methods

2.2.1.1. Adsorption

Adsorption techniques have gained favour due to their efficiency in the removal of pollutants that are mostly stable to conventional methods. Choy et al. [1999] reported that adsorption produces a high quality product, and is a process which is economically feasible.Decolourization is a result of two mechanisms: adsorption and ion exchange [Slokar and Le Marechal, 1997], and is influenced by many physico-chemical factors, such as, dye/ sorbent interaction, sorbent surface area, particle size, temperature, pH, and contact time.

2.2.1.1.1. Activated carbon

Raghavacharya [1997] reported that activated carbon is very effective for adsorbing cationic, mordant and acid dyes and to a slightly lesser extent, dispersed, direct, vat, pigment and reactive dyes. Performance is dependent on the type of carbon used and the characteristics of the wastewater. Removal rates can be improved by using relatively large doses, although its regeneration or re-use results in a steep reduction in performance, and efficiency of dye removal becomes unpredictable and depends on the carbon dose used.

2.2.1.1.2. Peat and wood chips

Poots and McKay [1976a] reported that peat requires no activation, unlike activated carbon, and also costs much less. Due to activated carbon's powdered nature, it has a much larger surface area, and hence has a better capacity for adsorption. Spent peat may be burned and utilized for steam raising, or, potentially, as substrate in solid state fermentation (SSF), for protein enrichment.

Wood chips show good adsorption capacity for acid dyes although, due to their hardness, it is not as good as other available sorbents [Nigam et al., 2000] and longer contact times are required. Adsorbed wood is conventionally burnt to generate power although there is potential for SSF of the dye adsorbed wood chips.

2.2.1.1.3. Fly ash and coal (mixture), and silica gel

An effective material for removing basic dyes, although with side reactions, such as air binding and air fouls with particulate matter, prevents these materials from being used commercially. A high fly ash concentration increases the adsorption rates of the

mixture due to increase in the surface area available for adsorption. This combination may be substituted for activated carbon, with a ratio of fly ash: coal, 1:1 [Gupta et al., 1990].

2.2.1.2. Membrane filtration

Mishra and Tripathy [1993] and Xu and Lebrun [1999] reported that this method has the ability to clarify, concentrate and, most importantly, to separate dye continuously from effluent. It has some special features unrivalled by other methods; resistance to temperature, an adverse chemical environment and microbial attack. The concentrated residue left after separation poses disposal problems, and high capital cost and the possibility of clogging, and membrane replacements are its disadvantages. This method of filtration is suitable for water recycling within a textile dye plant if the effluent contains low concentration of dyes, but it is inefficient to reduce the dissolved solid content, which makes water re-use a difficult task.

2.2.1.3. Ion exchange

Both cation and anion dyes can be removed from dye-containing effluent by passing the wastewater over an ion exchange resin until the available exchange sites are saturated. Advantages of this method include no loss of adsorbent on regeneration, reclamation of solvent after use and the removal of soluble dyes. A major disadvantage is cost. Organic solvents are expensive and the ion exchange method is not very effective for disperse dyes [Mishra and Tripathy, 1993]. Further, ion exchange has not been widely used for the treatment of dye-containing effluents, mainly because ion exchangers may not accommodate a wide range of dyes [Slokar and Le Marechal, 1997].

2.2.1.4. Irradiation

Sufficient quantities of dissolved oxygen are required for organic substances to be broken down effectively by radiation. The dissolved oxygen is consumed very rapidly and so a constant and adequate supply is required, which adversely affectsthe cost of this process. This method showed that some dyes and phenolic molecules can be oxidized effectively at a laboratory scale only [Hosono et al., 1993].

2.2.2. Chemical methods

2.2.2.1. Oxidative processes

This is the most commonly used method of decolourization by chemical means, which is also simple to apply. The main oxidizing agent is usually hydrogen peroxide (H_2O_2). Slokar and Le Marechal[1997] reported that many methods of chemical decolourization vary depending on the way in which the H_2O_2 is activated. Chemical oxidation removes the dye from the dye-containing effluent by oxidation resulting in aromatic ring cleavage of the dye molecules [Raghavacharya, 1997].

2.2.2.2. Ozonation

Oxidation by ozone is capable of degrading chlorinated hydrocarbons, phenols, pesticides and aromatic hydrocarbons [Lin and Lin, 1993; Xu and Lebrun, 1999]. The dosage applied to the dye-containing effluent is dependent on the total colour and residual COD to be removed with no residue or sludge formation and no toxic metabolites [Gahr et al., 1994]. Ozonation leaves the effluent with no colour and low COD suitable for discharge into environmental waterways. This method shows a preference for double-bonded dye molecules [Slokar and Le Marechal, 1997]. One

major advantage is that ozone can be applied in its gaseous state and therefore does not increase the volume of wastewater and sludge. Chromophore groups in the dyes are generally organic compounds with conjugated double bonds that can be broken down forming smaller molecules, resulting in reduced colouration [Peralto-Zamora et al., 1999]. A disadvantage of ozonation is its short half-life, typically being 20 min. This time can be further shortened if dyes are present, with stability being affected by the presence of salts, pH and temperature. Under alkaline conditions, ozone decomposition is accelerated that requires careful monitoring of the effluent pH. Better results can be achieved using irradiation or with a membrane filtration technique [Lopez et al., 1999]. One of the major drawbacks with ozonation is cost; continuous ozonation is required due to its short half-life [Xu and Lebrun, 1999].

2.2.2.3. Photochemical

This method degrades dye molecules to CO_2 and H_2O [Yang et al., 1998] by UV treatment in the presence of H_2O_2. Degradation is caused by the production of high concentrations of hydroxyl radicals ($OH^{\bullet}$). UV light may be used to activate chemicals, such as H_2O_2, and the rate of dye removal is influenced by the intensity of the UV radiation, pH, dye structure and the dye bath composition. Depending on initial materials and the extent of the decolourization treatment, additional by-products, such as, halides, metals, inorganic acids, organic aldehydes and organic acids, may be produced. There are advantages of photochemical treatment of dye-containing effluent; no sludge is produced and foul odours are greatly reduced. UV light activates the destruction of H_2O_2 into two hydroxy radicals as shown in equation (2.1).

$$H_2O_2 + hv \rightarrow 2OH* \qquad (2.1)$$

2.2.2.4. Sodium hypochloride (NaOCl)

This method attacks at the amino group of the dye molecule by the Cl^+. It initiates and accelerates azo bond cleavage. This method is however unsuitable for disperse dyes. An increase in decolouration is seen with an increase in chlorine concentration. The use of chlorine for dye removal is becoming less frequent due to the negative effects it has when released into waterways [Slokar and Le Marechal, 1997].

2.2.2.5. Cucurbituril

Cucurbituril is a cyclic polymer of glycoluril and formaldehyde [Karcher et al., 1999a, 1999b]. The name cucurbituril is because of its pumpkin shape (a member of the plant family Cucurbitaceae). The uril, indicates that a urea monomer is also part of this compound. Buschmann [1992] demonstrated very good sorption capacity of cucurbituril for various types of textile dyes. Cucurbituril is known to form host-guest complexes with aromatic compounds [Mock, 1995] and this may be the mechanism for reactive dye adsorption. Another proposed mechanism is based on hydrophobic interactions or the formation of insoluble cucurbituril dye-cation aggregates since adsorption occurs reasonably fast. To be industrially feasible, cucurbituril would need to be incorporated into fixed bed sorption filters. Like many other chemical methods, cost is a major disadvantage.

2.2.2.6. Electrochemical destruction

This technique has some significant advantages for use as an effective method for dye removal. There is little or no consumption of chemicals and no sludge build up. The breakdown metabolites are generally not hazardous leaving it safe for treated

wastewaters to be released back into water ways. It shows efficient and economical removal of dyes and a high efficiency for colour removal and degradation of recalcitrant pollutants [Ogutveren and Kaparal, 1994]. Relatively high flow rates cause a direct decrease in dye removal and the cost of electricity used may be comparable to the price of chemicals.

2.2.3. MicrobialTreatment Processes

The treatment of textile wastewater by purely biological processes may be possible even without the inclusion of other carbon sources, e.g. municipal wastewater. Such a situation was predicted by McKay [1979] who concluded that "decolourization through biological systems would receive increased attention in the future". Several microbial cultures have been tested or are implicated in textile dye decolourization as follows.

2.2.3.1. Bacterial degradation of textile dyes

Bacterial cultures capable of degrading azo dyes were first reported for *Bacillus subtilis*, *Aeromonas hydrophila* and *Bacillus cereus*. Extended periods of adaptation in chemostat conditions were needed to isolate the first two *Pseudomonas* strains capable of dye decolourization [Kulla, 1981]. An azo reductase enzyme was responsible for the initiation of the degradation of the Orange II dye by these strains and substituting any of the groups near the azo group's chemical structure hindered the degradation [Zimmermann et al., 1982]. Several other decolourizing *Pseudomonas* and *Aeromonas* species were then reported by a Japanese group [Ogawa and Yatome, 1990]. Haug et al. [1991] described a bacterial consortium capable of mineralizing the sulfonated azo dye Mordant Yellow. In a review, Groff and Kim [1989] described a

host of bacterial cultures with capabilities to carry out decolourization, including a *Rhodococcussp*., *Bacillus cereus*, a *Plesiomonas sp*. The presence of sulfo groups on the aromatic component of some azo dyes seemed to significantly inhibit the biodegradability of the sulfonated azo dyes by bacteria. A *Rhodococcus sp*. capable of effectively decolourizing two sulfonated azo dyes, Orange II and Amido Black, was used to clone DNA fragments coding for azoreductase into a mutant which had lost decolourization capability, thereby conferring sulfonated azo-dye decolourization ability [Heiss et al., 1992]. Several other actinomycete stains have been reported with a capability to decolourize reactive dyes, including anthraquinone, phthalocyanine and azo, through adsorption of dyes to the cellular bid mass without any degradation [Zhou and Zimmermann, 1993]. Other Cu-based azo dyes, such as formazan-copper complex dyes, were completely decolourized through degradation by the same actinomycete strains. *Pseudomonas luteola*, bacteria, with the ability to remove the colour of reactive azo dyes, such as Red G, RBB, RP2B and V2RP, has been isolated from dyeing-wastewater-treatment sludge. Complete degradation was observed for the latter three dyes, while only azo-bond cleavage was observed for Red G dye in this culture. Other bacterial strains of *Klebsiella pneumoniae* RS- 13 and *Acetobacter liquefaciens* S-1 capable of decolourizing Methyl Red (MR) have also been reported as suitable for future applications in azodyes- containing industrial effluents [Wong and Yuen, 1996].

2.2.3.2. Decolourization with algal cultures

There has been only one report of algae capable of degrading azo dyes through an induced form of an azo reductase [Jinqi and Houtian, 1992]. Several species of *Chlorella* and *Oscillatoria* were capable of degrading azo dyes to their aromatic

amines and to further metabolize the aromatic amines to simpler organic compounds or CO_2. Some were even capable of utilizing a few azo dyes as their sole source of carbon and nitrogen. Using such algae was proposed by the authors for use in stabilization ponds, as they can play a role in aromatic amine removal.

2.2.3.3. Biodegradation by fungi

Several fungi have been shown to be capable of decolourization. *Neurospora crassa* was reported to decolourize diazo dyes by Corso and coworkers[1981]. *Schizophyllum commune* also decolourized wastewater from a bagasse-pulping plant. A strain of a *Trichoderma sp*., belonging to the fungi imperfecti, was also shown to decolourize lignin-containing hardwood-extraction-stage leachplant effluent [Prasad and Joyce, 1991]. Up to 85% colour removal was achieved after 3 days cultivation. Enrichment procedures designed to obtain microbial agents suitable for decolourizing dye-containing wastewater, by Mou and co-workers [1991], resulted in the isolation of several strains of fungi capable of decolourization. These included strains of *Myrothecium verrucaria* and of *Ganoderma sp*. Up to 99% decolourization was observed after 48 h incubation, which was mainly through adsorption to the fungus mycelium and was effective for a wide range of dyes. *Myrothecium verrucaria* was shown to have a very strong binding affinity to some azo dyes, which were recoverable by extraction with methanol, suggesting a hydrophobic-hydrophilic interaction in the dye binding mechanism [Brahimi-Horn et al., 1992]. A strain of *Aspergillus sojae* B-10 was also shown to be able to decolourize the azo dyes Amaranth, Congo Red and Sudan III in nitrogen-poor media after 3-5 days incubation. Other facultative anaerobic fungi capable of growth on dyes as sole carbon sources have been reported but they do not seem to be able to carry out

decolourization [Marchant et al., 1994]. They appear to cleave some of the bonds in these dyes to use as carbon sources, yet do not affect the chromophore centre of the dyes.

2.2.3.3.1. *Phanerochaete chrysosporium*

Bio-decolourization of lignin-containing pulp and paper wastewater, as measured by the decrease in colour absorption, using two white-rot Basidiomycete fungi *Phanerochaete chrysosporium* and *Tinctoporia sp*., was reported in1980 [Fukuzumi, 1980]. Both were clear examples of colour removal through microbial degradation of polymeric lignin molecules. Since then, the wood-rotting *P.chrysosporium* in particular has been the subject of intensive research related to the degradation of a wide range of recalcitrant xenobiotic compounds, including azo dyes. The mechanism of colour removal involves a lignin peroxidase and Mn dependent peroxidase or laccase enzymes [Michel et al., 1991]. The decolourization of three polymeric dyes Polymeric B-411, Polymeric R-481 and Polymeric Y-606 (Sigma) by *P.chrysosporium* was confirmed by Glenn and Gold [1983]. Their results suggested that the decolourization was a secondary metabolic activity linked to the fungus' ligninolytic-degradation activity. *Phanerochaete chrysosporium* was also shown to biodegrade the azo- and heterocyclic-dyes Orange II, Tropaeofin O, Congo red and Azure B [Cripps et al., 1990]. The extent of colour removal varied depending on the dye complexity, nitrogen availability in the media and ligninolytic activity in the culture. Veratryl alcohol is believed to stimulate the ligninase activity, which seems to be linked to decolourization [Paszczynski & Crawford, 1991]. Capalash and Sharma [1992] tested the biodegradation of 18 azo dyes using *P. chrysosporium* and only eight were degraded, with 40-70% colour removal. This degradation was mainly

through the lignin-degrading enzyme system or adsorption to cell mass. The use of high lignin-peroxidase producing medium for growing *P. chrysosporium* immobilized on polyurethane foam significantly improved the decolourization achieved in olive-mill wastewater. In the majority of the above cases it was generally observed that nitrogen-limitation increased ligninolytic activities through increased lignin peroxidase and Mn-dependent peroxidase and therefore enhanced decolourization. Contrary to that, however, Chao and Lee [1994] reported higher decolourization rates when their strains were pre-grown in nitrogen-rich media, while slower or no decolourization occurred with strains pre-cultured in low-nitrogen media.

2.3.3.2.P. *chrysosporium* ligninase enymes

Lignin peroxidase (LiP) and manganese peroxidase (MnP) have been demonstrated to be major components of the lignin degradation system of *P. chrysosporium*. Since their discovery, these two enzymes have been purified and extensively characterized biochemically. The enzyme is present as a series of glycosylated isozymes with pHs ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDa. Each isozyme contains 1 mol of iron heme per mol of protein [Gold et al., 1989]. MnP enzyme exists as a series of glycosylated isozymes with pHs ranging from 4.2 to 4.9 and with molecular masses ranging from 45 to 47 kDa. Each isozyme also contains 1 mol of iron heme per mol of protein [Glenn and Gold, 1985]. Detailed electro paramagnetic resonance, resonance Raman, electronic absorption and nuclear magnetic resonance spectral studies demonstrate that these enzymes have similarities to horseradish peroxidase (HRP). The enzymatic reactions involve a mechanism that the initial one-electron oxidation of susceptible aromatic nuclei by an oxidized enzyme intermediate to form a substrate aryl cation radical then it can undergo a

variety of nonenzymatic reactions to yield a wide range of final products. Redox potential determines whether an aromatic nucleus is a substrate for LiP. The ability of LiP to oxidize lignin nonspecifically to generate cation radicals which undergo a variety of nonenzymatic reactions accounts for the variety of metabolic products observed. Manganese peroxidase catalyzes the H_2O_2-dependent oxidation of lignin and lignin derivatives and a variety of phenolic lignin model compounds. It has been demonstrated that Mn(II) is the preferred substrate for MnP. The enzyme oxidizes Mn(II) to Mn(III), which diffuses from the enzyme surface and in turn oxidizes the phenolic substrate. Organic acids, such as oxalate and malonate, which are produced by *P. chrysosponium*, activate the MnP system by chelating Mn(III) to form stable complexes with high redox potentials and by facilitating the dissociation of Mn(III) from the enzyme [Wariishi et al., 1989]. Thus Mn ion participates in the reaction as a diffusible redox couple rather than as an enzyme-binding activator. The initial reaction of Mn(III) with a phenol is a one-electron oxidation to form a phenoxy radical intermediate. Subsequently, alkyl-phenyl cleavage, Ca-C cleavage, or benzyliccarbinol oxidation yields the variety of products observed.

2.3. Computer Simulation onFungal Biomass Growth and Enzyme Secretion:

Computer simulation provides exact information on model systems in a controlled manner. One could check the accuracy of the approximations made in the analytical treatment and at the same time one could identify the precise interaction responsible for an observed experimental phenomenon. Thus computer simulation not only provides exact information, but also bridges the gap between theory and experiment. There are quite a number of numerical techniques found to be very useful for

computer simulation of model systems. Monte Carlo (MC) simulation is one of the most useful techniques used to study complex systems.

In a MC simulation [Landau and Binder, 2009], the time evolution of a model for which change, or growth, does not proceed in some rigorously predefined, but rather in a stochastic manner which depends on a sequence of random numbers generated during the simulation. With a different sequence of random numbers the simulation will not give identical results, but will yield values which agree with those obtained from the first sequence to within some 'statistical error'. Simplicity of the underlying principle of the technique enables its application to a wide range of problems including biological problems.

However, in literature, there are very few numerical or computer models for describing growth of fungus. For instance, the growth model described by Peilin et al. [2003] is based on the Monod- Jacob operon model and takes into account the production of repressors and mRNA for the estimation of secondary metabolites. However, the substrate depletion and growth rate were estimated using 1^{st} and 2^{nd} order differential relationships with biomass itself. It doesn't describe the change in the biomass growth behaviour caused by physico-chemical parameters. The model provided by Sugden et al. [2007] details on the mycelia growth of filamentous fungi and it mainly depends on the accumulation of microtubule transported vesicles containing nutrition as well as their distribution near the hyphae in *N. crassa*. Dynamics of growth as well as substrate depletion was studied by Osorio et al. [2008] with an unstructured Monod and Leudeking-Piret model on *A. flavipes*, which is however restricted to the determination of kinetic parameters of the system. In order

to study the time evolution of a system with many different time scales for its sub-processes, stochastic approach can be more useful over the deterministic models considered mostly in the literature.

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Chapter-3

Biomass growth and enzyme secretion by *P. Chrysosporium*

The white rot fungus *Phanerochaete chrysosporium* is well known for its ability to degrade a variety of hazardous pollutants including textile dyes owing to its lignin degrading enzyme system [Kirk et al., 1978]. It is a basidiomycete and grows with the formation of basidiospores when cultured in liquid medium [Kirk and Farrell, 1987]. Reactor as well as batch shake flask studies with the fungus confirms that the production of the enzymes is dependent on physico-chemical parameters [Linko and Haapala, 1993, Kirk et al., 1981] of the system such as media composition, temperature, pH, agitation etc. Among the enzymes produced by this fungus, lignin peroxidase (LiP) has been shown to be important. Secretion of this enzyme is understood to be mainly dependent on nutrient limiting conditions and biomass growth and other physico-chemical factors such as agitation [Leisola et al., 1984] and temperature [Linko, 1992], which are also responsible for the biomass growth. However, there is no clear study undertaken to elucidate variability in biomass growth and enzyme secretion kinetics of this environmentally important fungus in relation with these physico-chemical factors, which may form the basis for improved performance of the fungus for textile dye decolourisation and other applications. Among the various physico-chemical factors, the easily assimilable carbon source by this fungus, i.e. glucose, which in form affects the enzyme secretion by *P. chrysosporium*.In order to clearly understand the manner by which glucose influences these two biological phenomena, this study was aimed at investigating the biomass growth and LiP secretion kinetics as a function of variation in the initial glucose concentration in the media. To accurately predict these two processes, Monte-Carlo method was adopted.

In order to study the time evolution of a system with many different time scales for its sub-processes, stochastic approach can be more useful over the deterministic models considered mostly in the literature. In the present work, a Monte Carlo method was developed to model three different processes such as glucose consumption, cell division and enzyme secretion by a non-specific fungus. The results obtained from the model were compared with that of the experiments and a good qualitative agreement was observed.

3.1.Experimental Methods

Phanerochaete chrysosporium MTCC 787, purchased from IMTECH Chandigarh, India, was maintained at 25 ºC on malt agar slants; for spore production, slants were maintained at 39ºC for 2-5 days in medium containing (g/L) glucose 10 , malt extract 10 , peptone 2 , yeast extract 2 , asparagine 1 , KH_2PO_4 2 , $MgSO_4.7H_2O$ 1 , thiamin-HCl 0.001 , agar 20 .

Medium optimized by Kirk et al. [Kirk et al., 1990] was initially used to subculture the fungus from spores and later modified to include only glucose as the sole carbon source along with other nutrients in the medium in order to study the effect of glucose and other parameters on the fungal growth and LiP secretion kinetics. Thus the medium used was composed of Basal III Medium (100 ml), glucose (10 gL^{-1}), 0.1 M 2,2-dimethyl succinate (1.46 gL^{-1}), thiamin (0.001 gL^{-1}), ammonium chloride (4.68 gL^{-1}), 1% tween 80 (50 ml) and trace elements ,where the basal III medium contains KH_2PO_4 (20 gL^{-1}) , $MgSO_4$ (5 gL^{-1}), $CaCl_2$ (1 gL^{-1}) and trace elements. Initial pH of the medium was set to 4.5.

During the experiments the fungal samples were collected at six hour intervals from the medium and centrifuged to remove fungal spores. Fungal Growth was measured by counting the spores using a haemocytometer [Morris and Nicholls, 1978].Biomass free supernatants were divided into two portions. While one portion was analyzed for LiP activity by the fungus, the other portion was analyzed for glucose concentration. For glucose analysis, di-nitro salicylate method was adopted by taking the absorbance of the colour developed at 540 nm [Bailey, 1988].LiPactivity was determined spectrophotometrically by the oxidation of veratryl alcohol to veratraldehyde [Kirk et al., 1990, 1986a, 1986b].

To determine the effect of initial glucose concentration, its level was varied in the range 5-25 gL^{-1} keeping the other media constituents at fixed level. All batch shake flasks experiments in the study were carried out by incubating the flasks in an orbital incubator shaker set at 30^{o}C and 150 rpm.

To avoid the experimental errors, all experiments were performed in replicates. Samples for further analysis were collected in triplicates and averages were reported. At the 95% level of significance, 2σ control charts were maintained to ascertain the output data. For all the experiments performed here, P value was kept < 0.05.

3.2. Results

It can be seen from Fig. 2 that the rate of consumption of glucose increase with initial glucose concentration in the medium. In Fig. 4, the spore count is plotted against time for the same initial glucose concentrations, which reveal that the whole growth process has three distinct phases with an initial lag phase during which the growth is

slow and the corresponding rate of glucose consumption is also less. It may be because of time taken to adapt to the new environment due to introduction of the mature spores to another medium. It can also be noticed that the duration of lag phase shortens as the initial glucose concentration in the medium is high, because low glucose concentration correspond to less availability of glucose to a spore to consume. During the intermediate phase and the end of the lag phase, a fast growth is observed corresponding to higher rate of glucose consumption, due to more number

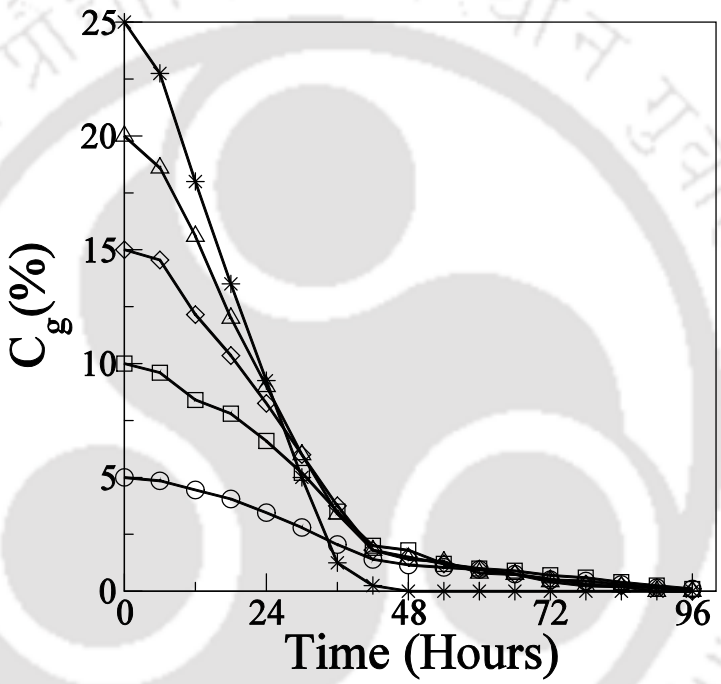

Figure 2: Residual glucose remained, Cg% (as % of the total medium) for different initial glucose concentration [0.5 (○), 1.0 (□), 1.5 (◊), 2.0 (Δ), 2.5 (✳) %w/v]

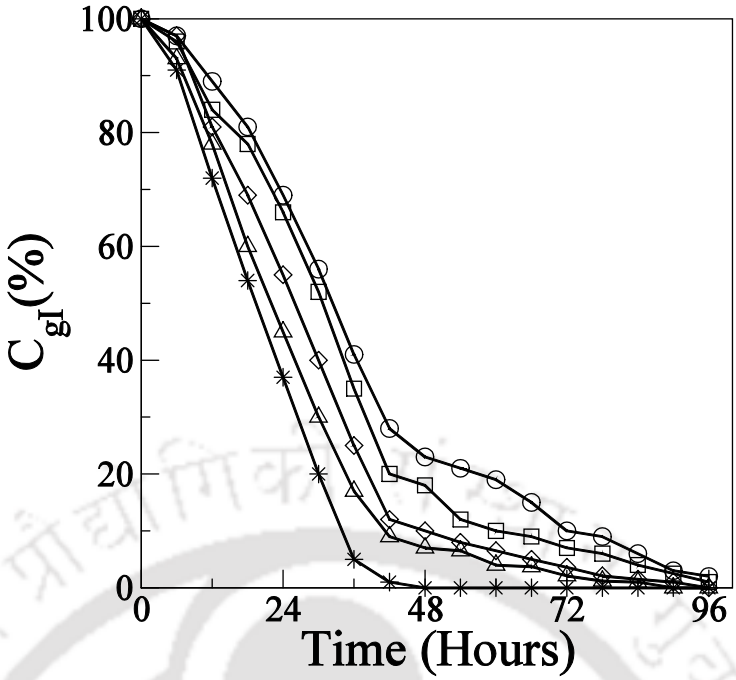

Figure 3: % glucose remained C_{gI}(%) (as % of initial glucose concentration) for different initial glucose concentration [0.5 (○), 1.0 (□), 1.5 (◊), 2.0 (Δ), 2.5 (✳) %w/v]

of mature spores available for cell division to occur, besides the availability of glucose in the medium.

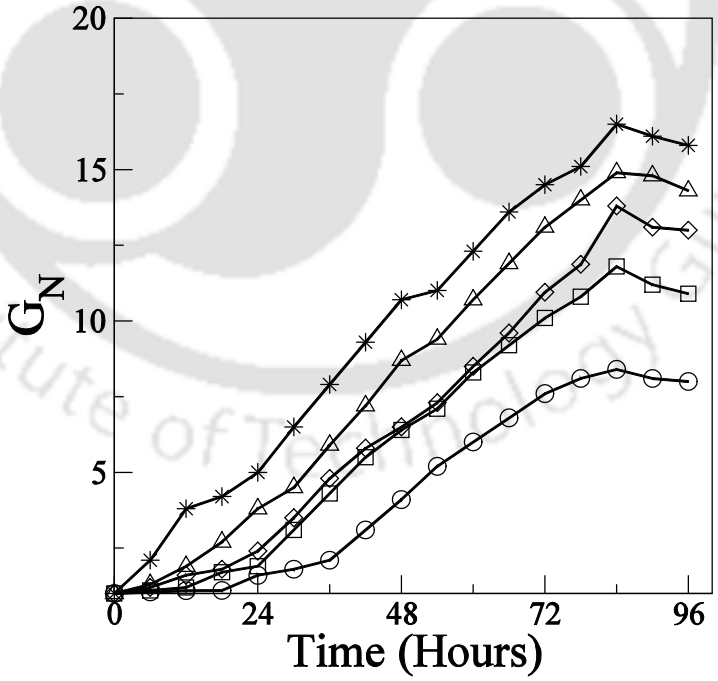

Figure 4: Growth in terms of generations of initial inoculums (G_N) for different initial glucose concentration [0.5 (○), 1.0 (□), 1.5 (◊), 2.0 (Δ), 2.5 (✳) %w/v]

During the last phase or the saturation phase, the spore count reaches a maximum value and remains almost constant over a longer period of time. By this time, the glucose supplied to the medium is completely consumed by the spores, with no further cell division possible. It is also observed that the maximum spore count is directly proportional to the initial glucose concentration.

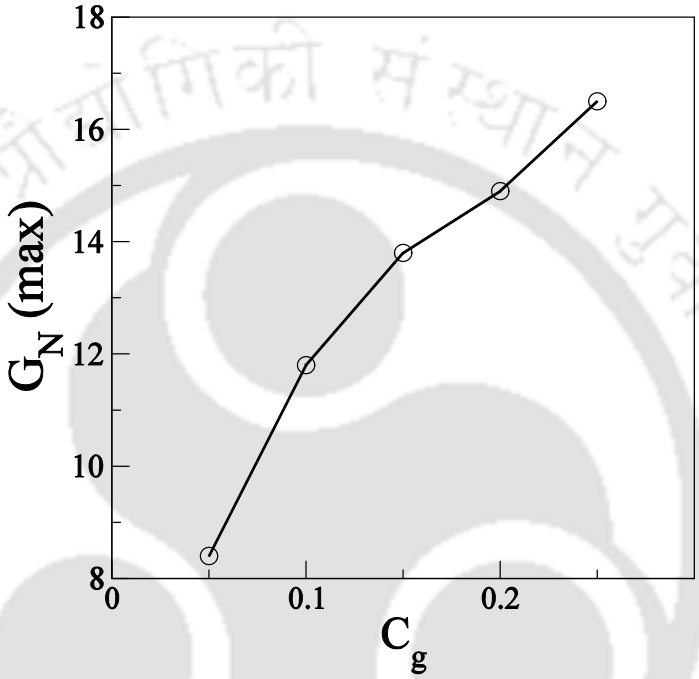

Figure 5: Maximum generations achieved $G_N(max)$ for different initial glucose concentrations.

The LiP activity by the fungal culture is plotted against time in Fig. 6, and where again three distinct phases can be observed. The first phase is of very low enzyme activity, which may be because spores take time to adapt to the new environment it was inoculated and also they start accumulating glucose before establishing growth. Hence, the production of enzymes was initially low. This phase is short in case of high initial glucose concentration in the medium that may be because of faster consumption of glucose, Fig.3. , which causes spores to secrete enzymes at a higher rate as scarcity of glucose reaches faster at higher initial concentration.

At the end of this phase enzyme activity increases rapidly denoting that most of the spores are producing enzymes. At the last phase, the activity saturates as there was no more glucose as well as the spore number is also not increasing.

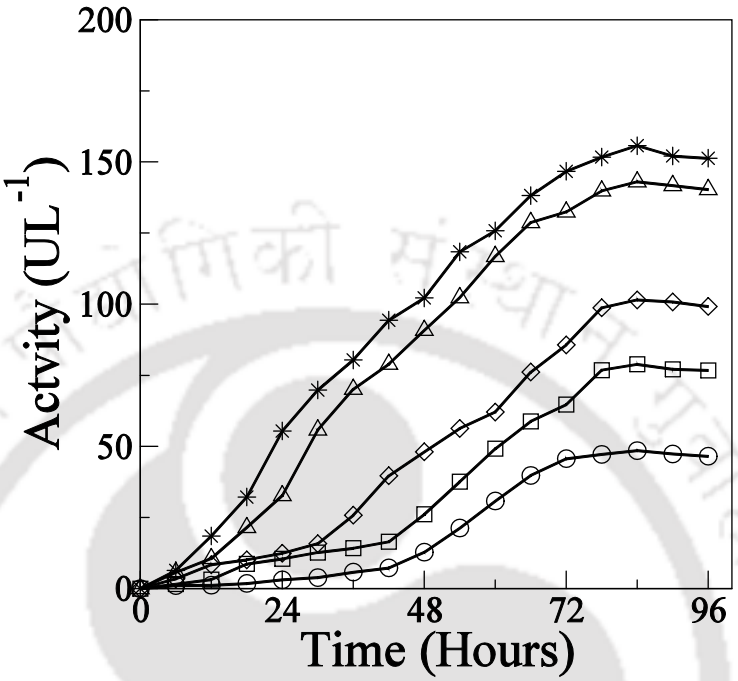

Figure 6: LiP activity (A_C) at different initial glucose concentration [0.5 (○), 1.0 (□), 1.5 (◊), 2.0 (Δ), 2.5 (✳) %w/v]

Though growth mechanism and LiP activity of *P. chrysosporium* is characterized from the results obtained in the controlled experiments, it is not clear what empirical laws governed the growth and enzyme activity of this fungus. In order to have some idea about these empirical laws behind the growth and enzyme activity, one needs to develop a model assuming certain growth law and check that the model results reproduce qualitatively the experimental results obtained.

3.3. Model

Based on the experimental findings, glucose seemed to influence both biomass growth and LiP secretion by the fungus. A stochastic model of fungal growth and activity of

the enzyme secreted by the fungus was developed in order to understand the experimental results obtained. The model was developed taking into account that glucose is the sole source of carbon. The entire process contains three major steps: (a) consumption of glucose, (b) cell division and (c) enzyme (LiP) secretion. A self-stabilized Monte Carlo algorithm [Sapoval et al., 1998, Mode, 2011] has been developed in order to study the time evolution of these processes. Initially, $N_s(0)$ number of cells and $N_G(0)$ number of glucose molecules were taken in a constant arbitrary volume V as the initial parameters of the model. Glucose molecules are consumed by the cells during the growth. As a consequence, the glucose concentration in the medium decreases with time t. The instantaneous glucose concentration in the medium at any time t is then given by:

$$G_t = G_0\left(1-\frac{n_G(t)}{N_G(0)}\right) \tag{3.1}$$

where $G_0 = N_G(0)/V$ is the initial glucose concentration and $n_G(t)=\sum_{i=1}^{t} n_G(i)$ is the number of glucose molecules consumed upto time t. Two main criteria for glucose consumption are: (i) availability of a glucose molecule to a cell which is directly proportional to the instantaneous glucose concentration G_t of the medium and (ii) intake probability $p_{\text{int}ake}$ of a glucose molecule by a cell which depends on the size of the cell. The size of a cell is defined as the number of glucose molecules present inside the cell at a given time. A matured cell contains g_c amount of glucose and has all active primary functions. A cell of size less than a mature cell is less probable to intake a glucose molecule than a cell of size more than that of a mature cell. The probability that a cell will intake a glucose molecule is assumed to be a Poisson's distribution and is given by

$$p_{intake} \propto g_r \exp(-g_r) \tag{3.2}$$

where, $g_r = g/g_c$ is the relative cell size. The probability of glucose consumption is then given by

$$p_{GC} = \Gamma G_t p_{\text{int}ake} \tag{3.3}$$

where, Γ is the molar ability of attachment of a glucose to a cell. In order to make glucose consume exactly with probability p_{GC}, a Monte Carlo technique is adopted. In this method, a random number r, uniformly distributed between 0 and 1, is called corresponding to that cell and if $r \leq p_{GC}$, the glucose molecule is consumed by the cell otherwise it is left out. The number of glucose molecules to be taken up by the cell is determined by the number glucose molecules present in the system per cell. All the cells present in the medium at that time were called for consumption of glucose.

Cell division is the process of release of daughter cells by the matured cells. It is assumed in the model that the division of a cell solely depends on the size of a cell. The probability of cell division is modeled by a sigmoidal growth probability distribution and is given as

$$p_{div} = \frac{1}{1+e^{-(g-g_c)/\pm_d}} \tag{3.4}$$

where $\pm_d$ is the capability of cell division of a matured cell . Again a Monte Carlo technique is adopted to perform cell division exactly with the probability p_{div} . A random number r was called and if $r < p_{div}$, the cell was allowed to divide otherwise no cell division occurred. All mother cells (cells of the previous time step) were called for cell division. It is known that *P. chrysosporium*, is a basidiomycete and grows by forming basidiospores (multiple spores released at a single ejaculation).

This phenomenon was incorporated in the model by the release of a number of daughter cells at a time by the mother cell. The number of daughter cells to be produced depends on its glucose content g. The glucose content of a cell is utilized as one glucose molecule for the release of every daughter cell, cell division cost and one glucose molecule for every new born daughter cell as initial glucose content.

It had already been established that *P. chrysosporium* produces LiP as secondary metabolite when glucose concentration in the medium is less [Kirk et al., 1981]. It is also known that the activity depends on the size of a cell for an under-developed cell [Gold and Alic, 1993]. In order to model the enzyme activity, the cells are classified as developed and under-developed cells. A cell is considered as a developed cell if it undergoes a cell division. The probability of an under-developed cell to produce enzyme is given by

$$p_E = \left(1 - G_t / G_0\right) \exp\left(-\Delta g_i^2\right) \tag{3.5}$$

where $\left(1 - G_t / G_0\right)$, is the fractional glucose consumed from the medium, $\Delta g_i^2 = \left[\left(g_C - g\right) / \sqrt{2}\pm_E\right]^2$, is the measure of deviation of size of a cell from the maturity and $\pm_E$ is the ability of enzyme production by a cell. For a developed cell, the probability to produce enzyme depends only on the scarcity of glucose in the medium and it is given by,

$$p_E = \left(1 - G_t / G_0\right) \tag{3.6}$$

The enzyme secretion is also modelled stochastically by adopting a MC technique. A random number r, uniformly distributed between 0 and 1, is called corresponding to a cell and if $r \le p_E$, certain amount of enzyme is released by the cell otherwise no enzyme is released. Certain unit of glucose is reduced from the glucose content g of

the cell as enzyme production cost on the release of one unit enzyme. All the cells were called for the production of enzyme. The three sub-processes, glucose consumption, cell division and release of enzyme constitute a single MC time step. The process stops on its own when either there is no glucose to consume or no further cell division occurs.

In order to measure the enzyme activity, a simplified enzyme kinetic reaction is considered here as $E + S \rightarrow E + P$, where E stands for enzyme, S stands for substrate and P stands for product. The enzyme-substrate reaction rate should be proportional to the concentrations of enzyme and substrate in the medium. Thus,

$$Rate = AC_E C_S \tag{3.7}$$

where A is a constant based on activation energy of the enzyme-substrate complex. As per the collision theory [House, 2007], the value of A is given by,

$$A = d^2 \text{®} \left(\frac{8k_B T}{\text{®«}} \right)^{1/2} \exp\left(\frac{\text{£}}{RT} \right) \tag{3.8}$$

Where ε> E_a , E_a is the activation energy for the current reaction, k_B is the Boltzmann constant, R is universal gas constant, T is the temperature, d is the average diameter of the two reacting species called as collision distance and « is the reduced mass of the enzyme-substrate complex. As the enzyme concentration C_E is known from the MC process, the rate could be calculated for a given substrate concentration C_s. The substrate concentration C_s is usually kept much larger than C_E in the enzyme assay and hence, C_s in the medium remains almost constant during the assay period. The same condition is also maintained here to calculate the enzyme production rate.

3.4. Model Results and Comparison withExperiment

The model of biomass growth and enzyme activity is now studied for the parameters listed in Table 1.

Table 1: Values of the parameters taken initially in the simulation

Parameter Name	Symbol	Value
Medium volume	V	10^7
Initial number of glucose molecules	$N_G(0)$	1-5 x 10^6
Initial number of spores	$N_S(0)$	10^3
Mature cell	g_c	64
Molar attachment ability of glucose	Γ	1
Capability of cell division of a matured cell	σ_d	12
Ability of enzyme production by a cell	σ_E	4

Results obtained from the model are presented in Figs. 7-11. The results are shown to 5000 MC steps to display the actual behaviour, though the process stops at more than 12700 MC steps. The drop in glucose concentration C_g in the medium is shown in Fig. 7 against the MC time steps.

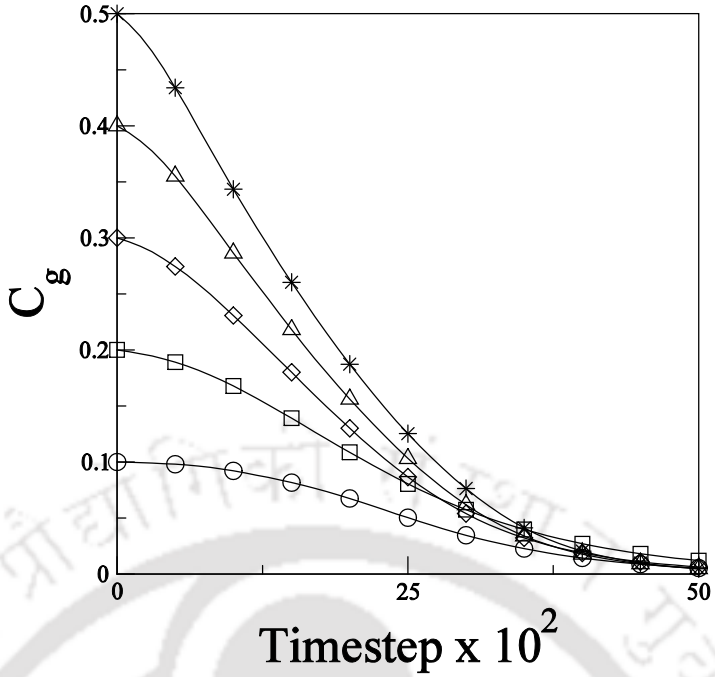

Figure 7: Plot of instantaneous glucose concentration (C_g) in the medium against MC time steps for different initial glucose [10 (○), 20 (□), 30 (◊), 40 (Δ), 50 (✳) %] concentrations.

The model result has depicted more or less the same nature of the experimental data.

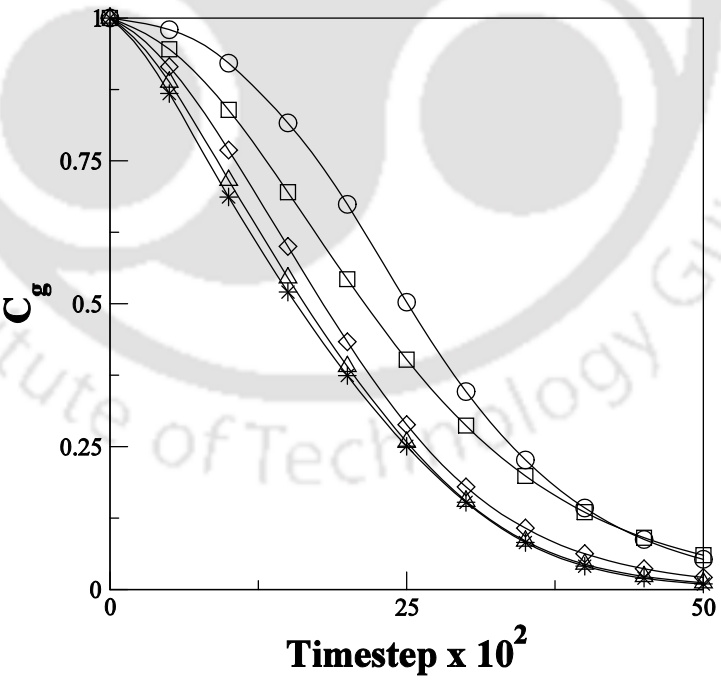

Figure 8: Plot of instantaneous glucose concentration fraction C_g = {C_g/Cg(I)} in the medium against MC time steps for different initial glucose [10 (○), 20 (□), 30 (◊), 40 (Δ), 50 (✳) %] concentrations.

It shows three different regimes in the consumption of glucose, slow, rapid and saturation. Initially, glucose consumption is found slow followed by a rapid consumption.As time passes, more and more cells are produced and consumption is increased. Finally, glucose concentration goes to a very low value. Glucose consumption rate is also found higher[Fig 8] for the higher initial glucose concentration in the medium as in the experiment. The growth of the cells at any time t is monitored by counting the number of cells per single initial spore G_N. It is plotted in Fig. 9 against the MC time steps.

The growth of the cells is also found similar to that of the experimental observations. However, it may be noticed that the growth form observed in the model has a better agreement to the experimental results at lower initial glucose concentration than that at higher initial glucose concentration.

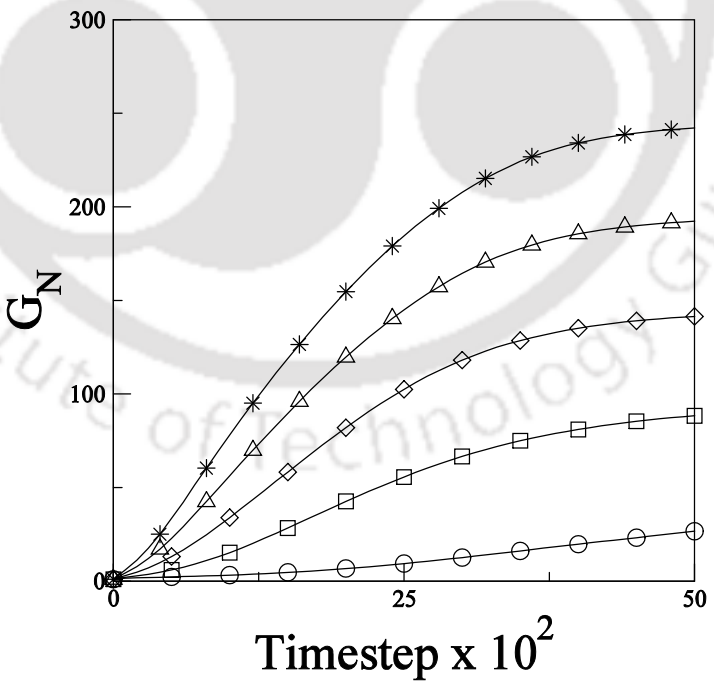

Figure 9: Plot of number of cells per single initial spore (G_N) against MC time steps for different initial glucose concentrations as [10 (○), 20 (□), 30 (◊), 40 (Δ), 50 (✳) %].

The growth process has an initial lag phase followed by the rapid growth and finally it saturates. Initial slow growth is due to less number of spores and saturation in growth is due to non-availability of glucose in the medium. The saturation values are also found directly proportional to the initial glucose concentration in the medium as in the experiment [Fig 10]. The results then confirm that the sigmoidal growth adopted here in a self-stabilized glucose medium is a suitable growth model for this fungus. One may notice that the saturation growth regime is achieved by nearly 5000 MC time steps for the given parameter values whereas such a regime is achieved in the experiment by about 96 hours. One MC step is then approximately 1 minute of experimental time.

In the experimental situation, enzyme activity is measured by performing enzyme assay.

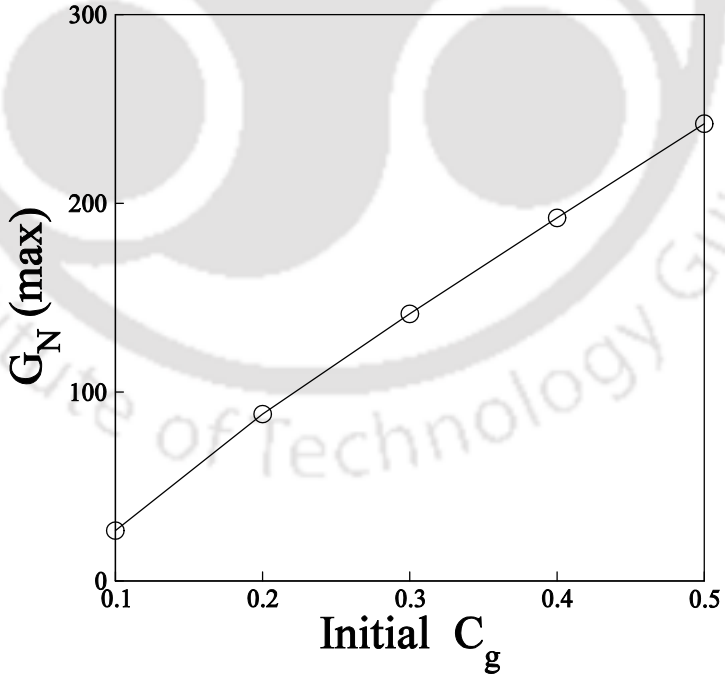

Figure 10: Saturation values for growth $\{G_N(max)\}$ against MC time steps for different initial glucose concentrations .

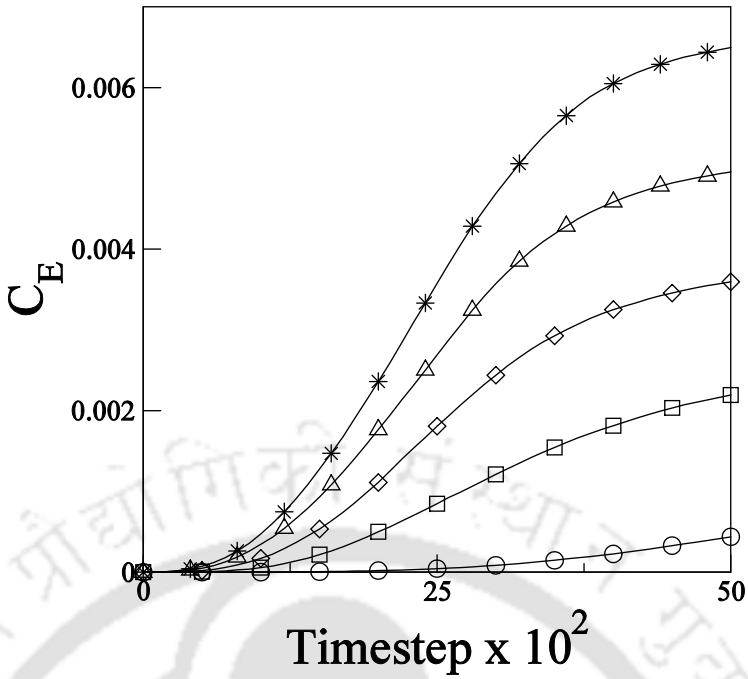

Figure 11: Enzyme concentration (C_E) obtained from the simulation is plotted against MC time step for different initial glucose concentrations as [10 (○), 20 (□), 30 (◊), 40 (Δ), 50 (✳) %].

However, enzyme activity here can be measured directly from the enzyme concentration of the medium applying the rate equation (3.7). The enzyme concentration C_E in the medium is plotted in Fig. 11 against the MC time steps. Since at room temperature, the product conversion rate per unit volume is directly proportional to the enzyme concentration C_E for a given substrate concentration C_S, Fig. 8 can represent the rate versus time plot if multiplied by an appropriate pre-factor. If the enzyme activity of 150 UL^{-1} obtained in the experiment correspond to enzyme concentration 0.4 per unit volume in the simulation, the pre-factor in the rate equation will be 10^{-4}. It may be noticed that enzyme concentration represents a similar behavior to that obtained in the experiment with three different phases of activity. Since the enzyme activity is qualitatively reproduced in this simulation, it may be concluded that the enzyme secretion is appropriately modeled for this fungus.

3.5. Discussion

The experimental results suggest that both biomass growth and LiP activity are dependent on the initial glucose concentration in the medium. However, higher concentrations (> 20 gL^{-1}) of glucose showed different effects. This can be attributed to the change in the cellular membrane capabilities of the cell [Gold and Alic, 1993]. The maximum biomass growth varies almost linearly with the initial glucose concentration of the medium, [Fig5]. LiP activity varies similarly with the initial glucose concentration, [Fig6]. By the model developed one can observe qualitatively similar behaviour as that observed in the experiments for varying initial glucose concentration. The constants should be adjusted according to the experimental condition to obtain quantitative agreement with the experimental data. Though the model produces results that qualitatively agree with the experiments, the model should be improved incorporating other details such as cell death, loss of enzyme, etc. The model can also be used for studying the behaviour of similar kind of fungal system by readjusting different parameters of the model.

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Chapter-4

Biomass growth and enzyme secretion by *P. chrysosporium* in presence of DR-80

Industrial wastes, such as textile dyeing effluent, are the main source of toxic pollutants in the environment mainly due to lack of proper treatment and discharge of such wastes. Biological treatment processes are frequently preferred to treat such effluents in comparison to other remediation methods [Korbahti andRauf, 2008, Aleboyeh et al, 2008, Hassan and Hawkyard, 2002, Linand Chen, 1997] as they seem more effective, economical and environment friendly. The white-rot fungus *Phanerochaete chrysosporium*, which belongs to a group of lignin-degrading basidiomycetes, has received considerable attention in the past for their bioremediation potential [Bumpus and Brock, 1988,Cripps et al., 1990, Bakshi et al.,1999] owing to its natural capability to degrade complex lignin using extracellular non-specific and non-stereo selective enzyme system composed of lignin peroxidases (LiP, EC 1.11.1.14), laccases (EC1.10.3.2), and manganese peroxidases (MnP, EC 1.11.1.13). The same unique non-specific mechanisms that give these fungi the ability to degrade lignin also allow them to degrade the toxic dyes present in textile dyeing effluents. In the previous chapter, the biomass growth and LiP secretion by the fungus was studied by varying the initial glucose concentration. The experimental results were also simulated by using a stochasticmodel for explaining this behaviour of fungus. However, in order to further establish the potential of the organism for textile dyeing effluent treatment purpose it is necessary to understand and model the biomass growth and enzyme secretion by the fungus in presence of textile dyes commonly found in such wastewaters. Therefore, the present study was aimed at investigating the effects of DR-80, a toxic azo dye widely used in modern textile and dyeing industries, on these two aspects of the fungus. Since media is also known to play a vital role on growth of the fungus and biodegradation of the dye [Pakshirajan et al.,

2009], effects of media constituents, mainly glucose concentration, on dye degradation were observed and modelled in the study.

Several models for the fungal growth have been observed in literature; however, kinetic models were preferably applied only to model dye decolourization for the procedures other than bioremediation [El-Dein et al., 2003]. Fluid dynamics modeling by numerical finite volume scheme has also been applied to study dye decolourization using titanium nano-photocatalysis [Mahmoodi et al.,2009]. Current study focuses on Monte Carlo (MC) based model for simulating biomass growth and enzyme secretion as well as DR-80 dye decolourization by *P. chrysosporium*.

4.1. Materials and Methods

4.1.1. Chemicals

The azo dye Direct Red - 80 (Figure 1, page #6) and veratryl alcohol were purchased from Sigma (St. Louis, Mo, USA); all other chemicals and solvents were purchased from High Media, Mumbai (India), SRL (India) or Merck (India), which were all of GR grade.

4.1.2. Microorganism and culture conditions

The fungus *P. chrysosporium* MTCC 787, used in this study was procured from IMTECH, Chandigarh, India, and was maintained at 25 ºC on potato dextrose agar (PDA) slants. For spore production, the slants were incubated at 39 ºC for 2 to 5 days in media containing glucose: 10 gL^{-1}, malt extract: 10 gL^{-1}, peptone: 2 gL^{-1}, yeast extract: 2 gL^{-1}, asparagine: 1 gL^{-1}, KH_2PO_4: 2 gL^{-1}, $MgSO_4 \cdot 7H_2O$: 1 gL^{-1}, thiamin-HCl: 0.001 gL^{-1} and agar: 20 gL^{-1} [Kirk and Tien, 1988]. The composition of media

used for studying DR–80 decolourization is mentioned in Table 1. All dye decolourization experiments in the study were performed using 250 ml flasks containing 100 ml media with initial pH 4.5; following inoculation, the flasks were incubated in an orbital shaker set at 30 ^{o}C and 150 rpm. Effect of initial concentration of glucose was studied in the range of 4-16 gL^{-1} by fixing the initial dye concentration at 0.02 gL^{-1}. To study the effect of initial dye concentration it was varied in the range 0.01-0.05 gL^{-1} keeping initial glucose concentration at 10 gL^{-1}. Fungal growth in the experiments was measured by counting the spores using a haemocytometer [Morrisand Nicholls, 1978].

Table 1: Composition of the media used for DR-80 decolourization by *P. chrysosporium*

Media Constituents	**Quantity (gL^{-1})**
Basal III medium	
KH_2PO_4	20
$MgSO_4$	5
$CaCl_2$	1
Trace elements solution	
$MgSO_4$	3
$MnSO_4$	0.5
NaCl	1.0
$FeSO_4.7H_2O$	0.1
$CoCl_2$	0.1
$ZnSO_4.7H_2O$	0.1
$CuSO_4$	0.1
$AlK(SO_4)_2.12H_2O$	0.01
H_3BO_3	0.01
$Na_2MoO_4.2H_2O$	0.01
Nitrilotriacetate	1.5
Ingredient	
Glucose	10
2,2-dimethyl succinate	0.1 *M*, pH 4.2
Thiamine	0.1, filter sterilized
Veratryl Alcohol	4 m*M* stock, filter sterilized
Ammonium chloride	4.68

4.1.3. Assays for LiP and DR-80

Samples collected at regular intervals were centrifuged at 10,000 ×*g* for 10 min at 4°C to remove the fungal biomass. After separation of the cells, one part of the supernatant containing lignin peroxidase enzyme was assayed by spectrophotometric method [Kirk et al., 1990], which was based on the oxidation of veratryl alcohol to veratraldehyde. For the enzyme assay, standard reaction mixture consisted of 1 ml of 125 mM sodium tartrate buffer (pH 3), 500 µl of 10 mM veratryl alcohol, 500 µl of 2 mM H_2O_2 solution and 500 µl of biomass free supernatant obtained as mentioned before. The reaction was initiated by adding H_2O_2 and the change in absorbance was monitored at 310 nm. Whilst the alcohol exhibits no absorbance at this wavelength, the aldehyde absorbs much strongly. One unit (U) of lignin peroxidase enzyme activity was defined as the amount that converts 1 mol of veratryl alcohol to veratraldehyde per minute per ml of the supernatant; the enzyme activities were expressed as UL^{-1} [Linko and Haapala, 1993]. The other part of the supernatant was used for determining the residual dye concentration by measuring its absorbance (λ_{max}) at 523 nm using a UV–visible spectrophotometer (Carry 100, Varian, USA). Percent (%) dye decolourization is calculated as follows

$$\%\ \text{dye decolourisation} = \left(1 - \frac{D_t}{D_0}\right) \times 100 \qquad (4.1)$$

where D_0 is the initial dye concentration and D_t is the dye concentration at any time t in the medium.

All experiments were performed in replicates to control the experimental errors. Samples for analysis were collected in triplicates and averages were reported. At the

95% level of significance, 2σ control charts were maintained to ascertain the output data. Experiments falling out of chart were rejected ($P<0.05$).

4.2. Results

Variation of initial concentration of glucose in the medium shows prominent effect on the growth of the fungus. Presence of the DR-80 (0.02 gL^{-1}), reduces the spore count initially due to its toxic nature [Fig 20]. The maximum biomass growth of fungus increases with the increase of initial concentration of glucose, whereas the growth rate remains same. The % decolourization of the dye increases [Fig 21] with increasing glucose as the LiP activity was also found to increase [Fig 22]. Changing the initial dye concentration at constant glucose (10 gL^{-1}), the fungus displays opposite behaviour with highest biomass growth in lowest concentrations of dye [Fig 23]. The reduction in initial inoculum is also observed here. Similarly the % dye decolourization as well as LiP activity is also reversed. It is found that the increment of the initial dye concentration decreases the % dye decolourization in the medium [Fig 24] and also the LiP activity at constant glucose [Fig 25].

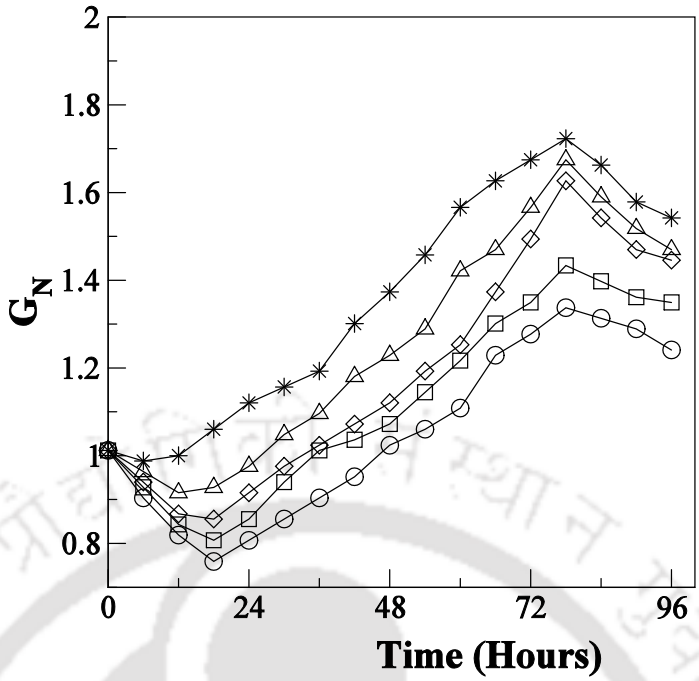

Figure 20: Growth of the fungus in terms of generations (G_N) with increasing initial glucose concentration [4 (○), 8 (□), 10 (◊), 12 (Δ), 16 (✳) gL^{-1}] at constant dye concentration [0.02 gL^{-1}]

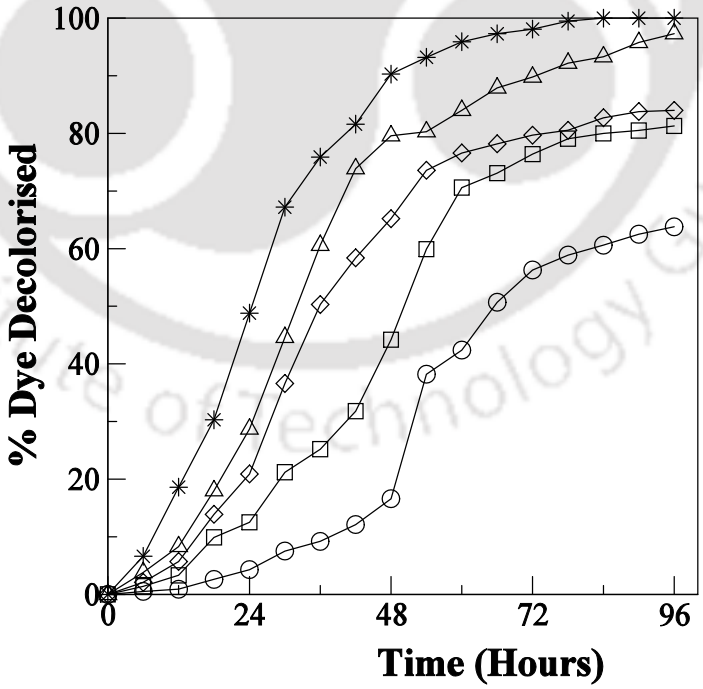

Figure 21: Percent dye decolourization in the medium at different initial glucose concentration [4 (○), 8 (□), 10 (◊), 12 (Δ), 16 (✳) gL^{-1}] at constant dye concentration [0.02 gL^{-1}]

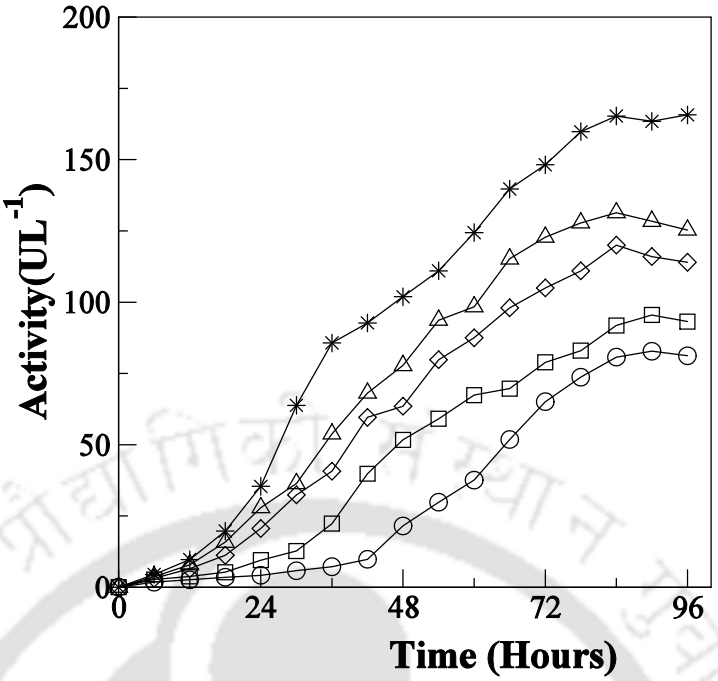

Figure 22: LiP activity with increasing initial glucose concentration [4 (○), 8 (□), 10 (◊), 12 (Δ), 16 (✳) gL^{-1}] at constant dye concentration [0.02 gL^{-1}]

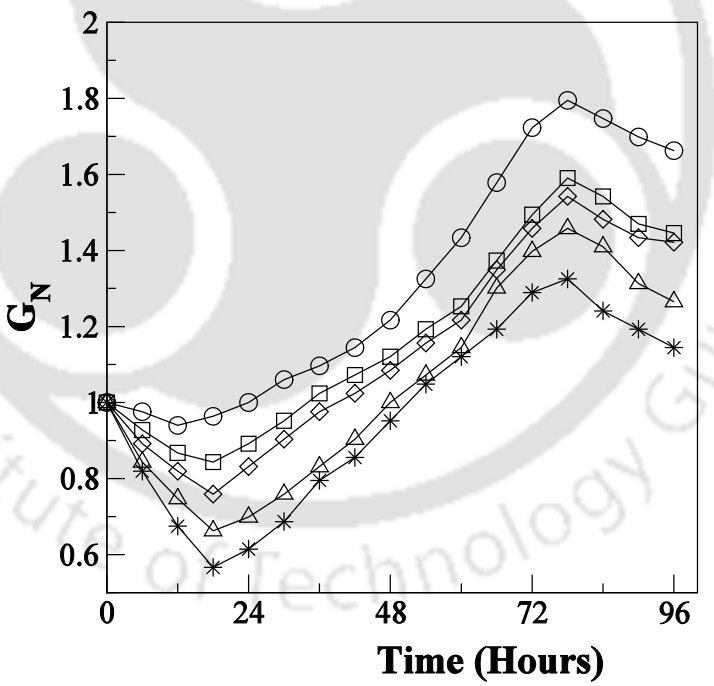

Figure 23: Growth of the fungus in terms of generations (G_N) with increasing initial dye concentration [0.01 (○), 0.02 (□), 0.03 (◊), 0.04 (Δ), 0.05 (✳) gL^{-1}] at constant initial glucose concentration (10 gL^{-1})

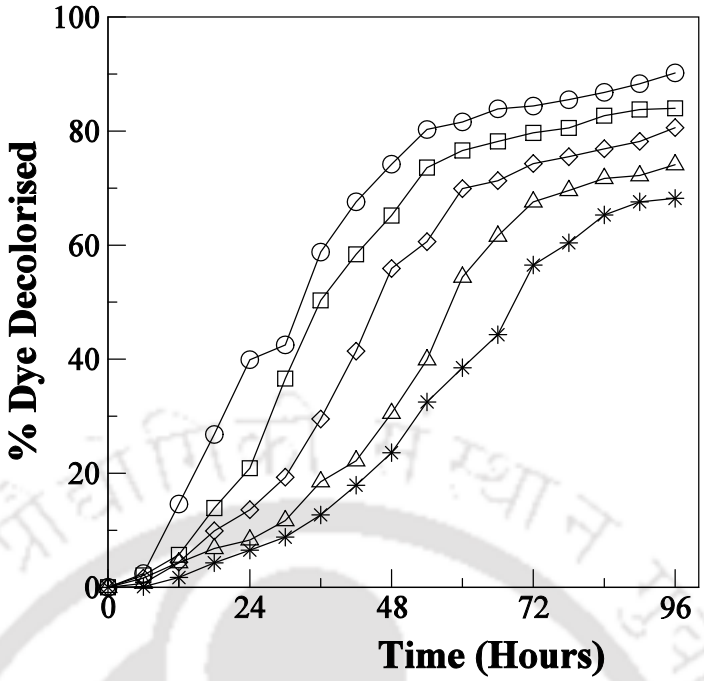

Figure 24: Percent dye decolourized in the medium at different initial dye concentration [0.01 (○), 0.02 (□), 0.03 (◊), 0.04 (Δ), 0.05 (✳) gL^{-1}] at constant initial glucose concentration (10 gL^{-1})

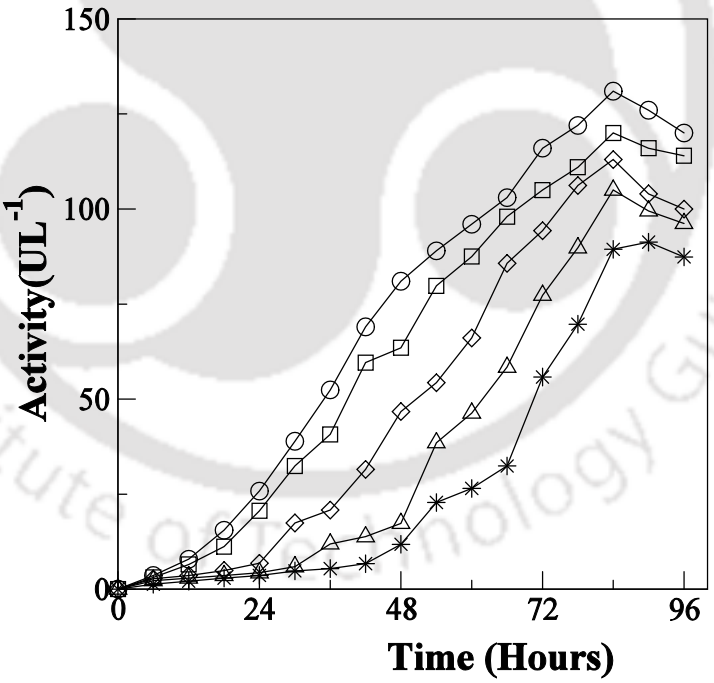

Figure 25: LiP activity with increasing initial dye concentration [0.01 (○), 0.02 (□), 0.03 (◊), 0.04 (Δ), 0.05 (✳) gL^{-1}]at constant initial glucose concentration (10 gL^{-1})

4.3. The Model

In the previous chapter, a stochastic model of fungal growth and enzyme secretion by the fungus was developed in order to understand the biomass growth and enzyme activity of *P. chrysosporium*based only on glucose consumption. The model successfully explains the experimental results obtained in a toxic pollutant free medium. In this study, the same model has been extended for studying the fungal growth and activity of the enzyme secreted by the fungus in presence of DR-80 as a toxic dye. The stochastic model developed, taking glucose as the sole source of carbon, contains three major parts: (a) consumption of glucose, (b) cell division and (c) secretion of enzyme. It was found in the experimental study performed here that the decolourization and biomass growth both increase with the increasing glucose concentration for a given dye concentration whereas they decrease with the increasing dye concentration for a given glucose concentration. In the present model, attempt has been made to reproduce the experimental results by controlling the interaction of the fungus with medium and the interaction of enzyme secreted by the fungus with the toxic dye. The immediate effect of addition of dye to the medium is the unavailability of certain amount of glucose to the fungus which is proportional to the dye concentration. The probability of glucose consumption is proportional to the instantaneous glucose concentration of the medium G_t and glucose intake probability of a cell p_i. G_t is given by

$$G_t = G_0\left(1-\frac{n_G(t)}{N_G(0)}\right) \tag{2}$$

where $G_0 = N_G(0)/V$ is the initially available glucose concentration and $n_G(t) = \sum_{i=1}^{t} n_G(i)$ is the number of glucose molecules consumed upto time t. The intake probability of a cell p_i is defined in terms of the cell size g and is given by

$$p_i \propto g_r e^{-g_r} \tag{4.3}$$

where $g_r = g/g_C$ is the relative size of the cell with respect to the mature cell size g_C. The glucose consumption probability is then given by

$$p_{GC} = \Gamma G_t p_i \tag{4.4}$$

where Γ is the molar ability of attachment of a glucose molecule to a cell. The probability of cell division is modeled by a sigmoidal growth probability distribution and it is given by,

$$p_{div} = \frac{1}{1+e^{-(g-g_C)/\pm_d}} \tag{4.5}$$

where $\pm_d$ is the capability of cell division of a matured cell.*P. chrysosporium* is known to produce LiP as secondary metabolite when glucose concentration in the medium is less. The probability of enzyme production by a cell is then directly proportional to the instantaneous glucose concentration of the medium. It is also a function of the cell size. The production of LiP by the fungus is modeled as

$$p_E = \frac{\sim \Delta G}{1+e^{-(g-g_C)/\pm_E}} \tag{4.6}$$

where $\Delta G = G_0 - G_t$, $\sim$ is the molar adaptability of the cell to the initial medium and $\pm_E$ is the enzyme production ability of a mature cell.

A Monte Carlo (MC) technique is adopted to implement different processes exactly with their respective probabilities [Mode, 2011]. For each process, a random number r, uniformly distributed between 0 and 1, is called corresponding to a cell. If $r \leq p$, $\left(p = p_{GC}, p_{div}, p_E\right)$ the probability of the corresponding process to occur, the cell is allowed to perform the respective process such as, consumption of glucose, cell division and enzyme production. The three sub-processes, glucose consumption, cell division and release of enzyme over all the cells constitute a single MC time step. To avoid accumulation of enzyme in the medium, lifetime [2] of enzyme is introduced. The number of enzyme decays exponentially as $e^{-\Delta t/2}$ where Δt is the time of presence of an enzyme in the medium.

In order to study the decolourization process, a simplified enzyme kinetic reaction between the enzyme and the dye is considered here as $E + D \rightarrow E + P$ [Agrawal et al., 2008], where E stands for enzyme, D stands for dye and P stands for dye degradation product. The rate of dye degradation can be estimated as

$$R = A\frac{N_E(t)N_D(t)}{\left(N_E(t) + N_D(t)\right)^2} \tag{4.7}$$

where A is a constant, $N_E(t)$ and $N_D(t)$ are instantaneous number of enzyme and dye molecules in the medium, respectively. Though A is independent of time, it is inversely proportional to the initial number of dye molecules per spore. In principle, A should be a function of temperature, reduced mass of two reacting molecules and the activation energy of the reaction. However, for a given reaction at room temperature, it is a constant and a prefactor is assigned accordingly.

4.4. Model Results

The model has been studied for two different initial conditions taking water volume equal to 10^7. First, for a given dye concentration (0.002), glucose concentration is varied from 0.08 to 0.12. It has been observed that the overall biomass growth is diminished due to the presence of dye whereas the growth is found to be enhanced with the increasing amount of glucose. The results plotted as generations, given by $G_N = \left(N_S(t)/N_S(0)\right)$, where $N_S(0)$ is the initial number of spores added to the medium and $N_S(t)$ is the number of spores present in the medium at any time t, with respect to the MC time step t in Fig. 26(a). The percent dye decolourization, calculated as $n_D = 100\left[N_D(0) - N_D(t)\right]/N_D(0)$, where $N_D(0)$ is the initial number of dye molecules and $N_D(t)$ is the number of dye molecules at any time t in the medium, with time is also estimated and the data are presented in Fig. 26(b). The maximum decolourization decreases with decreasing initial glucose concentration in the medium as it is observed in the experiment. The time represents the MC time steps only. It seems that 20,000 MC steps is equivalent to 96 hours of experiment since all the process considered here saturates by this time.

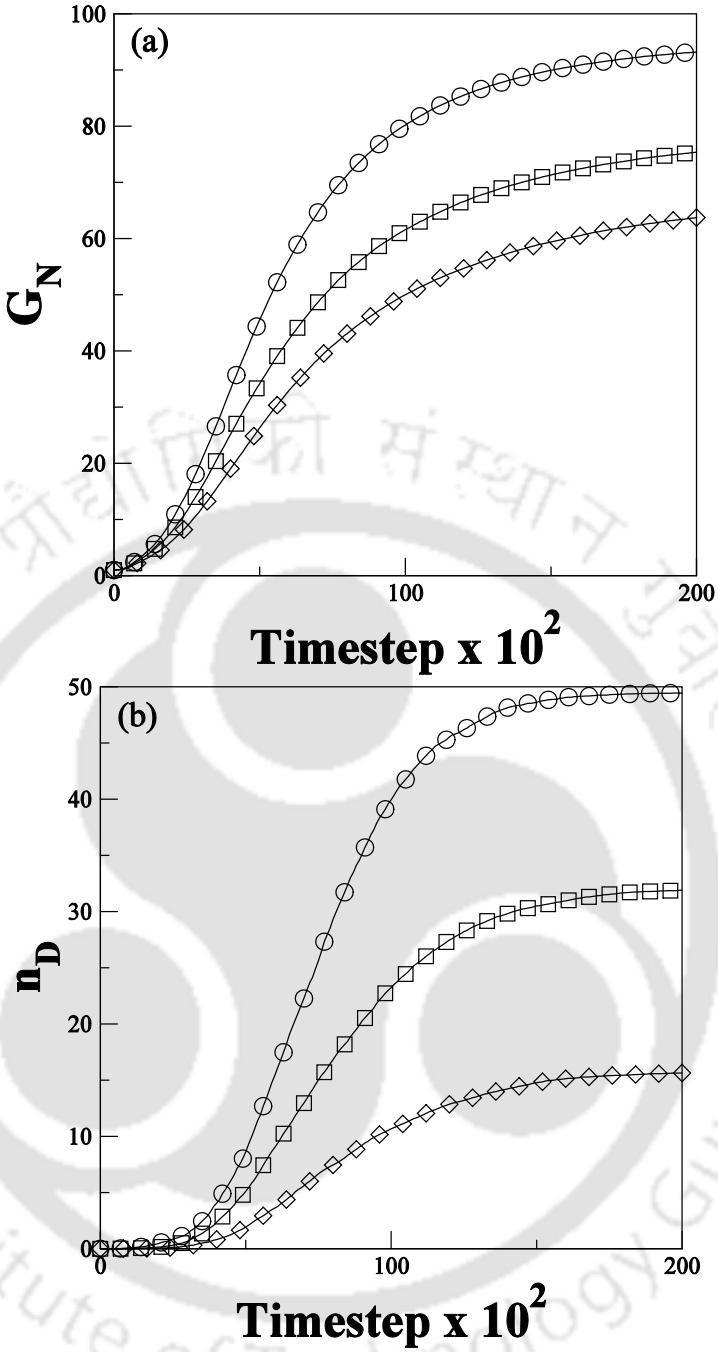

Figure 26: (a) Growth in terms of generations (G_N) of initial inoculums with respect to MC time steps in model with constant initial dye concentration [0.02] at different initial glucose concentration [0.10(○), 0.20(□), 0.30 (◊)] and (b) Percent dye decolourized (n_D) in the medium at different initial glucose concentration [0.10(○), 0.20(□), 0.30 (◊)] at constant dye concentration [0.02]

Secondly, the model is studied varying the dye concentration in medium for a given initial glucose concentration (0.1). Both biomass growth as generations (G_N) and

percent dye decolourization (n_D) are estimated and the data are plotted in Fig. 27(a) and (b) respectively with respect to time t. For a given glucose concentration, biomass growth decreases as the dye concentration in the medium increases, similar to the results found from experiments. The percent dye decolourization (n_D) is also found decreasing with increasing dye concentration. The latter is due to the toxic effect of dye on the fungus. Usually, the cellular processes are affected due to the presence of toxic pollutant in the medium. However, in the model only interaction of the cells with the external medium is considered. The effective interaction with the medium by the cell can be considered as an overall effect of all the modified cellular processes.

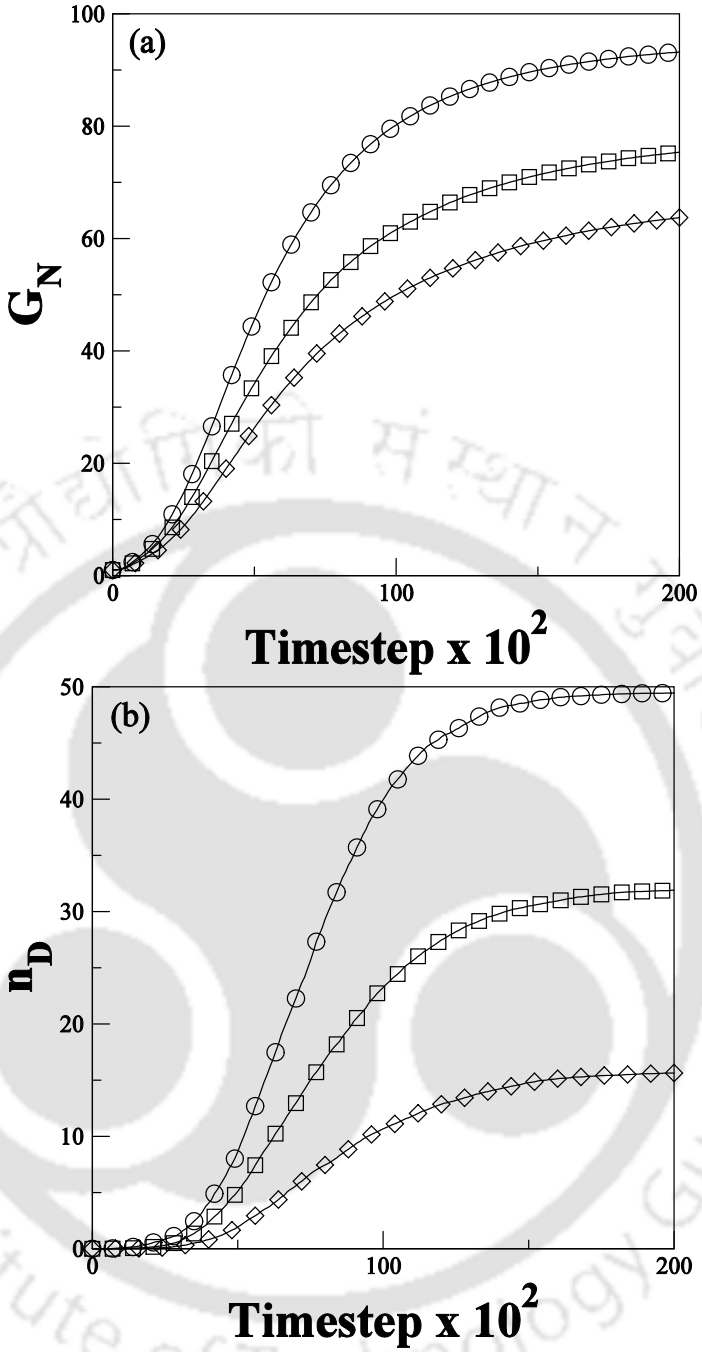

Figure 27: (a) Growth in terms of generations (G_N) of initial inoculums with respect to MC time steps in model with constant initial glucose concentration [0.1] at different initial dye concentrations [0.001(○), 0.002(□), 0.003(◊)] and (b) percent dye decolourized (n_D) in the medium at different initial dye concentration [0.001(○), 0.002(□), 0.003(◊)] at constant initial glucose concentration [0.1]

4.5. Discussion

Toxic effects of the dye result in reduction in biomass growth of the fungus. Increment of glucose concentration in the medium at constant dye concentration, however, increases the biomass growth. Whereas, increasing the dye concentration reduces it. Presence of dye in the medium reduces the initial glucose availability to the fungus. Incorporation of this effect in the model, predicted the change in biomass growth similarly. The dye decolourization in the medium was increased due to the increase in glucose concentration at constant dye but decreases when dye concentration is increased at constant glucose concentration. Similar effects are predicted by the model developed. Thus, careful quantization of the parameters of the model can predict the behaviour of the fungus for other types of dyes as well as other fungal systems with similar function.

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Chapter-5

Effect of Physico-Chemical Parameters on Biomass growth and Enzyme secretion by *P. chrysosporium*

Textile dyeing industries use different synthetic dyes and discharge several tons of dye based wastewaters worldwide every year into the environment[Kumar et al., 2009]. Discharge of such coloured effluents into rivers, lakes and other aquatic environment reduces the availability of oxygen and absorbs sunlight, thus threatening the life of photosynthetic plants and aquatic biota [Banat et al., 1996]. Textile dyes and effluents have toxic effect on germination rates and biomass concentration of several plant species[Wang, 1991,Kapustka and Reporter, 1993]. Many dyes are reported to be carcinogenic [Kalyuzhnyi and Sklyar, 2000]. Dyes with azo-based chromophores are the largest group of synthetic dyes known and the most common group found in wastewaters [Ertugrul et al., 2008]. Azo dyes that remain unutilized (10-15%) in the process are finally discharged in the effluent [Selvam et al., 2003, Wesenberg et al., 2003]. Dye containing wastewaters can be treated by physical and/or chemical treatment processes [Lin and Peng, 1994, Lin and Liu, 1994, Lin and Peng, 1996] (adsorption, coagulation-flocculation, oxidation and electrochemical methods). Although quite efficient in removing colour, these methods suffer from secondary sludge generation problems, high cost etc [Mishra and Tripathy, 1993, Yin and Dan-Li, 2004]. Biological methods are considered more sustainable and eco-friendly [Hong et al., 2000]. The white-rot fungus, *Phanerochaete chrysosporium*, is known for its ability to degrade xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs), dyes etc [Glenn and Gold, 1983, Cripps et al., 1990, Moreira et al., 2000, Tekere et al., 2001]. This fungus secretes nonspecific oxidoreductases during secondary metabolism in response to nutrient limitation, and the enzymes lignin peroxidase (LiP, EC 1.11.1.14) [Tekere et al., 2001, Olfat et al.,2000,Sue et al.,2000,Hatakka, 2001,Tien and Kirk,1983,Glenn and Gold,1983] plays a crucial role

in degradation of these compounds. In the earlier Chapters (3 and 4) the effect of change in initial carbon source concentration on biomass growth and LiP secretion by the fungus was shown in absence and in presence of DR-80. Besides the carbon source glucose, other physico-chemical parameters, particularly temperature, agitation speed, initial pH of the medium, may play significant role on the behaviour of *P. chrysosporium* in degrading such toxic dyes. Therefore, this chapter presents investigation on the effect of these parameters on biomass growth and LiP secretion by the fungus in absence and in presence of DR-80.

5.1. Materials and Methods

5.1.1. DR-80 and other Chemicals

The azo dye DR-80 and veratryl alcohol (3, 4-dimethoxybenzyl alcohol, 96% pure) were purchased from Sigma Chemicals (St Louis, MO, USA). All other chemicals and solvents were of reagent grade and purchased from Merck® India Ltd.

5.1.2. Microorganism and Culture Conditions

P. chrysosporium (MTCC 787) was obtained from IMTECH, Chandigarh, India. Stock culture of organism was grown on potato dextrose agar (PDA) at 25°C and maintained at 4°C, and refreshed in every 30-40 days. For spore production, slants were incubated at 39°C for 2-6 days in medium [Hatakka, 2001] (glucose, 10; malt extract, 10; peptone, 2; yeast extract, 2; asparagine, 1; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 1; thiamin-HCl, 1; and agar, 20 g/l). Media[28] used for the experiments composed of basal medium (KH_2PO_4, 20; $MgSO_4$, 5; and $CaCl_2$, 1 g/l), trace elements ($MgSO_4$, 3; $MnSO_4$, 0.5; NaCl, 1; $FeSO_4 \cdot 7H_2O$, 0.1; $CoCl_2$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; and $CuSO_4$, 0.1 g/l; $AlK(SO_4)_2 \cdot 12H_2O$, 10; H_3BO_3, 10; and $Na_2MoO_4 \cdot 2H_2O$, 10 mg/l; and

nitrilotriacetate, 1.5 g/l) and other ingredients [glucose, 100 g/l; 2,2-dimethylsuccinate, 0.1 M (pH 4.2); thiamine, 100 mg/l (filter sterilized); veratryl alcohol, 4 mM (filter sterilized); and NH_4Cl, 4.68 g/l].

5.2. Effect of various physico-chemical parameters

Effect of various physico-chemical parameters - temperature, agitation and initial pH of the medium- on the fungus growth and LiP secretion were studied by varying the levels of these variables one-at-a-time. All experiments were performed using Erlenmeyer flasks (250 ml) containing the afore-mentioned media and inoculated with 10% (v/v) fungal spores (optical density ~ 1.0). Effect of these variables was first studied in absence of DR-80 and later in its presence at 20 mg/L initial concentration. The levels of these parameters varied in this study were as follows: temperature: 25-60 ^{o}C; agitation speed: 0-200 rpm and initial medium pH: 3-7. Samples were taken during the experiments and analyzed for spore count using a haemocytometer.LiP activity and any residual dye concentration in the samples were determined as detailed further.

5.3. Analytical methods

For determining LiP activity, samples obtained were centrifuged at 10,000 × g for 10 min at 4°C to remove fungal biomass. The supernatant obtained was assayed for LiP activity at 310 nm using a UV-visible spectrophotometer (Carry 100, Varian, USA), which is based on the oxidation of veratryl alcohol to veratraldehyde [Singh and Pakshirajan, 2010]. One unit of the enzyme is defined as the amount that oxidizes l mM of substrate per min and its activity is reported in U/L. Residual concentration of

DR-80 in the media wasanalyzed using the supernatant obtained as above and by measuring its absorbance at 528 nm (λ_{max} of DR-80).

5.4. Results and Discussion

5.4.1. Influence of various parameters in the absence of DR-80

5.4.1.1. Effect of initial pH of the medium

Fig. 28 shows that the biomass growth decreased above pH 4, which can be attributed to altered cell membrane properties due to this change in the pH. Nutrient intake through the fungal cell membrane may also be altered due to change in the external pH, which therefore affects its cellular activity. In a manner similar to the biomass growth, the LiP activity was also found to decrease above pH 4 [Fig 29]. From the literature, it is known that the fungus shows maximum LiP activity at an optimum initial medium pH of 4. Besides LiP synthesis, transport of this enzyme via the membrane may also be altered by the pH change, which could have resulted in a reduced availability of the enzyme for the assay. Fig. 30 shows that the medium pH during the experiment was observed to fall upto 3 except in the case of pH 7 suggesting the formation of acidic products that may alter the cellular functions of the fungus, which,however, needs to be verified by performing experiments aimed at identifying these products.

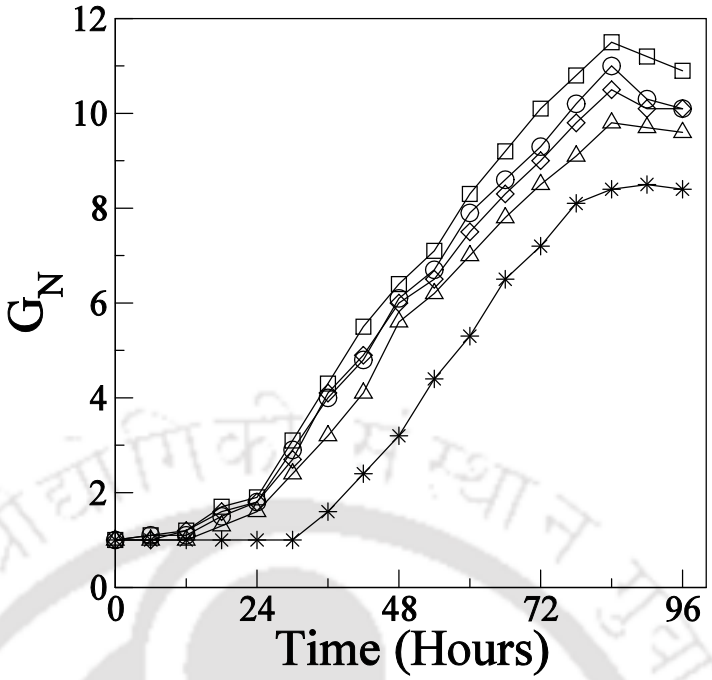

Figure 28. Growth in terms of generations (G_N) for different initial pH of the medium [3(o), 4(□), 5(◊), 6(Δ), 7(✳)]

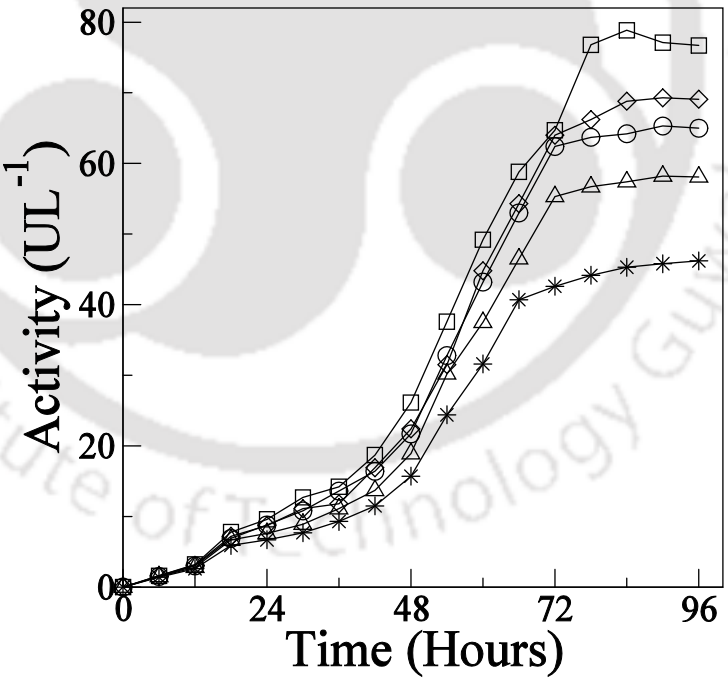

Figure 29. LiP activity for different initial initial pH of the medium the medium [3(o), 4(□), 5(◊), 6(Δ), 7(✳)]

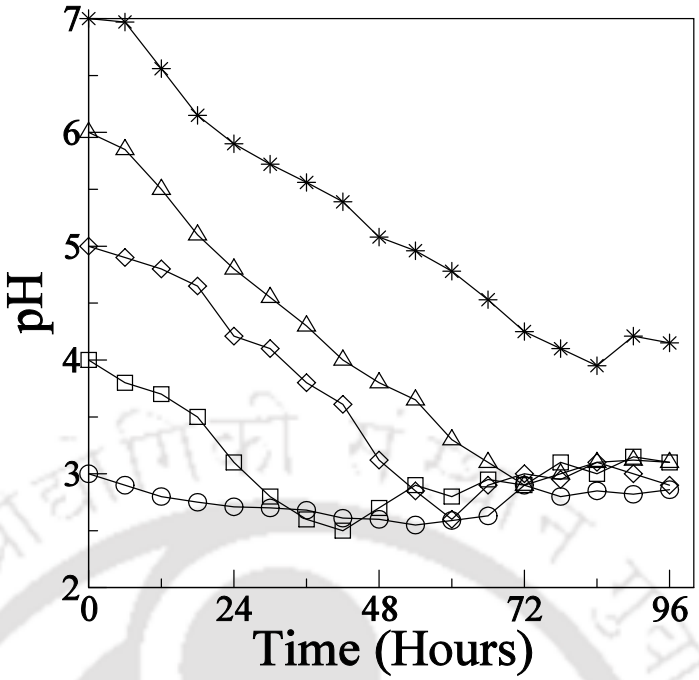

Figure 30.pH profile of the medium during the experiment for different initial pH of the medium [3(o), 4(□), 5(◊), 6(Δ), 7(✳)]

5.4.1.2. Effect of temperature

Fig. 31 shows the effect of different culture temperatureon spore count of the fungus, which clearly reveals that the biomass growth decreases rapidly above 30°C. This could be due to the destruction and alteration of cellular proteins at an elevated temperature [Sami and Radhouane, 1995].A high temperature during the culture can interfere with regular cellular processes leading to inhibition in the growth of the cells. The temperature effect on the LiP activity was also observed in a similar manner. Increase in medium temperature reduces the LiP activity rapidly [Fig 32] mainly due to the reduced biomass growth. Also, the produced enzyme can be denatured quickly due to a high medium temperature.

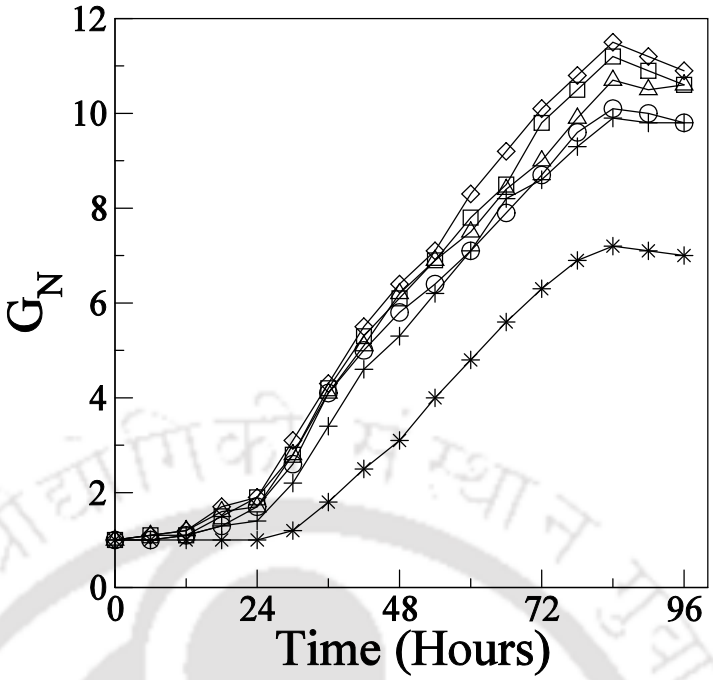

Figure 31. Growth in terms of generations (G_N) for different temperature [25(o), 30(□), 40(◊), 50(Δ), 60(✳)]

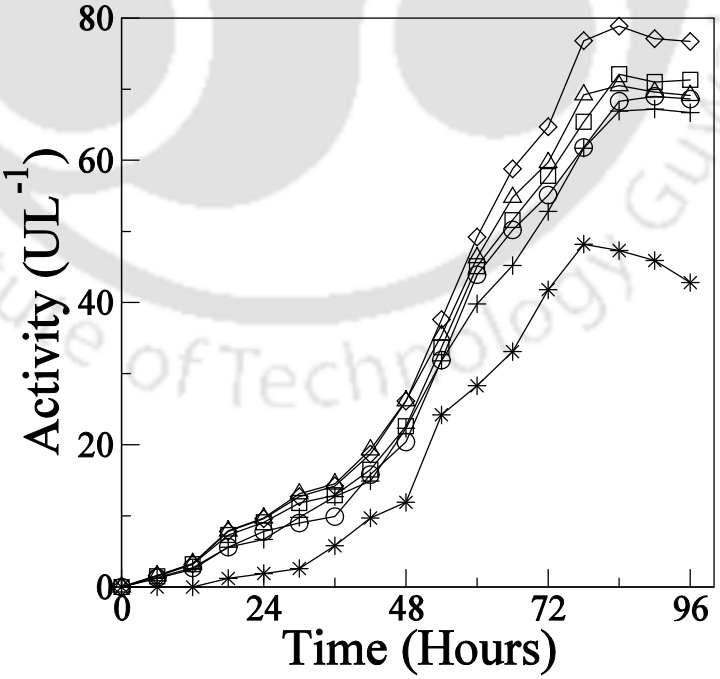

Figure 32. LiP activity for different temperature [25(o), 30(□), 40(◊), 50(Δ), 60(✳)]

5.4.1.3. Effect of agitation

Agitation of the culture medium ensures sufficient contact between the cells and the nutrients in the surrounding medium. Thus, an increase in the biomass growth of the fungus was apparent with increasing agitation from 0 to 200 rpm [Fig 33]. However, further increase in agitation above 200 rpm reduces the growth, which may be attributed to some form of biomass loss, for e.g. loss of cellular constituents, which,however, needs to be ascertained. LiP activity also increased with increasing agitation speed [Fig 34], which is mainly attributed to an increased biomass growth. Higher agitation similarly showedreduced activity of the enzyme.

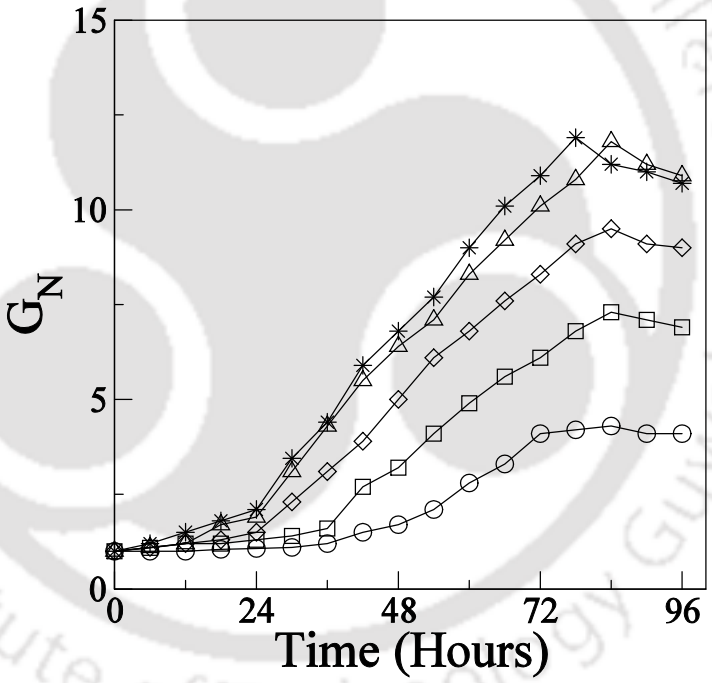

Figure 33. Growth in terms of generations (G_N) for different agitation speed [0(o), 50(□), 100(◊), 150(Δ), 200(✳)]

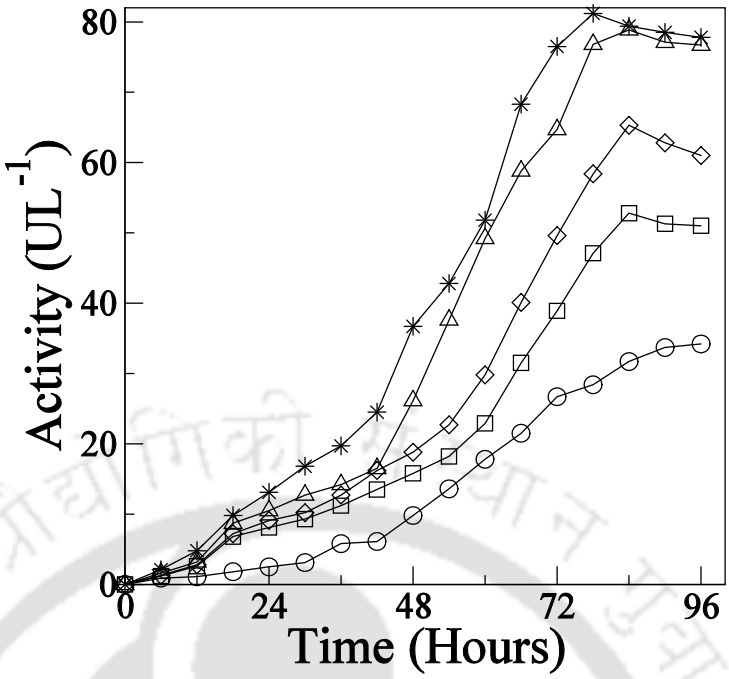

Figure 34. LiP activity for different agitation speed in rpm [0(o), 50(□), 100(◊), 150(Δ), 200(✳)]

5.4.2. Influence of various parameters in the presence of DR-80 in the medium

5.4.2.1. Effect of pH

Varying the initial pH of the medium in the range 3-7showedthat the biomass growth was more at a lower pH than at a high pH [Fig 35]as it affects the biochemical reactions in the cell.This may also be due to lower activity of cellular proteins on the both sides of their optimum pH. The same effect was observed for LiP activity [Fig 36] as well as for % DR-80 decolourization by the fungus [Fig 37]. pH is also another reason known to increase the cellular toxicity of the dyes, as it destabilizes the membrane of the fungal spores making the dye easier to penetrate and impart toxicity to the cells. This may also cause an increase in death of the initially added cells as the inoculum.

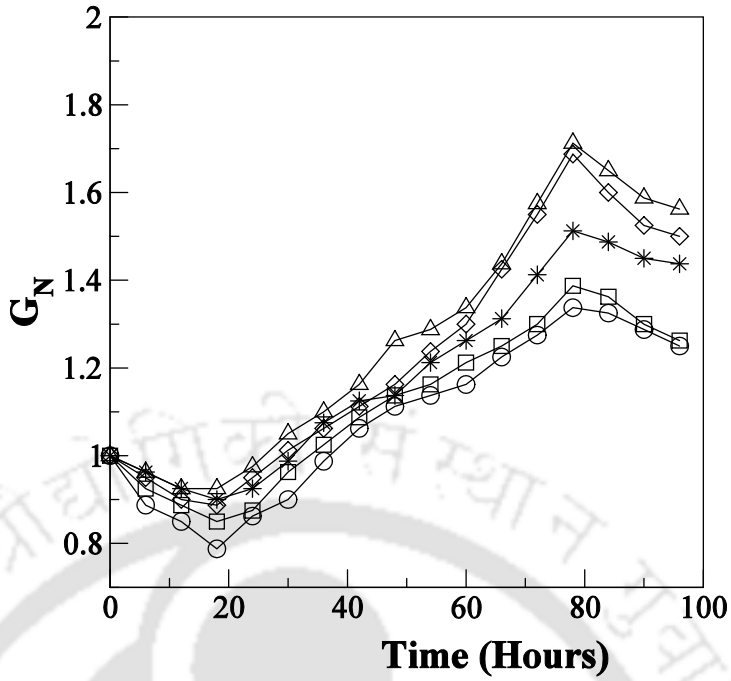

Figure 35: Growth in terms of generations (G_N) for different initial pH of the medium and in presence of DR-80 [3(o), 4(□), 5(◊), 6(Δ), 7(✳)]

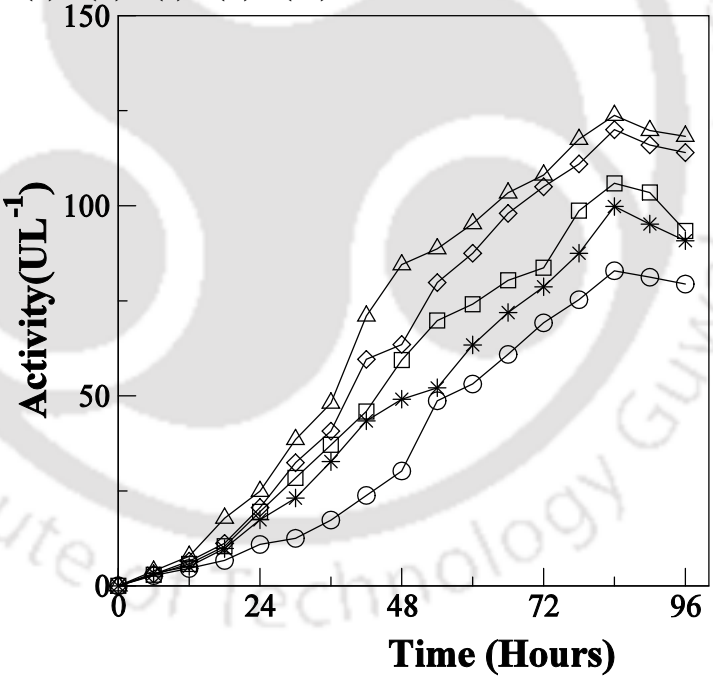

Figure 36. LiP activity for different initial pH of the medium and in presence of DR-80 [3(o), 4(□), 5(◊), 6(Δ), 7(✳)]

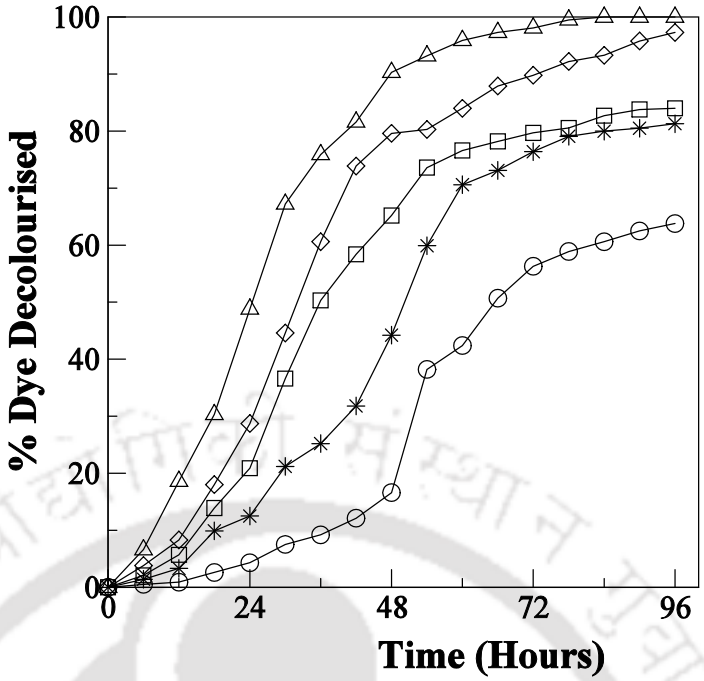

Figure 37.% DR-80 decolourization shown by the fungus for different initial pH of the medium [3(o), 4(□), 5(◊), 6(Δ), 7(✳)]

5.4.2.2. Effect of Temperature

Increasing the culture temperature showed similar effect like the pH. Increase of temperature reduced the biomass growth [Fig38] but only above a certain optimum value (30°C), which could be attributed to the alteration of protein and other cellular functions at higher temperature.As a consequence of reduced fungal biomass growth, LiP activity was also observed to be decreased at high temperature [Fig39].Due to the reduction in biomass growth as well as the LiP activity at higher temperature, % DR-80 decolourization was affected with a rise in the temperature above 30 ^{o}C(Fig. 40).

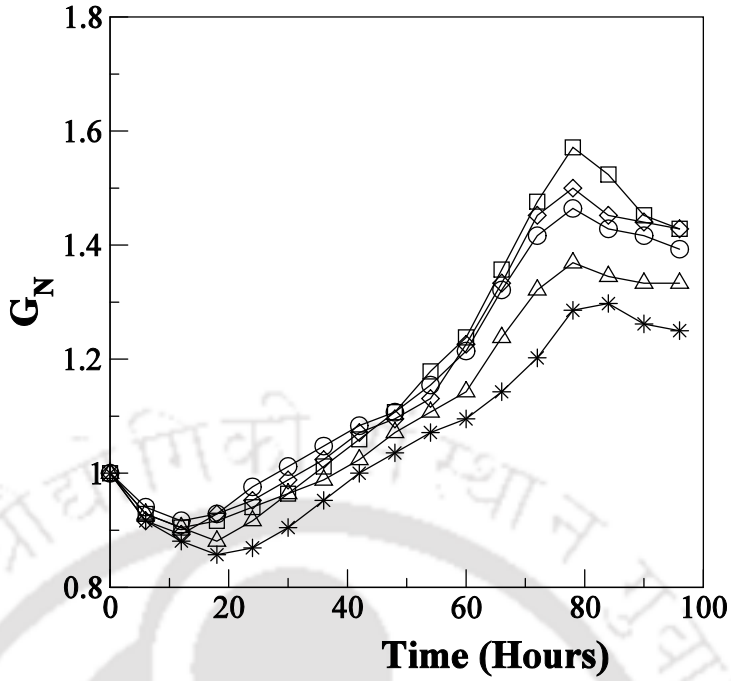

Figure 38. Growth in terms of generations (G_N) for different temperature and in presence of DR-80 [25(o), 30(□), 40(◊), 50(Δ), 60(✳)]

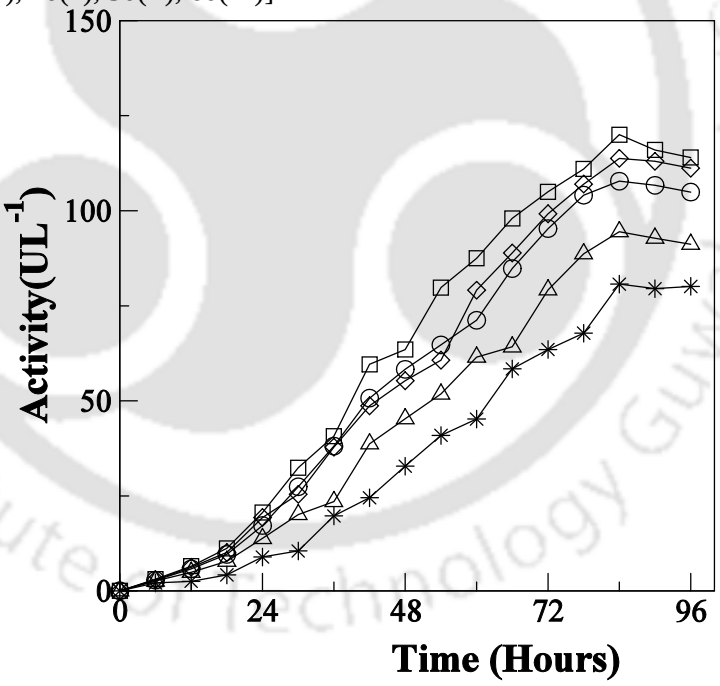

Figure 39. LiP activity for different temperature and in presence of DR-80 [25(o), 30(□), 40(◊), 50(Δ), 60(✳)]

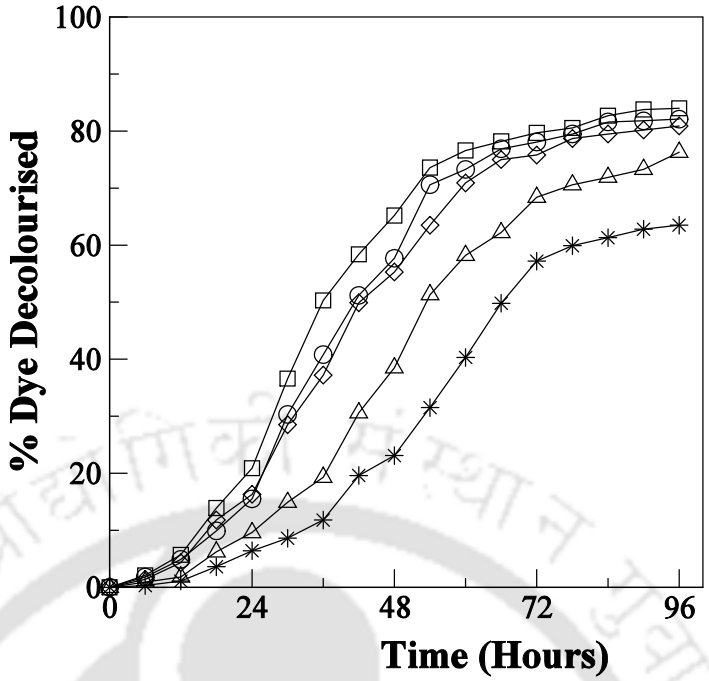

Figure 40. % DR-80 decolourization shown by the fungus for different temperature and in presence of DR-80 [25(o), 30(□), 40(◊), 50(Δ), 60(✳)]

5.4.2.3. Effect of agitation

The effect of agitation in presence of DR-80 was significant on the biomass growth [Fig 41]. Increase in agitation from 0 to 200 rpm yielded higher biomass growth. This can be attributed to the better contact between the spores and the available nutrient in the medium at higher agitation speeds, as observed in an earlier experiment. LiP activity was also found to be high at higher agitation speeds [Fig42], which may be due to enhanced biomass growth resulting in scarcity of nutrients particularly, glucose, necessary for enzyme secretion by the fungus. Due to this increase in LiP activity at high agitation speed, % DR-80 decolourization was also enhanced [Fig 43]. Increased bioavailability of the dye at higher agitation to the degrading enzymes can also be attributed towards this result.

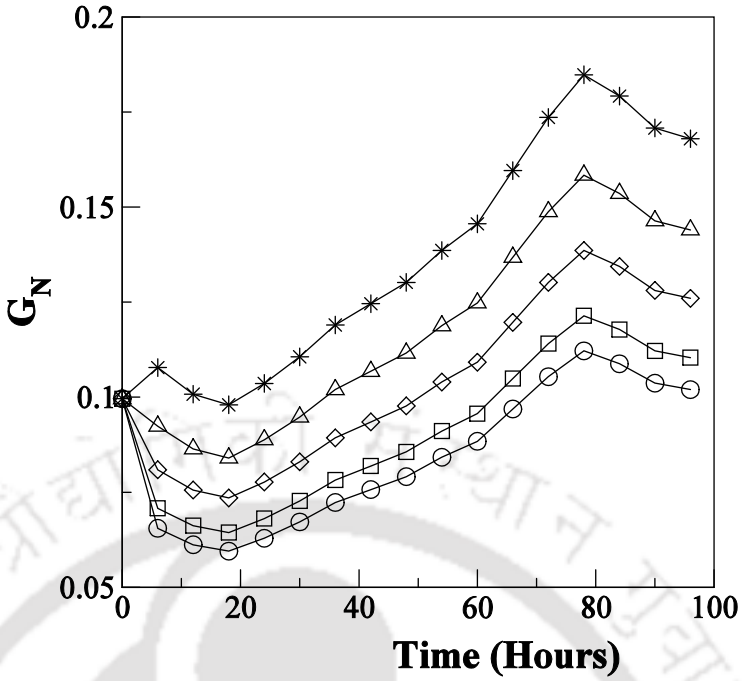

Figure 41. Growth in terms of generations (G_N) for different agitation speed and in presence of DR-80 [0(o), 50(□), 100(◊), 150(Δ), 200(✳)]

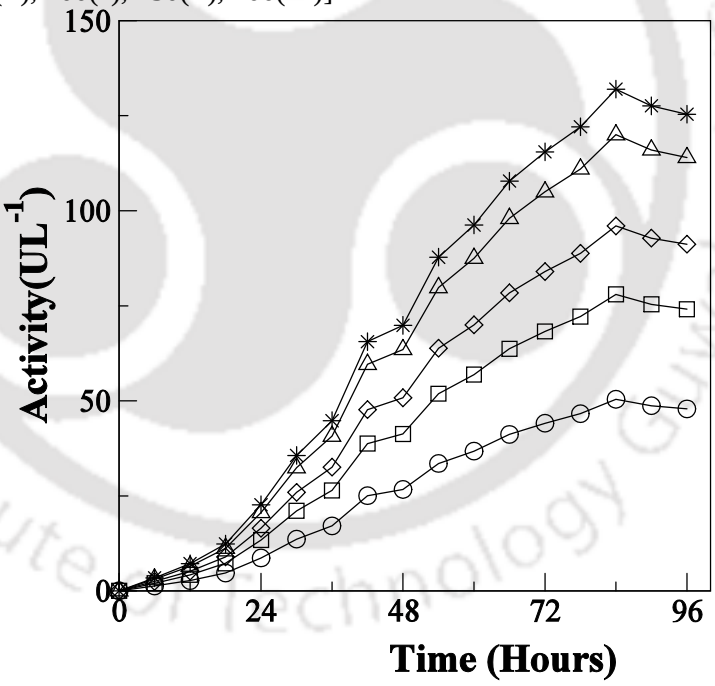

Figure 42. LiP activity for different agitation speed and in presence of DR-80 [0(o), 50(□), 100(◊), 150(Δ), 200(✳)]

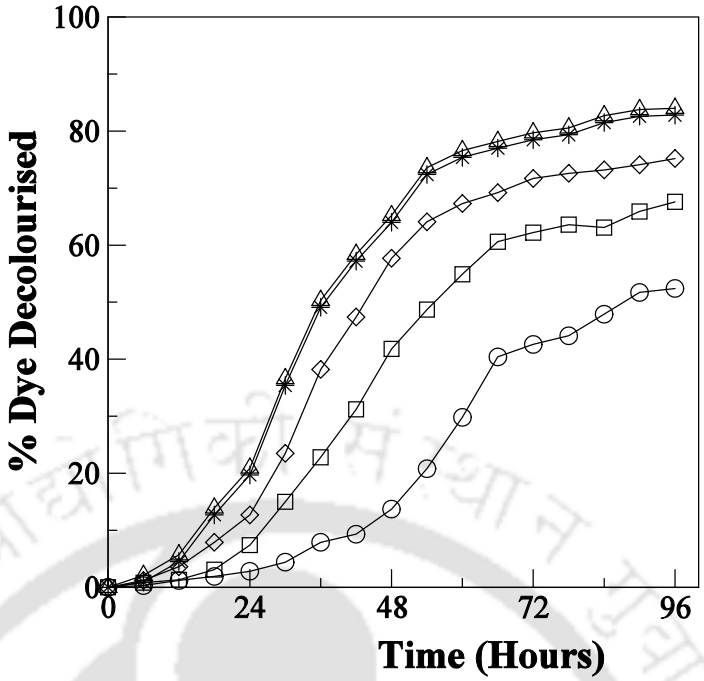

Figure 43. % DR-80 decolourization shown by the fungus for different agitation speed and in presence of DR-80 [0(o), 50(□), 100(◊), 150(Δ), 200(✳)]

Among the various physico-chemical parameters investigated, all the three factors, i.e initial pH of the medium, temperature and agitation, showed significant effect on biomass growth as well as LiP activity by *P. chrysosporium*. In presence of DR-80, the profiles of biomass growth and LiP activity by the fungus due to variation in these parameters were similar. Variation in these parameter values also highly influenced the dye decolourization ability of the fungus, which was directly associated with its biomass growth and LiP activity. Optimum values of these parameters for DR-80 decolourization by *P. chrysosporium*were found to be: temperature = 30 ^{o}C, initial pH of the medium = 4 and agitation speed = 200 rpm. This study proved very good potential of the ligninase producing fungus in decolourization of textile dyeing wastewaters.

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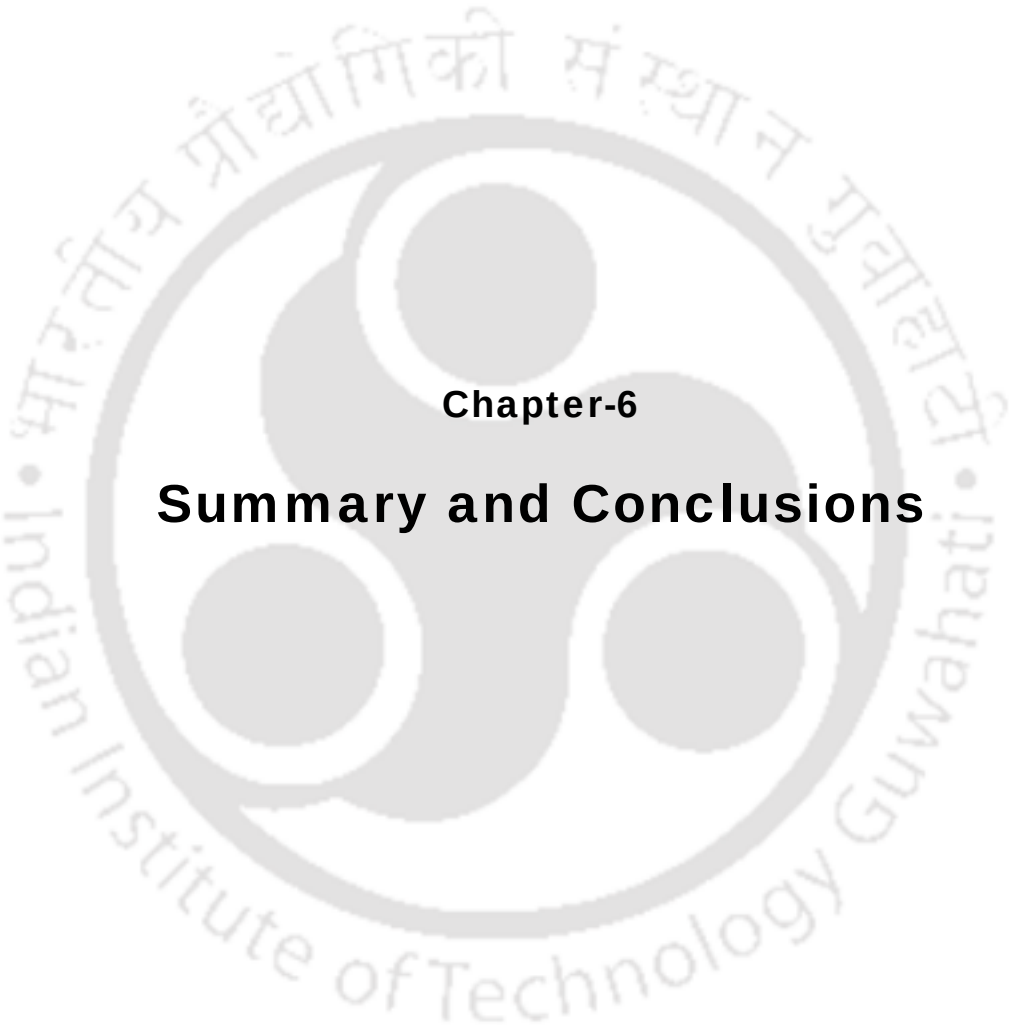


Chapter-6

Summary and Conclusions

Textile dyeing industries consume large volumes of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products. As a consequence of this, wastewater originating from textile dyeing industries is a complex mixture of potentially polluting substances which can impose serious threat to the receiving environment, if the wastewater is discharged without proper treatment. Wastewater treatment, particularly its decolourization can, however, be achieved by chemical and physical methods including adsorption, coagulation-flocculation, oxidation and electrochemical methods. Although these methods are quite efficient, high operational cost and other disadvantages like sludge generation severely limit the practical utility of these conventional methods. Further, while decolourization under anaerobic conditions can lead to toxic degradation products, exposure to oxygen may cause reverse colourization of the degradation products. On the other hand, microbial decolourization process offers to overcome all these drawbacks by reducing the complex colour components in the wastewater into simple compounds like carbon dioxide, ammonia and water in a cleaner and safer way compared to the conventional methods.

Among the various microorganisms, the white rot fungus *Phanerochaetechrysosporium* is known for its ability to degrade a wide variety of xenobiotics and lignin due to the non-specific ligninases (i.e., manganese peroxidases (MnP), E.C. 1.11.1.13; lignin peroxidases (LiP), E.C. 1.11.1.14 and laccases (Lac), E.C. 1.10.3.2) produced by the fungus. Among these ligninases, LiP has been identified to be an important enzyme for degradation of a large number of

environmental pollutants, including toxic azo dyes. However, being a secondary metabolite, it is essential for the fungus to grow in presence of such toxic dyes, and in such cases while immature fungal cells cannot survive toxic effects of the pollutants, mature cells have the capability to secrete LiP for degrading the xenobiotics. Hence, growth conditions of the fungus must be suitable to produce and secrete LiP to the medium for effective degradation of dyes in the wastewater. In addition to the presence or absence of toxic dyes, various physico-chemical factors may affect biomass growth and LiP secretion by the fungus. Thus, in the present thesis, all these aspects were addressed for its successful applicationpotential in textile dyeing effluent treatment. Further, for accurately describing biomass growth and enzyme secretion by this fungus, a stochastic based model was carefully applied.

Significant and Major findings from this research can be summarized as follows.

The effect of various physico-chemical parameters, viz. carbon source, nitrogen source, agitation, pH and temperature on biomass growth and enzyme secretion by the fungus was studied. Experimental results clearly revealed that glucose had a significant effect on biomass growth of the fungus. LiP activity of the fungus was found to be high for an increase in its biomass growth. On the other hand, nitrogen source showed very little or no effect on biomass growth, but scarcity of it resulted in an enhancement in LiP activity by the fungus. Agitation up to 150 rpm clearly showed a stimulatory effect on both biomass growth and the enzyme activity, but agitation rates higher than 150 rpm improved neither biomass growth nor LiP secretion. Similar observations were found for the parameters pH and temperature and optimum values of pH 5 and 40 °C were obtained in the study.

Selecting Direct Red-80 as a toxic pollutant of concern from textile dyeing industries, its effect on biomass growth and LiP secretion by the fungus was studied.In these experiments, initial spore concentration decreased from the beginning itself. Both efficiency and rate of dye degradation reduced with a raise in initial concentration of the dye in the media. LiP activity was also found to be less and affected due to the presence of DR-80 in the media. Similar patterns of biomass growth and LiP secretion were observed in presence of the dye by varying the parameters temperature, pH and agitation.

In order to gain understandingof the experimental results obtained in the study, a stochastic model of fungal growth and activity of the enzymes released by the fungi was developed. The model was initially formulated by considering glucose as the sole source of carbon. The entire process was also considered to consist of following three major steps: (a) consumption of glucose, (b) cell division and (c) release of enzyme. The enzyme activity of a cell is modeled by considering the glucose concentration in the medium and maturity of the cell. A self-stabilized Monte Carlo algorithm was thus developed to estimate the biomass growth and LiP secretion by the fungus.This model could able to satisfactorily explain the experimental results obtained in the study.

The effect of toxic pollutant on growth and enzyme activity was modeled by incorporating suitable inhibition effects as a function of dye concentration at all stages of growth, i.e glucose intake and cell division.The probability of enzyme production was also inhibited exponentially by dye initial concentration in the media and its degradation rate depended of the number of enzyme molecules per molecule of the

toxic dye. The model also qualitatively reproduced the effect of inhibition on growth and enzyme activity on the dye degradation.

From the model developed in the study, the inhibitory effect of dye on biomass growth and enzyme activities by the fungus were also explained in terms of delayed consumption of glucose due to an increase in the dye initial concentration. However, in model simulation, as no death is incorporated for the spores it shows growth even when the dye is fully degradedand similar results were obtained when LiP activity was plotted against time. On the other hand, fractional dye degradation showed complete qualitative agreement with the experiments, i.e. at high dye initial concentration in the media only a small amount of the dye was found degraded by the spores at the same glucose concentration. Although the stochastic model developed showed exact qualitative agreement with the experiments, the initial assumptions made to develop the model may restrict the usage of the model. This can be adjusted if the constants used in the equations are predicted/estimated before running the simulation. As the model was developed based solely on the observations made from the experiments, for every other factor in the experiments constants are to be recalculated. Thus, the model developed in this work can be used to predict the behavior of the fungus in presence of other toxic pollutants in textile dyeing wastewater.

Scope for Future Work

- Collection, characterization and treatment of textile dyeing industry wastewater by *P. chrysosporium* and other suitable microorganisms
- Reactor experiments on real textile wastewater treatment using *P. chrysosporium* and other suitable microorganisms
- Textile wastewater treatment process by select microorganisms identified for the purpose under optimized process parameter conditions
- Modelling behavior of the fungus in degrading other pollutants
- Testing the applicability of the MC model developed in this study for other fungal systems as well as for different pollutants

Appendix-I

C-Code for the study of biomass growth and enzyme secretion by *P. chrysosporium* in absence of toxic pollutant

Parameters used: V - Volume of the medium, Ng- Initial number of glucose molecules, Ns - Initial number of spores, Csz - Final number of cells, Cs - Critical size of the cell, DVCST - Cell division cost, MXST – Maximum timestep allowed, SMPL – number of samples, GAMMA - Constant for glucose availability, gamma -Constant for glucose consumption, sgg- sigma for glucose consumption, sdd - Sigma for division, sgE - Sigma for enzyme production

Sub-function used: ran2 (iseed); a random number generator excluding 0 and 1, iseed is a large negative number.

```
main()

{
static long i,j,k,en;
  static int
g[Csz],x,tstep,count[MXST],Pdcount[MXST],nb,hgstp,N,Np,n,NoE,nmr,dnr,modulo,dvd;
  static   float
Nt,gcrit,volume,Go,Gt,conc[MXST],gen[MXST],enz[MXST],Pt[MXST],nsp,pg1,r;
  static   float ab,bb,pg2,w,cc,cd,pd,nspi,nspf,spore,ec,pe,Et,m,xxx,yyy,slp,ASV,glu,gludif;
static   double rgen;
static   long seed=-99999999;
  FILE *fp1,*fp2,*fp3,*fp4;
  fp1=fopen("Concn_50.d","w");
  fp2=fopen("Growth_50.d","w");
  fp3=fopen("Enzme_50.d","w");
  fp4=fopen("Prod_50.d","w");

hgstp=0;
for (x=0;x<SMPL;x++)
   {
for (i=0;i<Csz;i++)
          {
          g[i]=0.0;
          }
     ASV=VOL;
for (i=0;i<MXST;i++)
          {
```

```
        conc[i]=0.0;
        gen[i]=0.0;
        enz[i]=0.0;
        count[i]=0;
        Pt[i]=0;
        Pdcount[i]=0;
        }
Nt=0;
gcrit=Cs;
volume=V;
    Go=Ng/volume;
Gt=Go;
    N=Ns;
spore=Ns;
tstep=0;
en=0;
for (i=0;i<N;i++)
        {
          r=ran2(&seed);
        if (r>=0.01&&r<= 0.32)
          {
        g[i]=(int)(100*r);
          }
        }
Et=en/volume;
conc[tstep]=conc[tstep]+Gt;
gen[tstep]=gen[tstep]+(N/spore);
enz[tstep]=enz[tstep]+Et;
count[tstep]=count[tstep]+1;
do
        {
        tstep++;
        Np=0;
        nsp=N;
        glu=Gt;
        for (i=0;i<N;i++)
          {

            //Glucose consumption
            pg1=GAMMA*Gt;
            r=ran2(&seed);
        if (r<pg1)
                {
                  pg2=gamma*(g[i]/gcrit)*exp(-g[i]/gcrit);
                  w=ran2(&seed);
                if (w<pg2&&Nt!=Ng)
                  {
                g[i]=g[i]+1;
                Nt++;
                  }
                }
            //Enzyme production
        if (g[i]>0)
                {
                ec=pow((g[i]-gcrit)/sgE,2);
```

```
            pe=(1-Gt/Go)*exp(-ec/2);
              r=ran2(&seed);
            if (r<pe)
               {
            en=en+1;
            g[i]=g[i]-1;
               }
            }
        //Cell division
if(g[i]>2)
            {
              cc=(g[i]-gcrit)/sdd;
            cd=exp(cc)+1;
            pd=1-(1/cd);
              r=ran2(&seed);
            if (r<=pd)
               {
            nb=(int)(g[i]/DVCST);
            g[i]=g[i]-nb*DVCST;
            nspi=nsp+1;
            nspf=nsp+nb;
            for (j=nspi;j<=nspf;j++)
                        {
                        g[j]=1.0;
                        }
            Np=Np+nb;
               }
            }
  }
   N=N+Np;
Et=en/volume;
rgen=(N/spore);
Gt=Go*(1-(Nt/Ng));
gludif=glu-Gt;
modulo=(tstep%100);
dvd=(int)(tstep/100.0);
if (modulo==0)
   {
conc[dvd]=conc[dvd]+Gt;
gen[dvd]=gen[dvd]+rgen;
enz[dvd]=enz[dvd]+Et;
count[dvd]=count[dvd]+1;
     //Enzyme activity
     m=(Smvol*Et);
     n=(int)((2*m)+1);
NoE=(int)(n/2.0);
if (NoE>=1)
            {
            kinetix(&tstep,&NoE,&nmr,&dnr);
            }
yyy=(nmr/ASV);
if (dnr!=0)
            {
            xxx=dnr;
            slp=(yyy/xxx);
```

```
                }
        if (slp>0)
                {
                Pt[dvd]=Pt[dvd]+slp;
                Pdcount[dvd]=Pdcount[dvd]+1;
                }
          }
        } while (gludif>0||Np>0);

if (hgstp<dvd)
        {
        hgstp=dvd;
        }
  }
for (k=0;k<=dvd;k++)
   {
if (count[k]>0)
        {
        fprintf(fp1,"%d %f\n",k,(conc[k]/count[k]));
        fprintf(fp2,"%d %10.2lf\n",k,(gen[k]/count[k]));
        fprintf(fp3,"%d %f\n",k,(enz[k]/count[k]));

        }
else
        {
        fprintf(fp1,"%d %f\n",k,count[k]);
        fprintf(fp2,"%d %f\n",k,count[k]);
        fprintf(fp3,"%d %f\n",k,count[k]);
        }
   }
for (k=0;k<=dvd;k++)
   {
if (Pdcount[k]==0)
        {
        fprintf(fp4,"%d %d\n",k,Pdcount[k]);
        }
else
        {
        fprintf(fp4,"%d %f\n",k,(Pt[k]/Pdcount[k]));
        }
   }

fclose(fp1);
fclose(fp2);
fclose(fp3);
fclose(fp4);
}
```

● ran2 () : program code taken from “Numerical recipes in c: The art of scientific computing”,

W. H. Press, S. A. Teukolsky, W. T. Vetterling and B. P. Flannery, 2ed ,Cambridge

University Press., 282

Appendix-II

C-Code for the study of biomass growth and enzyme secretion by *P chrysosporium* in presence of toxic pollutant

Parameters used: V - Volume of the medium, Ng - Initial number of glucose molecules, Nd -Initial number of dye molecules, Ns - Initial number of spores, Csz - Final number of cells, Cs - Critical size of the cell, DVCST - Cell division cost, DVCST1 (DVCST+1) - Cell division cost plus initial glucose for daughter cell, ENCST – Enzyme release cost, MXST – Maximum timestep allowed, SMPL – number of samples, GAMMA - Constant for glucose availability, gamma -Constant for glucose consumption, sdd - Sigma for division, sgE - Sigma for enzyme production

Sub-function used: ran2 (iseed); a random number generator excluding 0 and 1, iseed is a large negative number.

```
main()

{
static long i,j,k,NoE;
  static int g[Csz],x,tstep,count[MXST],nb,hgstp,N,Np,n,nmr,dnr,modulo,dvd,st,er,ngc,nc,d,Dy;
  static float
Nt,gcrit,volume,Go,Gt,conc[MXST],gen[MXST],enz[MXST],nsp,pg1,r,Avsp,ppsp,dye[MXST],alpha,Ng
0,Rt,CT,a,Nd0,nr,en,AA;
  static float ab,bb,pg2,w,cc,cd,pd,nspi,nspf,spore,ec,ed,pe,Et,m,xxx,yyy,ASV,glu,gludif,slp,pmg;
static double rgen;

  FILE *fp1,*fp2,*fp3,*fp4,*fp5,*fp7,*fp8;
   fp1=fopen("Cg_N1k_g02_D2t1.d","w");
  fp2=fopen("Gr_N1k_g02_D2t1.d","w");
  fp3=fopen("EC_N1k_g02_D2t1.d","w");
  fp4=fopen("DC_N1k_g02_D2t1.d","w");


hgstp=0;
for (x=0;x<SMPL;x++)
   {
for (i=0;i<Csz;i++)
          {
          g[i]=0.0;
          }
```

```
for (i=0;i<MXST;i++)
        {
        conc[i]=0.0;
        gen[i]=0.0;
        enz[i]=0.0;
        dye[i]=0.0;
        count[i]=0;
        }
    N=Ns;
for(i=0;i<N;i++)
        {
         r=ran2(&seed);
        g[i]=8*r+1;
        }
Nt=0;
gcrit=Cs;
volume=V;
alpha=10*Nd/volume;
    Ng0=(1-20*alpha)*Ng;
    Go=Ng0/volume;
Gt=Go;
Dy=Nd;
    Nd0=Nd;
    CT=(Ng0+10*Dy)/volume;
spore=Ns;
tstep=0;
en=0;
modulo=0;
dvd=0;
    AA=0.01*spore/Nd0;
Et=en/volume;

conc[tstep]=conc[tstep]+Gt;
gen[tstep]=gen[tstep]+(N/spore);
enz[tstep]=enz[tstep]+Et;
dye[tstep]=dye[tstep]+Dy/Nd0;
count[tstep]=count[tstep]+1;

do
        {
        tstep++;
        Np=0;
        nsp=N;
        glu=Gt;
        modulo=(tstep%100);
        dvd=(int)(tstep/100.0);
        for(i=0;i<N;i++)
          {
        if (Nt<Ng0)
                {
                 pg1=gamma*(g[i]/gcrit)*exp(-g[i]/gcrit);
                 pg1=GAMMA*Gt*pg1;
                 r=ran2(&seed);●
                if (r<=pg1)
                  {
                g[i]=g[i]+1;
                Nt=Nt+1;
                  }
                }
          /* Enzyme production */
```

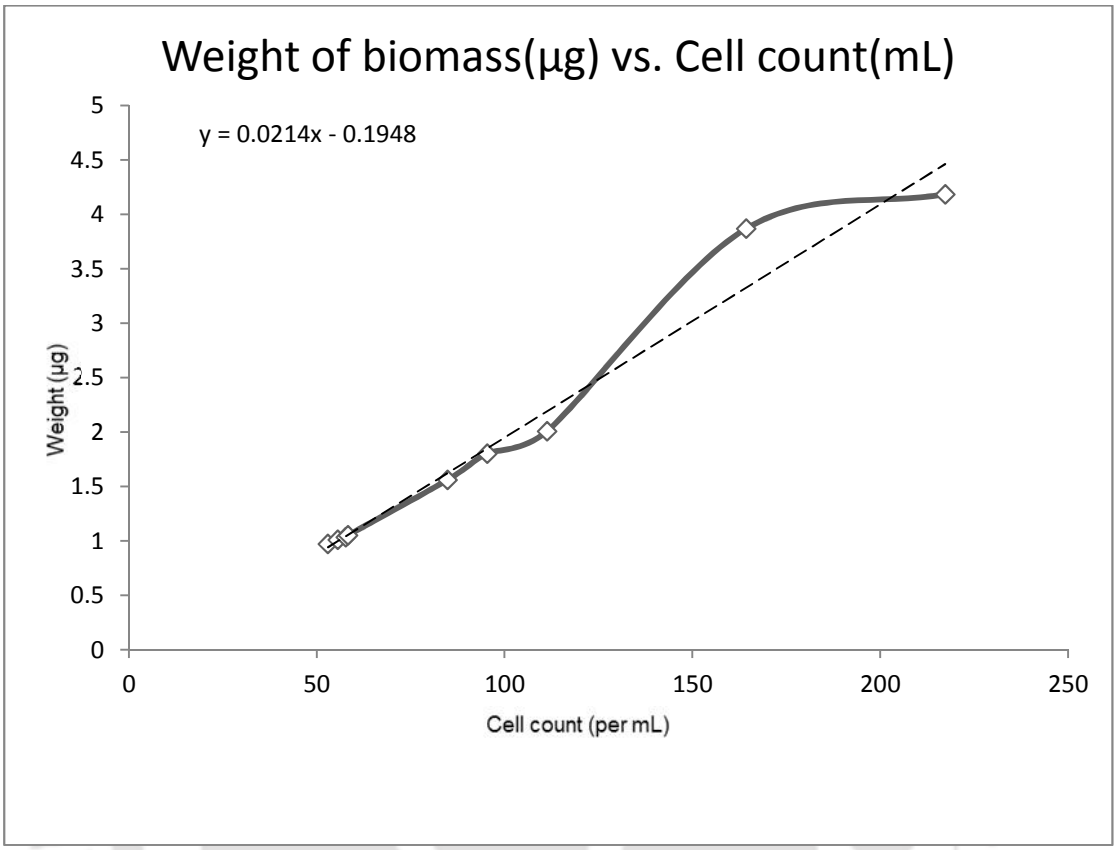
```
if(g[i]>ENCST)
            {
            ec=(g[i]-gcrit)/sgE;
            pe=1.0/(1+exp(-ec));
            pe=Go*pe*(1-Gt/Go);
              r=ran2(&seed);
            if (r<=pe)
               {
            nr=(int)((g[i]-1)/ENCST);
                w=ran2(&seed);
            nr=nr*w+1;
            g[i]=g[i]-nr*ENCST;
            en=en+nr;
               }
            }

     /* Cell division */
if(g[i]>DVCST)
            {
              cc=(g[i]-gcrit)/sdd;
            pd=1.0/(exp(-cc)+1);
              r=ran2(&seed);
            if(r<=pd)
               {
            nb=(int)((g[i]-1)/DVCST1);
                w=ran2(&seed);
            nb=nb*w+1;
            g[i]=g[i]-nb*DVCST1;
            nspi=nsp+1;
            nspf=nsp+nb;
            for(j=nspi;j<=nspf;j++)
                        {
                        g[j]=1.0;
                        }
            Np=Np+nb;
               }
            }
   }
  N=N+Np;
rgen=(N/spore);
Gt=Go*(1-Nt/Ng0);
Rt=AA*Dy*en/pow((en+Dy),2);
   d=0;
for(k=1;k<=Dy;k++)
  {
    w=ran2(&seed);
if (w<=Rt)
     {
          d=d+1;
     }
  }
Dy=Dy-d;
Et=en/volume;
 CT=((Ng0-Nt)+10*Dy+100*en)/volume;
gludif=glu-Gt;
printf("\n%d %f %f %d %f %d",tstep,Gt,Et,N,Rt,Dy);
if (modulo==0)
    {
conc[dvd]=conc[dvd]+Gt;
gen[dvd]=gen[dvd]+rgen;
```

```
            enz[dvd]=enz[dvd]+Et;
            dye[dvd]=dye[dvd]+Dy/Nd0;
            count[dvd]=count[dvd]+1;
                }

            } while (gludif>0||Np>0);
if(hgstp<dvd) hgstp=dvd;

   }
for (k=0;k<=hgstp;k++)
   {
if (count[k]>0)
            {
            fprintf(fp1,"%ld %10.6f\n",k,(conc[k]/count[k]));
            fprintf(fp2,"%ld %10.6lf\n",k,(gen[k]/count[k]));
            fprintf(fp3,"%ld %10.6f\n",k,(enz[k]/count[k]));
            fprintf(fp4,"%ld %10.6f\n",k,(dye[k]/count[k]));

            }
else
            {
            fprintf(fp1,"%ld %d\n",k,count[k]);
            fprintf(fp2,"%ld %d\n",k,count[k]);
            fprintf(fp3,"%ld %d\n",k,count[k]);
            fprintf(fp4,"%ld %d\n",k,count[k]);
            }

   }
 //================================================
fclose(fp1);
fclose(fp2);
fclose(fp3);
fclose(fp4);
}
```

● ran2 () : program code taken from “Numerical recipes in c: The art of scientific computing”, W. H. Press, S. A. Teukolsky, W. T. Vetterling and B. P. Flannery, 2ed ,Cambridge University Press., 282

Appendix-III

Change in Biomass growth with respect to the cell count of the fungus



As observed in earlier experiments, almost a linear relationship exists between the biomass growth and cell count of the fungus under the given experimental conditions. However, huge variations also observed at the extremities. They were controlled strictly to maintain the correlation between the biomass growth and spore count throughout the work.

Appendix-IV

ANOVA tables of biomass growth and enzyme activity by *P chrysosporium*

Every single set of experimental data have been analysed and P and F values were calculated follows,

For growth, in variable initial glucose medium in absence of toxic pollutant,

Anova:
Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Glu 5%	17.00	75.14	4.42	8.86
Glu 10%	17.00	105.60	6.21	16.22
Glu 15%	17.00	116.72	6.87	20.83
Glu 20%	17.00	140.10	8.24	25.85
Glu 25%	17.00	165.41	9.73	27.22

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	276.17	4.00	69.04	3.49	0.01	2.49
Within Groups	1583.92	80.00	19.80			
Total	1860.08	84.00				

For enzyme activity,

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Glu 5%	17.00	364.22	21.42	409.00
Glu 10%	17.00	613.30	36.08	925.87
Glu 15%	17.00	844.22	49.66	1465.16
Glu 20%	17.00	1411.11	83.01	2812.66
Glu25%	17.00	1599.17	94.07	3030.61

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	64729.01	4.00	16182.25	9.36	0.00	2.49
Within Groups	138292.67	80.00	1728.66			
Total	203021.68	84.00				

As, we can see the P factors are well below the limit. The experiments showing higher P values have been discarded and repeated. Same method was employed for all the experiments with and without toxic pollutant.

LIST OF PUBLICATIONS

Published/Accepted in Refereed International Journals

1. **Sen, K.,** Pakshirajan, K. and Santra, S.B. (2012) Modeling the biomass growth and enzyme secretion by the white rot fungus *Phanerochaete chrysosporium*: a stochastic-based approach Applied Biochemistry and Biotechnology, 167: 705–713.

2. **Sen, K.,** Pakshirajan, K. and Santra, S.B. (2012) Modeling the biomass growth and enzyme secretion by the white rot fungus *Phanerochaete chrysosporium* in presence of a toxic pollutant. Journal of Environmental Protection, 3: 114-119.

3. Vinod Kumar Yata, **Kausik Sen**, MattaparthiVenkataSatish Kumar and Siddhartha SankarGhosh (2012), Interaction studies of *E. coli* uracil phosphoribosyltransferase with 5-fluorouracil for potent anti-cancer activity, Med. Chem. Res., 21:1149–1155

Manuscript under Preparation

1. **Sen, K.,** Pakshirajan, K. and Santra, S.B. Effect of physico-chemical parameters on biomass growth and enzyme secretion by *Phanerochaete chrysosporium* in presence and in absence of DR-80 as a toxic pollutant.

In International Conferences

1. Pakshirajan, K.and**Sen, K.** (2007) Prediction of coliforms in surface waters based on easily estimable parameters using artificial neural networks. International Conference on New Horizons in Biotechnology, November 26 - 29, 2007, Trivandrum, India.
2. B. R. Meher, M. V. Satish Kumar and **Kausik Sen**, (2008), Pressure induced conformational dynamics of HIV-1 protease: A Molecular Dynamics simulation study, International Conference on Information Technology.