A comparative study on the degradation of azo dyes by fungal strain and silver nanoparticles

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In

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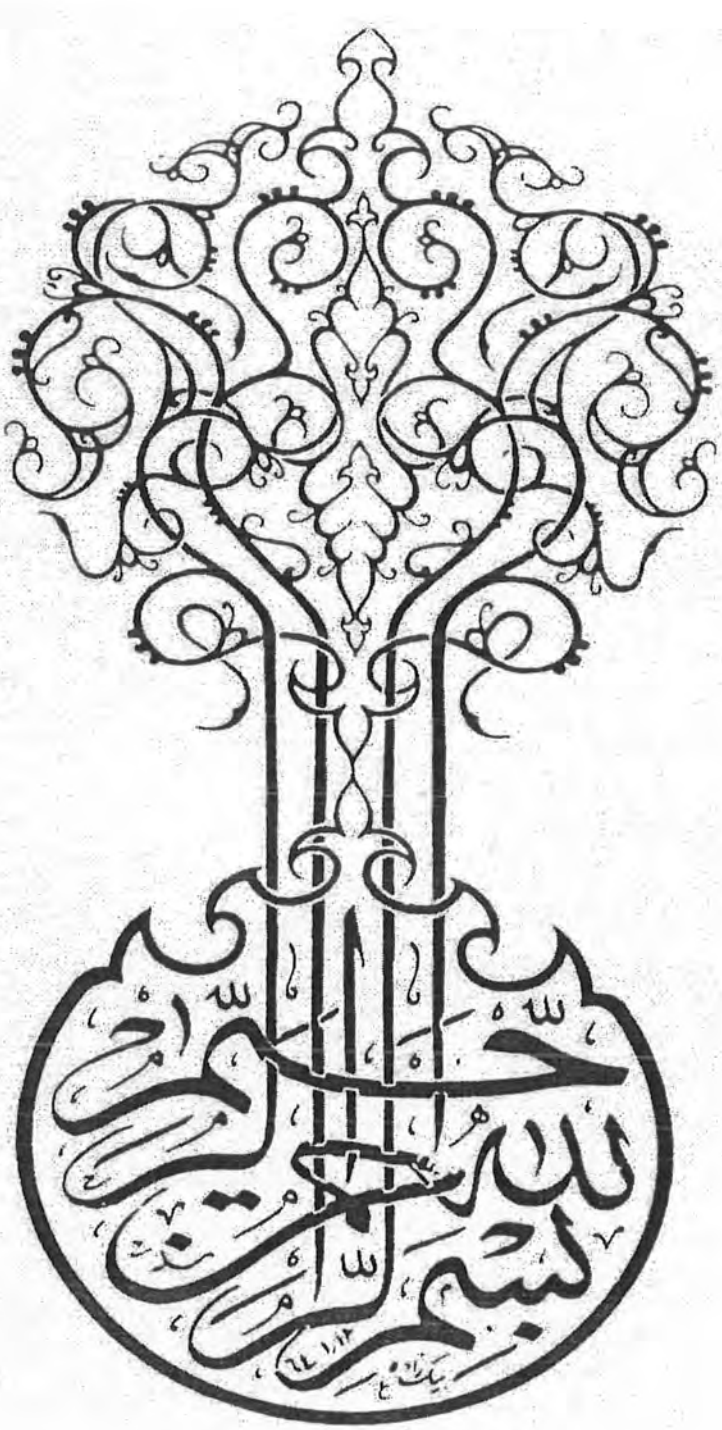
بسم الله الرحمن الرحيم

I humbly dedicate this piece of work to my

Father (late) & Mother

Whose selfless life and great efforts with unceasing prayers has enabled me to reach the present position in life.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Afshan Hina Naeem

Certificate

This thesis submitted by *Afshan Hina Naeem* is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, and Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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List of Acronym/abbreviations

%	Percentage
°C	Degree Celsius/Centigrade
µ	Micro
A. niger	*Aspergillus niger*
Ag^0	Silver nanoparticle
AR 151	Acid red 151
ATR	Attenuated total reflectance
B Ag^0NPs	Biologically synthesized silver nanoparticles
C Ag^0NPs	Commercially available silver nanoparticles
C. I.	Color Index
$CaCl_2$	Calcium chloride
COD	Chemical oxygen demand
Conc.	Concentration
$CuSo_4$	Copper Sulphate
DNA	Deoxyribonucleic acid
$FeCl_3$	Ferric chloride
Fig.	Figure
FTIR	Fourier transform infrared spectroscopy
HPLC	High performance liquid chromatography
hrs	hours
hrs.	Hours
i.e.	That is
IR	Infrared

KH_2PO_4	Potassium dihydrogen phosphate
KNo_3	Potassium Nitrate
l	Liter
LiP	Lignin peroxidase
M	Molar solution
$MgSO_4$	Magnesium sulphate
min	Minute
mM	Milli molar
MnP	Manganese peroxidase
Na_2Co_3	Sodium Carbonate
$NaHCO_3$	Sodium bicarbonate
nm	nanometer
NP(s)	Nanoparticle(s)
OD	Optical density
Or II	Orange II
PDB	Potato Dextrose Broth
RNA	Ribonucleic acid
Rpm	Revolution per minute
SDB	Sabrourd Dextrose Broth
Sp.	Species
STE	Simulated textile effluent
UV-Vis	Ultra violet-Visible
W/V	Weight volume ratio
λ	wavelength

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Abstract

Synthetic textile dyes have found to be recalcitrant and xenobiotic compounds in nature. Development of ecofriendly and efficient processes for biodegradation of these azo dyes is an important step in field of applications of nanotechnology. Present research work is focused on the treatment of two commonly used textile azo dyes acid red 151 (monoazo) and orange II (diazo) by silver nanoparticles (Ag°NPs) synthesized by *A. niger*. In present study silver nanoparticles (Ag°NPs) were used as photocatalyst in degradation process and were compared with its plain culture *(A. niger)* and also with commercial silver nanoparticles (Ag°NPs). Combined treatment was also applied to determine the extent of decolorization in which culture and nanoparticles both were used synergistically. First, different physiological reaction conditions (e.g. pH, temperature, initial concentration of dye and catalyst dosage) were optimized for maximum degradation of both dyes As a result the optimum conditions for degradation of dye obtained were pH 3 & 9, 30 °C, 50 mg/l for AR 151, 20 mg/l for Or II and 200 mg/l of Silver nanoparticles (Ag°NPs). The laboratory synthesized *A. niger* silver nanoparticles (Ag°NPs) efficiently decolorized the both dyes within 24 hours of incubation time while its plain culture (*A. niger*) takes more than 48 hours for the same practice. Laboratory synthesized silver nanoparticles (10–20 nm) showed similar decolorization abilities as commercial silver nanoparticles. When dye decolorization was tested for both dyes, maximum decolorization was observed in case of AR151. Degradation of both azo dyes was investigated by UV-Vis Spectrophotometer and Fourier Transform Infrared Spectroscopy (FTIR) and high performance liquid chromatography (HPLC). Cytotoxicity and phytotoxicity assessment of biodegradation products were carried out using brine shrimp bioassay and radish seed germination bioassay which confirmed that the treated dye samples were very slight toxic to water and soil organism.

Chapter 1

INTRODUCTION

INTRODUCTION

Various synthetic dyes are widely used in textile dyeing, paper, pulp, plastic, color photography, pharmaceutical, food, cosmetic and other industries (Ren *et al.*, 2006). Several reports have mentioned that there are about 100, 000 different kinds of dyes commercially available with an estimated production of up to 700,000 tons worldwide annually (Gao *et al.*, 2010). At present, more than 10,000 dyes have been effectively commercialized that are used at large scale (Leena *et al.*, 2008). All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, oxidizing agents, and microbial attack. Synthetic dyes comprise an important part of industrial effluents, as they are discharged in abundance by many manufacturing industries. The impact of these dyes on the environment is a major health concern because they are potentially toxic and carcinogenicin nature (Parson *et al.*, 2004). Most of commercial azo dyes are chemically stable and difficult to remove from wastewater as they are stable and resistant to oxidizing agents, light and heat (Mamdouh *et al.*, 1991). Therefore cause environmental concern because of their color, biorecalcitrance, potential toxicity and carcinogenicity to animals and human beings (Raju *et al.*, 2007; Siddiqui *et al.*, 2009). Dyes are generally classified on basis of their structure, nature, color, method of application, and their source in the color index (C.I.) which has been revised continuously since 1924. Dyes can be classified in several classes depending on the presence of specific choromophores. These include azo dyes, acridine dyes, arylmethane dyes, nitro dyes, anthroquinone dyes, quinine amine dyes and xanthenes dyes (Rauf *et al.*, 2010).

Azo dyes comprise a major group of synthetic dyes (60-70 %) because of their wide range, low cost, and ease of their synthesis. These dyes are used in all major industries such as textiles, foodstuffs, leather, papers, cosmetics, and laser printing etc. (Olukanni *et al.*, 2006). So the wastewater originated from these industries always carries a major fraction of dyes with other contaminants. The removal of dyes from waste waters has become a serious issue during last few years because of their toxic and recalcitrant nature (Mamdouh *et al.*, 1991). Xenobiotic nature of azo dyes makes them difficult to be degraded by conventional biological and chemical treatments including activated sludge system. Although many of these dyes are banned for

consumption, but still a large number of these dyes are commonly used due to their low cost and ease of application in the textile manufacturing (Pandey *et al.*, 2007).

All azo dyes contain nitrogen to nitrogen double bonds (–N=N–) that are mostly attached to two moieties of which one or both can be aromatic such as benzene or naphthalene. Examples of few azo dyes includes; Methyl Orange, Acid Orange 7, Acid red 151, Methyl Red, Reactive red 2,Congo red, Direct blue 160, Remazol Brilliant Orange 3R, etc. Azo dyes can be classified as monoazo (Methyl red, Orange G), diazo (Amido Black 10B), triazo (Procion red MX-5B, etc.) depending upon number of –N=N–groups in bonds and their associated auxochromes and chromophoresthat determine the color of azo dyes. Reductive cleavage of these azo bonds by any treatment leads to decolorization of azo dyes (Rauf *et al.*, 2009). It is estimated that (30-40%) of the dyes are lost in effluent due to inefficiencies of dyeing processes(Olukanni *et al.*, 2006; Ali *et al.*, 2008; Gao *et al.*, 2010). Azo dyes released in waste water proved to be a great environmental threat as they reduce sunlight penetration and oxygen dissolution in water thus effecting aquatic life. Most of synthetic dyes are toxic, cancerous and mutagenic in nature as they cause allergies, skin irritation and dermatitis in human beings causing serious health problems (Wesenberg *et al.*, 2003; Ofomaja, 2009; Dos Santos *et al.*, 2007).

Conventional techniques used to decolorize colored effluents, do not result in complete decolorization, and thus enhance the need of secondary treatments. While commonly used biological treatments do not have high removal efficiencies (Sharma *et al.*, 2009). Different types of physiochemical processes are being used for a long time. Most important process is coagulation process (Zhao *et al.*, 2007), but it has some serious drawbacks such as requirement of large number of chemicals and production of significant volume of sludge. In adsorption process (Gao *et al.*, 2010; Nassar *et al.*, 1997), contaminants are transformed from aqueous to solid phase which however, requires further treatment or degradation of the solid waste. Membrane filtration techniques are although very efficient but they are not cost effective (Gomez *et al.*, 2006). Numerous oxidation techniques such as UV/H_2O_2 (Chang *et al.*, 2006), ozonation, (Fanchiang *et al.*, 2009) Fenton reaction (Rosales *et al.*, 2009) and photo fenton may prove to be effective in decolorization process, but their operational cost are very high. Besides this biological methods such as treatment by microbes for

example *Shewanella decolorationis* NTOU1 and *Dichomitus squalens* laccase isoenzymes can decolorize some dyes, but long term degradation process restrict their application (Susla *et al.,* 2007). On the other hand, some dyes can undergo anaerobic decoloration that results in formation of very harmful byproducts which are potential carcinogens (Neill *et al.,* 2000). Another newer and more powerful set of techniques called Advanced Oxidation Processes (AOPs) has been established and used to treat waste water effluent containing dyes. In these processes strong oxidizing agent such as hydroxyl radicles are produced in situ which carry out a sequence of reaction that breaks down macromolecules to smaller and less toxic substances, and sometimes macromolecules are completely mineralized into water and carbon dioxide (Rauf *et al.,*2009).

These advanced oxidation processes have proved to be more efficient, therefore have drawn huge attention from scientific community because these are easy to manage and less residual are produced as compared to conventional approaches (Elmorsi *et al.,* 2010). Techniques which are used in AOPs are Fenton process (Masarwa *et al.,* 2005; Bouasla *et al.,* 2010), photo-Fenton process (Modirshahla *et al.,* 2007; Monteagudo *et al.,* 2010; Abdessalem *et al.,* 2010; Tehrani *et al.,*2010), the UV photolytic technique (Gul *et al.,* 2009; Al-Hamedi *et al.,* 2009; Elmorsi *et al.,* 2010), ozonation process, sonolysis (Song *et al.,* 2007; Ghodbane *et al.,* 2009; Merouani *et al.,* 2010), photocatalytic techniques (Bukallah *et al.,* 2007; Habib *et al.,* 2007; Zhang *et al.,* 2009; Rauf *et al.,* 2010; Xu *et al.,* 2010) and radiation induced degradation of dyes (Dajka *et al.,* 2003; Chen *et al.,* 2008; Mohamed *et al.,* 2009; Vahdat *et al.,*2010). In spite of the reasonable efficiency of these wastewater treatment techniques such as electrocoagulation and fenton (Mollah *et al.,* 2004; Hasani*et al.,* 2009; Azizi *et al.,* 2010; Elmorsi *et al.,* 2010), these methods have shown to face some drawbacks including cost and energy consumption (Pandey *et al.,* 2007), generation of large amounts of sludge which require safe disposal (Supaka *et al.,* 2004) and interference with other wastewater constituents (Van der Zee and Santiago, 2005).

Therefore, biological processes which are certainly environment friendly and cost effective alternatives to the physicochemical treatment of wastewaters have excessively been applied within the last decades (Sirianuntapiboon and Yommee,

2006;Garcia*et al.*, 2008; Yang*et al.*, 2009). Thus inexpensive, efficient and locally available decolorization techniques are mandatory. Recently a developing interest in photocatalysis assisted over semiconductor has been witnessed. It is a branch of Advanced Oxidation technologies for breakdown of organic compounds into less toxic smaller compounds (Chatterjee *et al.*, 2005; Qamar *et al.*, 2005; Nezamzadeh-Ejhieh *et al.*, 2011). In this technique redox transformations and finally decomposition of dye molecules occur. In photocatalytic process a semiconducting material absorbs light energy equal to or greater than its band gap, thus generating electrons and holes, which further generate free radicles to oxidize dye molecules. These resulting free radicals can efficiently oxidize organic matter (Kamat *et al.*, 2003; Kabra *et al.*, 2004). To date, nanotechnology has provided initiative for synthesis and manipulation of different materials in range of 1-100 nm. These nanomaterials are currently applied in variety of electronic applications, water remediation technologies (Tratnyek *et al.*, 2006), imaging and medical apparatus nano medicines, fabrics and cosmetics (Mornet *et al.*, 2008). These nanocrystalline particles are widely applied for degradation of organic compounds (Ralph., 1992; Seo *et al.*, 2001; Xiaodan *et* al., 2006).

Photocatalytic degradation by Titanium dioxide (TiO_2) in nanometer size have attracted immense interest because of its advantages which includes complete removal of organic impurities from waste water, which is mainly because of its several qualitiessuch as chemical stability, optical-electronic properties, non-toxicity and low cost (Shiping*et al.*, 2010; Soutsas *et al.*, 2010). $TiO_2$and ZnO nanomaterials have been particularly investigated in photocatalytic degradation of several pollutants because of their high photosensitivity, chemical stability and non-toxic nature (Neppolian *et al.*, 1999; Akyol *et al.*, 2005; He *et al.*, 2006; Anandanab *et al.*, 2006; Liang *et al.*, 2008; Habibi *et al.*, 2007; Ni *et al.*, 2007; Mahmoodi *et al.*, 2009; Jun *et al.*, 2010). It has been reported that utilization of TiO_2 nanoparticles has serious drawback that they show photocatalytic activity only in presence of UV light instead of natural solar light. Besides, this, nanotechnology has enabled the development of metallic nanoparticles with novel and distinguishing physio-chemical properties and alsotheir broad range of application in scientific and technological fields (Moore,2006).

Among various nanoparticles silver nanoparticles are most widely studied because of their optical, chemical, biological, thermal and electrical properties (Fayaz *et al.*,

2010). They have been used to synthesize various products such as antibacterial wound dressings, surgical gloves and masks, textile, furniture, anti-odor clothes and many houses hold products (Wang *et al.*, 2008).

In present study the biologically prepared Ag^0 nanoparticles are used as photocatalyst in degradation of AR 151 and Or II which are organic dyes. AR 151 and Or II are heterocyclic aromatic compounds with molecular formula $C_{22}H_{15}N_4NaO_4S$ and $C_{16}H_{11}N_2NaO_4S$ respectively (Rajaguru *et al.*, 2000). Or II is also known as Acid Orange7, commonly used in textile, cosmetics, weaving and paper industry at large scale. Therefore these dyes are seen in waste water effluent (Méndez-Paz *et al.*, 2005). But, due to extensive consumption of AR 151 & Or II in dying industries and regard to their environmental aspects, it is vital to discover a more efficient method to treat the waste waters polluted with these dyes. In this work we studied the role of biologically synthesized and commercial Ag^0NPs that resulted in fast reaction rate, less production of polycyclic compounds and degradation of organic pollutants in range of ppm.Which are important qualities that make this technique superiors to previous methods (Ioannis *et al.*, 2004; Wang *et al.*, 2000).

Aims and Objectives

The aim of the present work is to investigate the comparative degradation of two azo dyes AR 151 and Or II by *A. niger* and *A. niger* mediated silver nanoparticles in separate and combined treatment. Present work is using interactive approach towards mineralization/degradation of different azo dyes using fungi and nanoparticles under different operational conditions in cheap, safe and efficient manner.

The detailed objectives are;

1. To evaluate the degradation of two different azo dyes AR 151 and Or II by biologically synthesized fungus mediated silver nanoparticles using photocatalytic degradation technique in comparison with fungal degradation.
2. To evaluate different parameters that affects degradation kinetics such as solution pH, reaction temperature, dosage of Ag^0 nanoparticles and initial concentration of dyes.
3. To determine concentration of residual dye in treated samples and formation of new products through
    - UV-Vis spectroscopy
    - FTIR
    - HPLC
4. To determine the toxicity level of two different azo dyes and their metabolites using brine shrimp bioassay and phytotoxic assessment test using radish seeds.

Chapter 2

Chapter 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Dyes and colorants are colored substances that are natural or synthetic in nature used to impart color to fabric material. They are generally resistant to degradation upon exposure to light, water, heat and different chemicals like oxidizing agents etc. (Rai *et al.,* 2005). In 1856, Wiliam Henery Perkin discovered the world's first commercially effective dye. There were more than 10,000 different dyes by the end of 19th century that were commercially produced and applied in different industries like leather, textile, food and beverages etc. (Robinson *et al.,* 2001). In addition to this, the development of the textile industry all over the world also favored an expanded consumption of synthetic dyes, and this resulted in a tremendous increase in pollution load due to release of unfixed dyes into the water bodies from the industrial units (Pandey *et al.,* 2007). Consumption of water and generation of waste water from the textile industry depend upon the treating procedures and techniques employed during formation of textile fabric (Dhanve *et al.,* 2008). Textile industry is generating largest quantity of liquid pollutants, because of using large quantity of water in dying process which finally goes to waste water along with toxic compounds. Generally fixation rate of dyes to the fiber and other material vary from 60-95 %, due to which a high load of dyes is added into the corresponding water bodies (Banat *et al.,* 1996). It is estimated that 280,000 tons of synthetic dyes are released in such industrial effluents worldwide each year (Jin *et al.,* 2007). Generally the persistent nature of the dyes creates huge problem in terms of removal whenever wastewater containing them is being put into any treatment facility. Considering this issue, a variety of treatment systems have been devised and employed worldwide though each facing some kind of drawback due to varying nature of dyes and associated waste water compositions. Stability of selected azo dyes has been tested and it has been concluded that the dye metabolites are persistent in aquatic environment and cannot be degraded by conventional waste water treatments (Ekici *et al.,* 2001). Dye containing waste water after some biological treatment showed increased mutagenicity in comparison to untreated samples (Fracasso *et al.,* 1992).

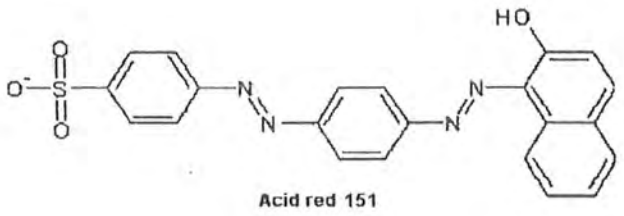
2.1 Classification of dyes

Dyes can be classified in different ways. Mostly they are classified on basis of their chemical structure, nature and commercial names. Dyes are unsaturated complex

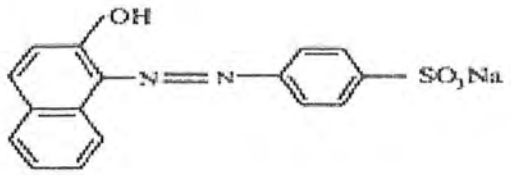
aromatic compounds having characteristics of resistance to fading, solubility, and intense color. Dyes can be well defined as coloring compounds which differ in chemical composition from each type of dye which are easily soluble in many solvents and are used for coloration of different fabric materials. http://www.dyespigments.net/types-of-dyes.html.

Table 2.1: Uses of dyes in different industries

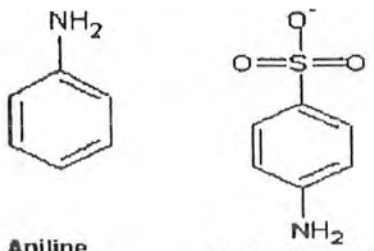
Group	Uses
Azo dyes, based on a -N=N- azo structure	Textiles
Arylmethane dyes Diarylmethane dyes, based on diphenyl methane Triarylmethane dyes, based on triphenyl methane	=
Anthraquinone dyes, derivatives of anthraquinone >C=O and>C=C	=
Acridine dyes, derivatives of acridine >C=N- and>C=C Textiles	Textile and leather
Cyanine dyes, derivatives of phthalocyanine	Leather
Nitroso dyes, are based on a -N=O nitroso function	=
Nitro dyes, based on the -NO2 nitro functional group	=
Diazonium dyes, based on diazonium salts	=
Xanthene dyes, derived from xanthene -O-C6H4-O	Wool, silk and cotton
Xanthene dyes, derived from xanthene -O-C6H4-O	=
Phthalocyanine dyes, derivatives of phthalocyanine >C=N	Paper
Oxazone dyes, derivatives of oxazone	Colored photography
Indophenol dyes, derivatives of indophenol >C=N-and>C=O	=
Thiazin dyes, derivatives of thiazin Oxazin dyes, derivatives of oxazin -C-N=C =C-O-C=	Calico printing
Thiazole dyes, derivatives of thiazole >C=N-and-S-O=	Intermediate
Rhodamine dyes, derivatives of rhodamine	
Fluorene dyes, derivatives of fluorene	
Pyronin dyes	



Acid red 151



Orange II



Aniline Sulfanilic acid

Fig. 2.1: Structure of Acid red 151, Orange II and related products (Ali *et al.*, 2007).

Table 2.2: General properties of AR 151 and Or II.

Name of dye.	Mol. formula	Mol. wt	C.I.	λ max
Acid red 151	$C_{22}H_{15}N_4NaO_4S$	454.43	26900	512 nm
Orange II	$C_{16}H_{11}N_2NaO_4S$	350.32	15510	486 nm

2.2 Color and stability of dyes

Dyes are complex colored substances that have affinity towards a substance to which they are applied, they are commonly applied in solvent system, and need a mordent for improving their color fastness. A dye is basically ionizing aromatic compound having aryl ring containing delocalized electrons in them, called chromophore. This part of dye molecule is responsible for absorption of the light of different wave lengths depending on the energy of electrons cloud. Chromophores can make changes in delocalized electron cloud of dye. These changes in electrons clouds invariably effects absorbing radiation in visible region of light. Through this way our eye can detect this absorption and can response to different colors. Electrons present in delocalized region may result in color removal. Any shift or removal of electron may cause the reversion of remaining electrons to local orbits. Schiff's reagent is good example of this. In reaction between sulphurous acid and Pararosaniline, sulphonic group attaches to compound central carbon atom. That hinders the conjugated double bond system of Quinoid ring that results in localization of electrons which causes ring destruction of Chromophore part that result in color removal. Presence of auxochromes in chromogen molecules is very important, because it is the only substance that provides cohesiveness and solubility to dye. An auxochromes is a collection of atoms attached with chromophore part of dye molecule which then alters the ability of chromophore to absorb light. These auxochromes include amino group, aldehyde group and hydroxyl groups which have the ability to intensify and modify the color of that dye. There are two types of auxochromes positively charged and

negatively charged (Cegarra *et al.*, 1992; Supriyanta *et al.*, 1992; Haarer *et al.*, 1994; Yoon *et al.*, 1996)

Important groups of dyes used in textile industry

- Acid dyes
- Azo dyes
- Direct dye
- Basic dyes
- Mordant dyes
- Reactive dyes
- Disperse dyes
- Sulphur dyes
- Solvent dyes
- vat dyes

2.3 Azo dyes

Azo dyes are also known as ice colors and ingrain colors which are water insoluble pigments. Only way of synthesis of azo dyes is coupling reaction between aromatic azo compounds and coupling component, these coupling components mostly contain an active hydrogen atom attached with a carbon atom. Of all the dyes synthesized, 60 % of dyes are manufactured by this way (Chudga and Oakes, 2003). Azo dyes are complex organic compounds that consist of functional group R-N=N-R', in which alkyl and aryl are present at position of R or R'. According to IUPAC definition azo compounds are derivatives of diazene HN=NH, where hydrogen atoms are substituted by aryl or alkyl groups (Nic *et al.*, 2006). Presence of two aryl groups provides more stability to N=N group which is called azo group. This name is derived from *azote* that is a French name used for nitrogen that is taken from Greek that means a (not), zoe (to live). http://www.dyespigments.net/types-of-dyes.html)

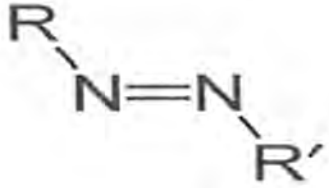


Fig. 2.2: General chemical formula of azo dye

Azo dyes are one of major groups of dyes that accounts for more than 70 % of all different types of dyes commonly used worldwide (Zollinger, 1987), so these dyes are commonly found in textile effluents (Chang *et al.*, 2001b; Saratale *et al.*, 2009a; Zhao and Hardin, 2007). This group of dyes comprises more than 3000 different types of dyes. These are commonly used because of their low cost of synthesis, their fastness, stability, and varieties available (Chang *et al.*, 2004). Industries where these dyes are used broadly, includes textile, paper, cosmetics, leather (Telke *et al.*, 2008). Textile waste water containing azo dyes when discharge into environment leads to severe contamination that result in reduction of sunlight penetration resulting reduced photosynthetic activity, water quality, oxygen concentration which have very lethal effects on aquatic life. Azo dyes effects badly water qualities in terms of total organic carbon (TOC) biological oxygen demand (BOD) chemical oxygen demand (COD) (Saratale *et al.*, 2009b).

2.4 Classification of azo dyes

Due to chemical nature of azo dyes which is characterize by presence of one or more azo groups (-N=N–), these dyes absorb in visible part of light spectrum (Chang *et al.*, 2000). Variation in types of azo dyes is mainly because of substitution of azo group with benzene or naphthalene which have different substituents like hydroxyl (–OH), carboxyl (–COOH), nitro ($–NO_2$), chloro (–Cl), amino ($–NH_2$) (Zollinger, 1987). In addition to number of azo groups, further subdivision of azo dyes can be done depending on first, the solubility of dye, secondly type of component used. In a group containing disazo dye, primary and secondary types of dyes are present. While in group of triazo and polyazo dyes, direct dyes are mostly present. These dyes are used for dyeing all types of substrates natural and synthetic materials such as cotton, wool, silk, leather, paper, acrylics, polyamides, polyolefin, polyesters and viscos rayons, foods, drugs, cosmetics, and printing. Some of these dyes contain more than one sulphonate groups that provide increased solubility in aqueous solution (Chudga and Oakes, 2003). Several synthetic dyes have very hazardous effects on seed germination, and growth rates of all plants which can disturb environment by affecting habitat provision, fertility of soil, and erosion protection, because these dyes and their metabolites are poisonous, oncogenic and mutagenic (Myslak and Bolt, 1998; Ghodake *et al.*, 2009a). Therefore, industrial effluents contaminated with azo

dyes and their metabolites should be treated essentially before their disposal into environment (Kapustka and Repor *et al.*, 1993).

2.5 Removal of azo dyes from textile waste water

Several procedures have been applied for the elimination of dyes from textile effluents. However, these methods are economically unfeasible, and are not able to entirely remove azo dyes and their metabolites from water. It finally results in generation of substantial amount of sludge causing further pollution problems (Forgacs *et al.*, 2004; Zhang *et al.*, 2004). In comparison to physio chemical methods microbial decolorization is cost effective and ecofriendly method of degradation that helps in less water consumption (Rai *et al.*, 2005; Verma and Madamwar, 2003).

Waste water effluent coming from textile industries contain only 0.7- 0.8 g/l of solid substances but contamination and hazardous effects it consequences, is mostly because of their stability and recalcitrance (Jadhav *et al.*, 2007). Therefore it is essential to develop efficient technologies for removal of these dyes. Several methods such as adsorption, precipitation, coagulation, electrochemical treatments and various oxidation reduction reactions have been used and these are reported in several previous studies (Dos Santos *et al.*, 2007; Wang *et al.*, 2008 a, b, c).

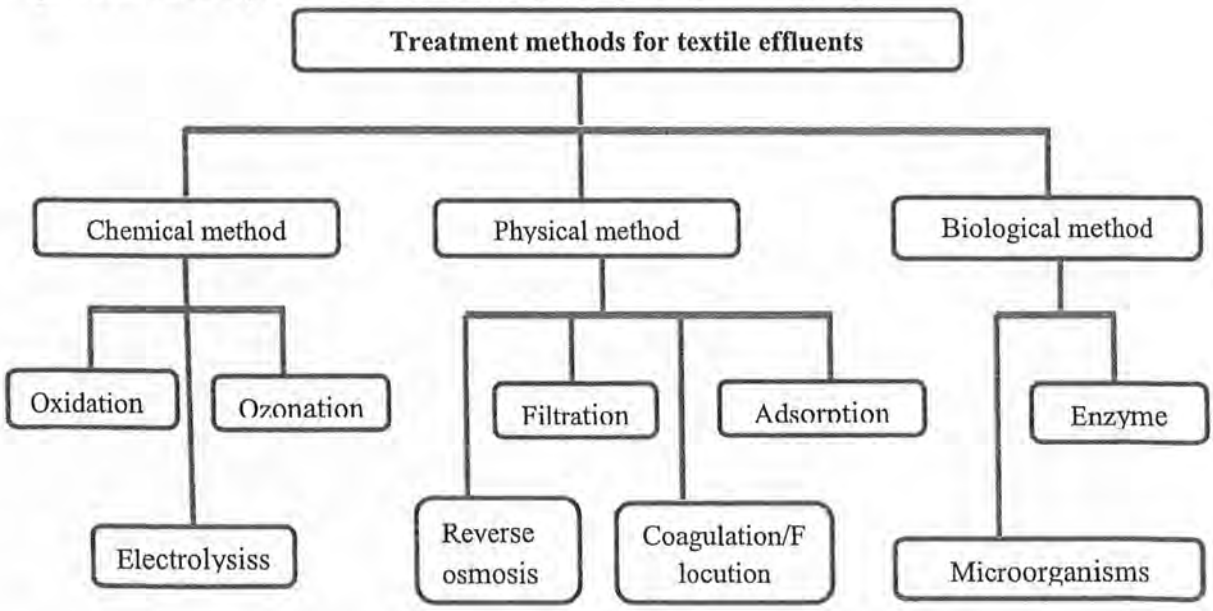


Fig. 2.3: **Physical, chemical and biological methods for the removal of dyes from waste water**

2.5.1 Chemical methods

Chemical oxidation methods use several oxidizing agents such as hydrogen peroxide (H_2O_2), ozone (O_3), and permanganate (MnO_4) and result in decomposition of complex dye molecules In presence of these oxidizing agents modification in chemical composition of dye molecules takes place which result in dye degradation. Ozonation process has shown some significant results in terms of dye decolorization due to its fast reaction with large number of dyes (Alaton *et al.,* 2002). But very small life time of ozone, ineffectiveness towards water insoluble and disperses dyes, high cost of ozone and low COD removal capacity limits its practical application technique (Anjaneyulu *et al.,* 2005). The Fenton reaction method is found to be relatively cheap, has high COD removal efficiency for both water soluble and insoluble dyes, (Chamarro *et al.,* 2001), but major drawback is high sludge generation (Robinson *et al.,* 2001). Electrochemical oxidation process is found to be very helpful in degradaing complex organic compounds and generating less harmful byproducts but much higher cost of electrical energy limits its practical application (Mollah *et al.,* 2004; Zhou *et al.,* 2007). In advanced oxidation techniques H_2O_2/UV is highly efficient technique due to significant level of color remove, high COD removal efficiency and lack of sludge generation (Wang *et al.,* 2009a, b, c). But this is less effective for dispersed or vat dyes, highly colored waste water and high cost of UV light used in process (Baran et *al.*, 2008; Chen and Zhu, 2007).

Above brief review of several physical and chemical methods commonly used for removal of dye from waste water reveals that all of them have some disadvantages such as high cost, incomplete removal of recalcitrant azo dyes because of their stability, recalcitrance and color fastness and generation of significant amount of sludge (Akpan *et al.,* 2000; Forgacs *et al.,* 2004; Zhang *et al.,* 2004).

2.5.2 Physical methods

Physical methods having basic mechanism of coagulation and flocculation are mostly effective for disperse and sulphur dyes but these methods are not efficient for acidic, reactive, direct and vat dyes. These methods have drawbacks of the larger amount of sludge production and low color removal efficiency (Fujita *et al.,* 2000). Adsorption methods have been used at larger scale for a wide range of dyes because of their higher efficiency. Characteristics such as affinity of pollutant towards adsorbing

substance, capacity of target substrate and extent of adsorbent regeneration help in selection of an adsorbent (kaewprasit *et al.*, 1998). Activated carbon is commonly used as an effective adsorbent for different types of dyes but this technique is high in cost (Robinson *et al.*, 2001; Martin *et al.*, 2003). Investigators have used low cost adsorbent materials such as polymeric resins, peat, fly ash, bentonite clay, ion exchangers, and some biological substance like wheat straw and maize stalks for the removal of dyes from waste water (Alzaydien, 2009). But problems faced by these methods such as their disposal and regeneration, less efficiency against wide range of dyes and high volume of sludge production have limited the practical application of these adsorbents .

Different types of filtration processes such as reverse osmosis, nanofiltration and ultrafiltration and have been used commonly (Torres *et al.*, 2010). The use of membranes helps in separation of dyestuffs that result in reduction of color, COD and BOD of water. The porosity of membrane and selection of type of membrane depends upon composition of textile effluent and temperature at which process was done. But these processes have some major drawbacks such as potential membrane fouling, high investment cost and secondary waste metabolites (dos Santos *et al.*, 2007; Robinson *et al.*, 2001).

2.5.3 Biological methods

Bioremediation is the process that involves application of microbes in order to deal with pollution present in environment. Microbes adapt to the toxic waste and in result resistant strains develop naturally. In microbial system mechanism of biodegradation of azo dyes depends upon biotransformation of enzymes (Saratale *et al.*, 2009a). Several enzymes reported associated with the degradation are laccase (Hatvani and Mecs, 2001, Zille et al., 2005), lignin peroxidase (Duran and Esposito, 2000; Revankar *et al.*, 2007), tyrosinase (Zheng et al., 1999), NADH-DCIP reductase (Bhosale *et al.*, 2006), aminopyrine N-demethylase (Salokhe and Govindwar, 2003), hexane oxidase (Saratale *et al.*, 2010). Use of bacteria, and sometimes in combination with specific physiochemical processes has attracted great interest in an eco-efficient manner. Microbial and enzymatic treatments have some advantages, they are eco-friendly, low in cost, produce less amount of sludge, yield less toxic byproducts, and require less water consumption (Banat *et al.*, 1996; Rai *et al.*, 2005). The efficacy of

microbial decolorization depends on the activity of selected microbes and their adaptation to environment. A large number of species has been tested for decolorization of wide range of dyes (Pandey *et al.*, 2007). Isolation of potent specie and then its application in degradation process is very interesting aspect of bioremediation (Chen, 2007; Naeem *et al.*, 2008).

2.5.3.1 Decolorization and degradation of azo dyes by yeast

Very little research has been done to find out decolorization ability of yeast, it has been considered mainly biosorption. Some actinomycetes have been used to carry out enzymetic biodegradation such as *Debaryomyces polymorphus, Candida tropicalis, Candida zeylanoides* (Yang *et al.*, 2009). Similarly *Issatchenkia occidentalis.* and *Saccharomyces cerevisiae MTCC*-463 were found to play a significant role in decolorization in Methyl Red and Malachite green (Jadhav and Govindwar, 2006; Jadhav *et al.*, 2007 Different yeast species perform as dye adsorbing agent and can uptake higher content of dye concentration (Safarıkova *et al.*, 2005) and besides this *Galactomyces geotrichum* MTCC 1360 can decolorize azo, triphenylmethane, many reactive dyes in high concentration (Jadhav *et al.*, 2008). Recently *Trichosporon beigelii* NCIM-3326 has been reported for decolorization of Navy Blue HER with enzymatic mechanism and biodegradation products were assessed for toxicity test (Saratale *et al.*, 2009a; Aksu and Tazer, 2000).

2.5.3.2 Degradation of azo dyes by algae:

Algae or cyanobacteria which are ubiquitous and abundant in nature are recently used in waste water decolorization. Many species of Oscillatoria and Chlorella can degrade azo dyes to their aromatic amines and then to simpler organic compounds (Acuner and Dilek, 2004). Decolorization of dyes by algae to be a series of steps involving biosorption, bioconversion and then biocoagulation. *Chlorella pyrenoidosa, Chlorella vulgaris* and *Oscillateria tenuis* can degrade more than 30 different types of azo dyes (Mohan *et al.*, 2002; Yan and Pan, 2004). Thus the previous outcomes could mean that algae can play a significant role in azo dyes degradation. These biosorption processes can help in practicable way for removal of colorants from waste water being cost effective and efficient manner (Banat *et al.*, 1996; Daeshwar *et al.*, 2007).

2.5.3.3 Decolorization and degradation of azo dyes by plants:

Phytoremediation is a new developing technique that is efficient and cost effective method that helps the bioremediation of soil and waste water contaminated with organic pollutants (Patil *et al.,* 2009). This technique is advantageous because it is an autotrophic system that needs less nutrient from environment, is easier to handle, and is safe in terms of environmental sustainability (Ghodake *et al.,* 2009a; Kagalkar *et al.*, 2009). Mbuligwe, (2005) reported 73-78% decrease in color in fields implanted with coco yam plants. Plants species including *Phaseolus mungo, Sorghum vulgare, and Brassica juncea* have been observed for the decolorization of reactive group of azo dyes. They showed 53 %, 57% and 79 % dye decolorization respectively Ghodake *et al.*, 2009a). Similarly, *Blumea malcommi,* a herb that can degrade Reactive Red 5B at high concentration (Kagalkar *et al.*, 2009). Enzyme system of hairy root cultures of *Tagetes patula L.* was determined to be effective in degradation of Reactive Red 198 (Patil *et al.*, 2009). However, application of phytoremediation at a large scale faces a large number of problems as plants cannot tolerate high level of pollutants and volatile organic compounds are evaporated in atmosphere, in addition to this large area is required to implant the process (Ghodake *et al.*, 2009a).

2.5.3.4 Bacterial decolorization and degradation of azo dyes

Different groups of bacteria can decolorize azo dyes under different conditions such as aerobic, facultative anaerobic and anaerobic conditions. The mechanism behind microbial decolorization involves the breakdown of azo bonds (–N=N–) using azoreductases under anaerobic environment that results in formation of harmful aromatic amines (Van der Zee and Villaverde, 2005). These intermediary compounds are degraded further aerobically or anaerobically (Joshi *et al.,* 2008).

Several studies focus on the application of microbial biocatalyst for dye removal from textile effluent (Chang *et al.,* 2004; Hu, 2001). There has been seen much interest in this technique for higher degree of biodegradation due to its effectiveness towards wide variety of dyes, cost and less amount of sludge production (Rai *et al.*, 2005; Saratale *et al.*, 2009c; Verma and Madamwar, 2003). Different types of synthetic dyes along with other chemicals like salts, acids, bases, and dispersants used in textile industry are discharged into water bodies that causes decrease in oxygen level, which is very lethal to local organisms thus results in increased mortality. Single bacterial

strain cannot degrade azo dyes completely, any the intermediary metabolites are more toxic compounds, which need further treatment (Joshi *et al.*, 2008; Ayed *et al.*, 2010). Treatment system having mixed microbial population perform a higher degree of biodegradation because of synergistic activities of microbial consortium (Chen, 2007; Saratale *et al.*, 2010b). In mixed microbial culture, different strains of bacteria attack the organic compound at different positions producing a variety of intermediate compounds, which other coexisting bacteria can decompose completely (Chang *et al.*, 2004; Forgacs *et al.*, 2004; Jadhav *et al.*, 2008b; Saratale *et al.*, 2009b). Initially reductive cleavage of azo bond occurs, which results in formation of aromatic amines, which are degraded by complementary organisms of microbial consortium reducing the concentration of dye and aromatic amines (Tony *et al.*, 2009). It has been reported that mixed cultures are more effective in this context than individual pure strain (Nigam *et al.*, 1996). Conversely, mixed cultures cannot provide complete insight about what is happening inside, so it is very difficult to interpret the results quite appropriately. Research has been started to separate pure bacterial culture since 1970s. *Bacillus subtilis*, *Aeromonas hydrophila* and in a *Bacillus cereus* are found to be helpful in for this purpose (Wuhrmann *et al.*, 1980).

Table 2.3: Advantages and disadvantages of commonly used physical and chemical treatment of dye removal from textile effluents courtesy (Andrea *et al.*, 2005)

Physical/Chemical methods	Advantages	Disadvantages
Ozonation	Applied in gaseous state	Very short half-life (20 min)
Fenton process	Effective for both soluble and insoluble dyes	Large amount of sludge generation
Activated Carbon	Effective for wide variety of dyes	High cost
Electrochemical destruction	Less hazardous break down compounds	High cost of electricity
Photochemical reactions	No sludge formation	Harmful by products
Peat	Good adsorbent due to cellular structure	Small surface area for absorption
Wood chips	Good for acid dyes	Large retention time
NaOCl	Initiates reduction and cleavage of azo bond	Release of aromatic amines

Disadvantages found in all such processes inspired us to find a technique that should be efficient, cost effective and ecofriendly. Therefore photocatalytic degradation has driven considerable interest in this regard.

2.6 Photocatalytic oxidation

Advanced oxidation processes (AOPs) which include photocatalytic and photochemical and reactions, oxidation mediators such as O_3 and H_2O_2 and heterogeneous photocatalysts are utilized, such as Mn, Fe, TiO_2, and ZnO_2, in the presence of an light source which produces hydroxyl radicals for the degradation of hazardous organic compounds including dyes (Forgacs *et al.,* 2004; Wang *et al.,* 2009; Qiu *et al.*, 2008; Joon *et al.*, 2006). Over the past years, heterogeneous photocatalytic degradation mediated by TiO_2 has received significant consideration, because it can completely degrade dye compounds to harmless molecules (Lucarelli *et al.*, 2000; Lechheb *et al.*, 2002; Stylidi *et al.*, 2003). Advanced oxidation processes, TiO_2 photocatalytic oxidation can be carried out in ambient environment where air can act as the oxidant (Vinodgopal *et al.*, 1996; Konstantinou *et al.*, 2004). Moreover, TiO_2 is found to be highly active non-toxic, inexpensive and stable under a wide range of chemical conditions, which makes it the best photocatalyst to be used for environmental remediation (Aryton *et al.,* 2006; Xu *et al.*, 2010).

2.6.1 Photocatalytic process

Photocatalytic decolorization is one of advanced technology which is considered as substitute for degradation of dyes (Meng *et al.,* 2008). Degradation based on semiconductor photocatalysis has attracted much attention as an alternative method among advanced oxidation processes (AOPs) a favorable technique for treating dye contaminated wastewater at low cost (Comparelli *et al.,* 2005; Zahraa *et al.*, 2006). In this technique semiconducting material act by absorbing light energy greater than or equal to its band gap which in turn generates electrons and holes, which results in generation of free radicals to oxidize substrates. These free radicals can oxidize organic compounds very efficiently. Nanocrystalline particles are widely applied currently as photocatalyst for degradation of organic compounds (Ralph *et al.,* 1992: Seo *et al.,* 2001: Xiaodan *et al.,* 2006). Different nanoparticles are extensively used and their photocayalytic abilities have been explored in many previous studies (Ioannis *et al.*, 2004). Photocatalysis has been proved to be efficient and cost effective

treatment for purification of dye containing waste water (Tafer and Boulkamh, 2008; Kansal *et al.*, 2009; Liang *et al.*, 2008). TiO_2 and ZnO have been studied for degradation of environmental pollutants due to their chemical stability, nontoxicity and high photosensitivity (He *et al.*, 2004; Habibi *et al.*, 2007; Ni *et al.*, 2007; Mahmoodi and Arami, 2008; Jun *et al* ., 2007). Several studies revealed the effects of several factors on the photocatalytic degradation of organic pollutants including textile dyes using TiO_2 mediated photocatalysts and concluded that various parameters, such as initial pH of the dye solution, presence of oxidizing agents and catalyst dosage may effect photocatalytic degradation of many dyes in aqueous solution (Nishio *et al.*, 2006; Akpan *et al.*, 2009; Wang *et al.*, 2004). But conversely research has articles, revealing that the TiO_2 nanoparticles show photocatalytic activity only in presence of UV irradiation rather than natural solar radiation. Therefore, some catalyst which can perform in sunlight should be searched out for safe degradarion of organic pollutants (Moore, 2006).

2.6.2 Mechanism of photocatalytic degradation:

Among all famous advanced oxidation processes, photocatalytic degradation is the most efficient technology for degradation of organic pollutants (Forgacs *et al.*, 2004; Fujishima *et al.*, 2000) because semiconductors and photocatalytic materials are economical and can mineralize a extensive variety of organic compounds (Stylidi *et al.*, 2004). In heterogeneous photocatalysis reactants in liquid phase are transferred on to catalyst surface, these reactants are adsorbed on surface of catalyst, reaction occur in step where adsorption occurs, then desorption takes place and final product is removed from catalyst surface. The photocatalytic degradation of organic compounds takes place according to following steps. When light from any source is exposed on surface of photocatalyst, electrons are moved from valance band to conduction band. This results in formation of electron hole pair (Konstantinou *et al.*, 2004). Photocatalytic degradation of dyes by Ag nanoparticles is similar to reaction mechanism of Ag/AgCl and Ag/TiO_2 plasmonic photocatalyst (Wang *et al.*, 2008). Surface plasmon state of silver nanoparticles is present in visible region of light. At surface of Ag^0NPs absorption of visible light takes place i.e., photons are absorbed which in turn leads to generation of electrons and holes. These electrons reduce the dissolved oxygen and produce superoxygen anionic free radicals O_2^-, $HO_2\cdot$ along with H_2O_2 may be formed in same process (Wang *et al.*, 2010; Hirakawa and Kamat,

(2004). These all entities then react with dye molecules in result of which oxidation of compounds occur.

Catalyst + hν (light) →electron e^- (cb) + hole $\hbar^+$ (vb)

These electrons and holes then transfer to catalyst surface, where they enter in redox reactions. In most cases holes react easily with H_2O and produce OH^- radicals.

$$H_2O + \hbar^+_{vb} \rightarrow OH^- + H^+$$

$$O_2 + e^-_{cb} \rightarrow O_2^-$$

Whereas electrons react with O_2 to produce superoxide anions radicals of oxygen. This reaction inhibits the combination of electrons and holes produced in previous step. The hydroxyl radicals and superoxide radical anions produced in this manner can further react with dye molecules and results in discoloration.

$$O_2^- + H^+ \rightarrow HO_2^{\cdot}$$

$$2HO_2^{\cdot} \rightarrow O_2 + H_2O_2$$

$$H_2O_2 \rightarrow 2^{\cdot}OH$$

$$^{\cdot}OH + \text{dye} \rightarrow \text{dye ox}$$

$$\text{Dye} + e_{cb}^- \rightarrow \text{reductions products}$$

Presence of water molecules and dissolved oxygen make possible the reactions involved in photocatalysis. There will be no formation of highly reactive hydroxyl radicals in absence of water molecules and the degradation of dye molecules would not occur (Wang *et al.*, 2010).

2.7 Important parameters influencing the photocatalytic degradation

2.7.1 Effect of dye concentration

The amount of dye adsorbed on the surface of photocatalyst is the most significant parameter that affects the extent of photocatalytic process. The initial concentration of dye in a given photocatalytic reaction affects the extent of dye adsorption. With increase in quantity of dye concentration percentage degradation of dye decreases (Wang *et al.*, 2008). This occurs because as the concentration of dye increases larger

numbers of dye molecules are adsorbed on the surface of catalyst, which results in reducing the number of photon reaching the surface of catalyst. So less number of hydroxyl radicals are formed that leads to reduction in degradation percentage.

2.7.2 Effect of catalyst amount

The quantity of photocatalyst and accumulation of catalyst particles influence the extent of dye degradation. With increase in catalyst dosage, dye degradation increase, which is distinguishing character of heterogeneous photocatalysis (Rauf *et al.*, 2009). Increasing the catalyst amount actually increase the count of active sites on surface of photocatalyst that results in the greater number of OH^- formation which take part in actual degradation of dyes. Beyond the specific limit of catalyst dosage, turbidity of reaction solution increases, which blocks light penetration thus hindering the reaction to progress. Thus percentage degradation value goes on reducing (Li *et al.*, 2008).

2.7.3 Effect of pH

The primary pH value of solution significantly affects photodegradation efficiency. Significant shift in surface charge and potential of catalyst particles occur by changing the pH of solution. Reaction rate is altered because extent of dye adsorption on to catalyst surface is changed. This alters the adsorption of dye molecules on to surface of catalyst causing a change in reaction rate. Under acidic or alkaline condition surface of catalyst gets pronated or depronated respectively as in following reactions (Konstantinou *et al.*, 2004).

$TiOH + H^+ \rightarrow TiOH_2^+$

$TiOH + OH^- \rightarrow TiO^- + H_2O$

The pH of reaction mixture determines the surface charge of photocatalyst used in process. When the solution pH is at the point of zero charge (isoelectric point) minimum amount of dye is adsorbed at catalyst surface. Above isoelectric point photocatalyst surface is negatively charged and have positive charge below this point. In acidic or alkaline medium, adsorption of dye is dependent on the nature of dye that is required to be degraded.

At low pH reductive cleavage of azo bond occurs by reduction of electrons that plays an important role in degradation of dyes (Baran *et al.*, 2008; Soutsas *et al.*, 2010). Acid Orange 7 which is an anionic dye maximum degradation occurs in acidic pH at pH 3 (Behnajady *et al.*, 2004). Similarly in alkaline medium, hydroxyl radicals are easier to be formed, which increase the decolorization efficiency (Qamar *et al.*, 2005). There are reported similar type of results regarding photocatalytic degradation of diazo and triazo dyes (Zielinska *et al.*, 2001; Guillard *et al.*, 2003).

2.7.4 Effect of irradiation time and light intensity:

Intensity and duration of light affects the extent of dye degradation (Daneshvar *et al.*, 2003; El-Bahy *et al.*, 2009). At low intensity of light (0–20 mW/cm^2), rate of degradation increases linearly as the light intensity increases. When the light of intermediate intensity is used, rate of degradation depends upon square root of light intensity (Herrmann *et al.*, 2010). While using light of high intensity, rate of degradation becomes independent of it. At low light intensity, formation of electron hole pair is predominant and recombination of electron hole pair is negligible. At higher light intensity a competition starts between electron hole combination and generation. It has been proved in literature that dye degradation initially increase with increasing light intensity (Sauer *et al.*, 2002; So *et al.*, 2002). The rate of reaction reduces with increasing irradiation time because this reaction follows pseudo first-order kinetics and competition between the reactants and intermediate products occurs and other reason for reduced rate of reaction is the short life time of photocatalyst whose active sites are covered by strong by products (Li *et al.*, 2008).

2.8 Toxicity assessment of byproducts of biodegradation

Molecular structure of dye is intensely related to mutagenic activity of aromatic amines. International Agency for Research on Cancer (IARC) in 1982 gave a report on suspected azo dyes chiefly amino substituted azo dyes, few sulphonated azo dyes, benzidine azo dyes. Azo dyes are being studied for their chronic and mutagenic effects. Researchers are focused on health hazards caused by colorants used in food and textile industry. In mammals azo compounds are metabolically reduced by bacteria present in anaerobic parts of lower gastrointestinal tract. Donlon *et al.*, (1997) demonstrated that by reducing the azo bond, dyes can be detoxified and reported that IC50 concentration of metabolic byproducts of Mordant Yellow 12 was more than 10

times of its parent compound. Byproducts of biodegradation can be assessed for their toxic or nontoxic level (jadhav *et al.,* 2008). When these end products are disposed of in environment they may prove to be toxic for plants and animals. In intestinal tract, after reduction of azo dyes, produced aromatic amines are adsorbed in intestine and may cause serious problems. These aromatic amines can cause serious health problems such as cancer, mainly bladder cancer. Mechanism of carcinogenesis involves formation of acyloxy amines that are produced by N-hydroxylation and N-acetylation of the aromatic amines followed by O-acylation. These resulting compounds can be transformed to carbonium and nitremium ions that can easily bind to DNA and RNA, which results in mutations and tumor formation (Brown *et al.,* 1993). There has been performed many different types of assays to check toxicity level of compounds produced after biodegradation. Jadhav *et al.,* (2008) have evaluated the phytotoxic level of the treated and untreated samples of biodegradation products of methyl red by *Glactomyces geotricum* MTCC 1360 in the concentration range 50-300 mg/l. test were performed according to the system of ISO (1993) on two kinds of seeds commonly utilized in Indian agriculture i.e., *Sorgum bicolor* and *Triticum aestivum.* Effect of treated and untreated dye metabolites on seed germination and root and shoot elongation was determined after 5 days. (Turker and Camper, 2002) used radish seeds to check the phytotoxicity of plant. A compound may not only hamper plant growth but after some time it phytotoxic due to accumulation of inorganic toxic compounds leading to reduction in soil productivity. Two classes of flowering plants can be used for phytotoxicity testing. These are monocots and dicots. Test and control compounds are analyzed by checking the yield of these plants.

(http://www.environment.gov.au/settelments/publications/waste/degradable/biodegradable/chapter6.html, 2007)

Pourbabaee *et al.,* (2006) used *Triticum aestivum* to check toxicity level of 150 ppm untreated effluent containing azo dye Reactive blue -59 and treated effluent and determined germination inhibition of seeds. For animal testing earthworms were used as representative organism of soil ecosystem and *Daphnia as* model organism of aquatic ecosystem, to check cytotoxic level of metabolites of different compounds. Acute toxicity test was performed by exposing earth worms to different concentration of test material. The cytotoxicity test was European test (OECD Guideline No. 207)

which involves exposing of earthworms to different concentrations of test compounds. The Daphnia cytotoxicity test can help in finding out detrimental effects posed to surface water bodies. Percentage mortality and survival of organism can be calculated after exposing test samples which helps in finding toxicity level of test material. Lieberman, (1999) performed cytotoxicity bioassay using brine shrimp (*Artemia franciscana)* for assessment of toxicity level of chemicals. There are various published whole –animal bioassays for assessment of compound toxicity (Helliwel *et al.,* 1996). Brine shrimp have been used as a bench top bioassay for detection and refinement of bioactive natural products (McLaughlin *et al.,* 1991) and they proved to be excellent select for elementary investigation of consumer products. Toxicity testing was carried out by adding various doses of test samples to small number of shrimps. Generally stated aromatic amines having benzidine moieties, and toluene, aniline, and naphthalene are responsible for genotoxicity. The structure of molecule and the location of the amino-group(s) strongly affect the toxicity level of aromatic amines. 1-naphthylamine is very less toxic as compared to 2-Naphthylamine, which is known to be serious carcinogen (Cartwright, 1983; Alam et al., 2010). Nature and position of other constituents also affect the toxicity of compounds. Substituents such as methyl, nitro, helogen or methoxy groups generally increase toxicity of compounds, while presence of some substituents such as sulphonate group and carboxyl groups reduce toxicity (Chung and Cerniglia, 1992).

Chapter 3

MATERIALS AND METHODS

MATERIALS AND METHODS

This study was carried out on the degradation of two azo dye Acid red 151 and Or II by using commercial grade and biologically synthesized silver nanoparticles Ag^0NPs and fungus *A. niger*. Besides, a synergistic approach was used where B Ag^0NPs along with fungus were used to check the degradation of the dyes. The experimental work was executed at Microbiology Research Laboratory (MRL) in Quaid-i-Azam University (QAU), Islamabad, Pakistan.

3.1 Dyes and chemical reagents

Acid red 151 (Di-azo), Orange II (Mono-azo) and their metabolic byproducts aniline and sulfanilic acid were purchased from Sigma chemicals.

3.2 Study design:

Previously, *A.niger* was screened out for the synthesis of silver nanoparticles (Ag^0NPs) (10-20 nm) under optimized conditions and then those silver nanoparticles were applied in the present study for the degradation of dyes. Dyes were initially treated with fungus. Then, these dyes were treated by biologically synthesized and commercial Ag^0NPs. Experiments were run by taking 50mg/l of dye in 100 ml of STE in 250 ml Erlenmeyer flasks and these flask were then incubated (30°C) in a shaker incubator (150 rpm) at 30 °C both for fungi and Ag^0NPs. Experimental conditions like pH, temperature, conc. of dyes and nanoparticles dosage were optimized during the study in order to achieve maximum degradation of dyes. Samples of treated dyes solutions were taken after every 24 hrs then centrifuged (10000 rpm) and filtered through pvdf filters (0.45 μm) for removal of solid particles. Treatment efficiencies of different setup were evaluated by using Ultraviolet-Visible (UV-Vis) spectroscopy, Fourier transform infrared spectroscopy (FTIR) and High performance liquid chromatography (HPLC) respectively.

3.3 Maintenance of fungal cultures:

Pure culture of *A.niger* was taken from Microbiology Research Laboratory (MRL), Quaid-i-Azam University, Islamabad. This culture was refreshed on monthly basis in Sabouraud dextrose broth medium in order to use, while, they were stored in slant position on the same solid at 4°C.

3.4 Preparation of fungal inoculum

Pure culture of *A.niger* was grown for 7 days in Sabouraud Dextrose Broth in shaker incubator (100 rpm) at 30°C and. Biomass produced after 7 days was filtered and then washed three times with distilled water. The fungal pallets (5 g 100 ml^{-1}) were homogenized with distilled water in a mixer for 5 minutes. This homogenized fungal biomass was later used as inoculum in experiments.

3.5 Componentsof simulated textile effluent (STE)

STE was prepared by adding per liter of distilled water; $(NH_2)_2CO$ 108.0 mg, $NaHCO_3$ 840.0mg, KH_2PO_4 67.0 mg, $MgSO_4.7H_2O$ 38.0 mg, $FeCl_3.6H_2O$ 7.0 mg, $CaCl_2$ 21.0 mg, glucose 6 g, Acetic Acid (99.9%) 0.15 ml (Luangdilok and Panswad, 2000).

3.6a Biodegradation studies of dyes with *A. niger*:

Decolorization of both Azo dyes (AR 151 and Or II) was carried out by *A. niger* in a shaker (150 rpm) incubator (30°C) at neutral pH for 7 days except in experiments performed for optimization of each parameter. Specific concentration of dye (20ppm for Or II and 50 ppm for AR 151) was dissolved in 100 ml of STE and it was taken in 250 ml of conical flask. Then 0.001% (w/v) fresh inoculum of homogenized fungal biomass was added in each flask. Initially pH and temperature was optimized for degradation of 50mg/l of dye. For optimization studies, pH range of 3-9 and temperature range of 25-37 °C was applied (Ali *et al.*, 2007) Further, different concentrations (10, 50, 100, 150, 200 mg/l) of both dyes were used to evaluate the maximum decolorization capabilities of fungi in comparison with silver nanoparticles.

3.6b Degradation studies of dyes with Ag^0 nanoparticles

An amount of 100mg $l^{-1}$of silver nanoparticles Ag^0NPs were separately added to 100 ml of 50 mg/l of AR151 and 20 mg/l of Or II (AR151 or Or II) solutions/STE set at an initial pH 7. Then the solutions were sonicated in dark in sonicator for 30 min to maintain adsorption equilibrium between dye solution and photocatalyst. Then the solutions were exposed to tungsten lamp of 60 watt mounted 15 cm away from it 1 hr Solutions were continuously stirred during irradiation. Different parameters such as pH, temp, initial concentration of dye and catalyst dosage were optimized using same experimental set up.

3.6c Degradation studies of dyes using both biologically synthesized Ag^0 nanoparticles and fungus *A. niger* in combination:

Batch shake flask experiments were carried out for the degradation two dyes using fungal strains *A. niger* with in combination with biologically synthesized Ag nanoparticles. 100ml of STE (pH 5) containing any of two dyes (50 mg/l) was taken in 250 ml Erlenmeyer flask. Biologically synthesized silver nanoparticles(100 mg/l) were added to above solution. Then, this solution was sonicated in dark for 30 m. This solution was inoculated with 0.001% W/V fungus*A. niger*. These experimental flasks were placed in shaker (150 rpm) incubator (30°C). Samples were drawn after every 24 hours in 2.5 ml eppendorff tubes up to incubation time of 240 hrs .Experiments were run in triplicate. Samples were centrifuged at 10000 rpm for 15, supernatants was filtered through pvdf filters (pore size 0.45µm) and then analyzed spectrophotometrically using UV Visible spectrophotometer at 486 nm for Or II and 512 nm for AR151. The decolorization efficiency (%) was expressed by the following equation;

Decolorization (%) = [(Initial Absorbance – Final Absorbance) / Initial Absorbance] × 100.

3.7 Characterization studies

To characterize degradation, following techniques were used:

i-Visual observations

ii-Ultraviolet-Visible (UV- Vis) Spectrophotometer

iii-Fourier transform infra-red spectroscopy(FTIR)

iv- High performance liquid chromatography(HPLC)

3.7a Visual observations of degradation of dyes

There was observed a gradual change in color of dye containing STE in each experiment during incubation. For AR 151, the color of untreated dye sample was dark red which changed to light red, pink, and greyish white during treatment. In case of Or II, color of STE changed from bright yellow to light yellow, and then greyish white after 48 hrs of incubation. No change in color was observed in untreated dyes (without Ag^0 nanoparticles or *A. niger)* containing STE.

3.7b Ultraviolet-Visible (UV-Vis) Spectrophotometric analysis ofsamples:

Samples were initial centrifuged and filtered as describe in the previous section then analyzedspectrophotometrically using Shimadzu UV Visible spectrophotometer at 486 nm for OrIIand 512nm for AR151.

The working range in this system was 190-1000 nm but in current study wavelength range of 200-800 nm was used for scanning of samples. In this spectrophotometer, "UV-Visible ChemStation Software" is attached for recording and analyzing data. STE was used as blank to correct base line of spectrophotometer. Absorption spectra of all treated samples were recorded then the mathematical data were plotted in Microsoft Excel sheet.

Standard curves of AR 151 and Or II

To prepare standard curve of dyes, stock solution 100mgl^{-1} (100 ppm) were prepared in STE. Then from this stock solution different concentrations viz10, 20, 30, 40, 50of each dye were prepared using formula $C1V_1=C_2V_2$.Optical density of different concentrations of AR 151and Or II was recorded at 512 nm and 486 nm respectively. The OD of samples of each dye was plotted against their respective concentrations. In treated dye sample residual concentration of dye was calculated by the following formula using Microsoft excel.

X=(Y-0.1459)/0.0213

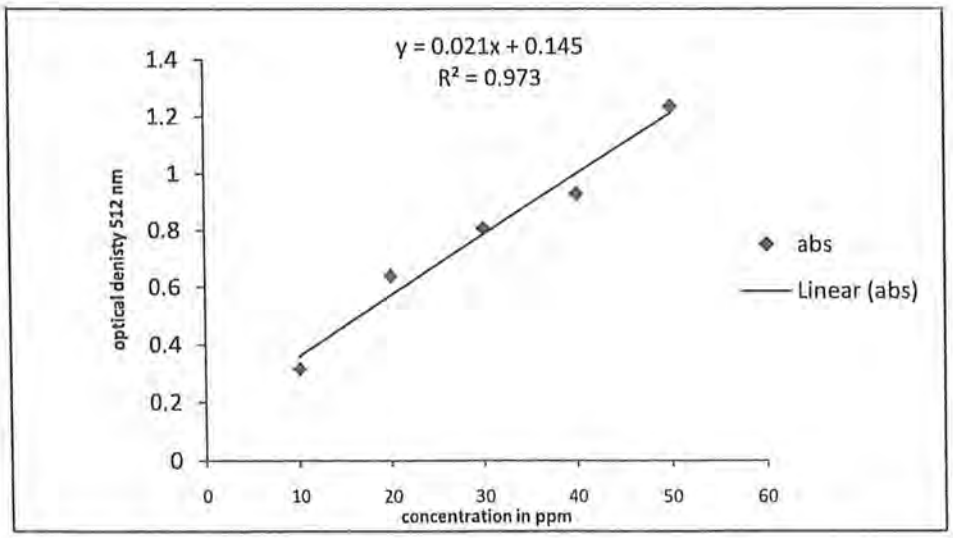


Fig. 3.2: Standard curve of AR151 for estimation of reduced dye

Y is the optical density of treated samples at 512 nm and X is the concentration of dye in treated dye samples.

X=Y-0.1338/0.0495

Y is the optical density of anonymous samples at 486 nm and X is the concentration of dye in treated dye samples.

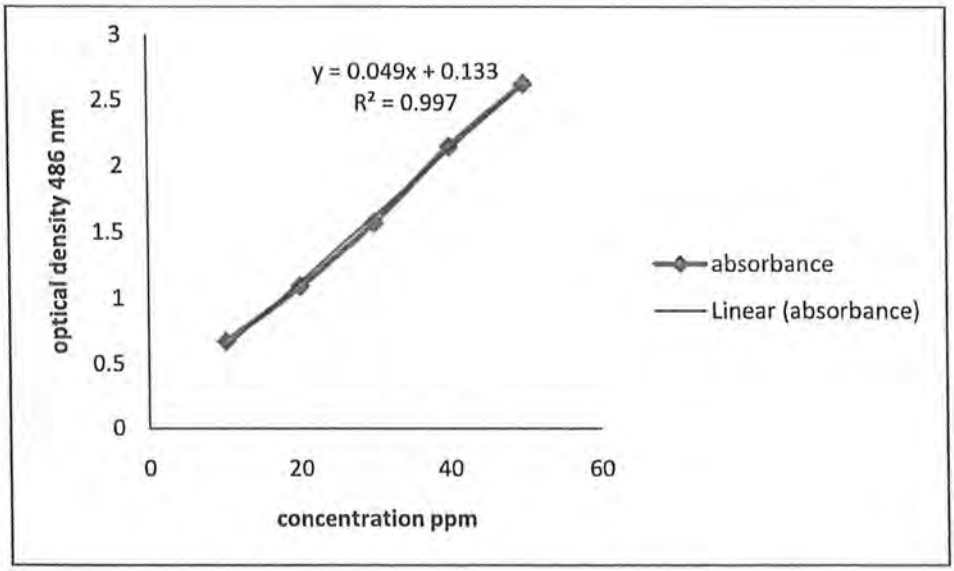


Fig. 3.2:standard curve of Or IIfor reduced dye estimation

3.7c Fourier transform infrared spectroscopy

To analyze degradation of parent compound and chemical changes occurring in treated samples, FTIR was performed by Perkin Elmer Spectrum 65 FTIR spectrometer equipped with ATR. It works on principle of detection of all types of changes happening in samples after treatment. On FTIR sample plate untreated and treated samples were placed and fixed. A spectrum in the range of 600-4000 wave number/cm was taken for each sample. To observe changes in different peaks, an overlay was formed which showed new peaks and significant changes occurring in treated samples as compared to control.

3.7d High performance liquid chromatography

Treated and untreated dye samples were also analyzed by Agilent 1100 HPLC with C18 column at a flow rate of 1 ml/min. Mobile phase used during the process was acetonitrile and H2O (30:70). This system was equipped with *Breeze* software Waters 717 Plus Auto sampler, Waters 1525 Binary HPLC Pump, Waters 2487 Dual λ absorbance detector, and Waters 2414 Refractive Index detector. The λ max of dye used during analysis was 512 and 225 for AR 151. Injection volume of 10 µl was used for standards while 20 µl of injection volume was used for treated samples. HPLC analysis was done in order to detect by products after different treatments of dye. Two types of by products were expected Sulfanilic acid and aniline. These two pure compounds were used as standards for their detection in treated samples.

3.8 Cytotoxicity Assay

Cytotoxicity of the compound was detected by Brine Shrimp Assay (Maridass, 2008). Procedure for assay is as follow.

a. Sample Preparation

Samples taken from experimental flask were filtered to remove any particulate matter. Control and treated dyes samples were analyzed for their toxicity.

b. Synthesis of Artificial Seawater

Preparation of artificial sea water was done by adding 34 g of commercial sea salt (Harvest Co. H. K.) in 1liter of distilled water with constant agitation. Aeration was done for two hours by vigorous stirring on magnetic stirrer

c. Hatching Shrimps

Eggs (Sera, Heidelberg, Germany) of brine shrimp (*Artemia salina*) were hatched in prepared sea water poured in narrow rectangular dish (22 x 32 cm). This dish was divided into two unequal portions by a plastic wall of 2 mm having many holes. The eggs were scattered in larger portion of dish that was wrapped by aluminum foil to protect it from light, while smaller portion was open to light. Egg started hatching within 24 hrs. After that Pasteur pipette was used to collect phototropic brine shrimp larvae named nauplii.

d. ***Assay Procedure***

An aliquotof 0.5 ml of treatedand untreated dye samples after 240 hrs interval was taken in vials and then methanol was evaporated with a vacuum. Residue was mixed in 2 ml of artificially prepared sea water. With help of Pasteur pipette ten shrimps were transferred to a separate vial then volume of vial was made up to 5ml with sea water. Each sample was tested in triplicate manner. The vials were kept at room temperature i.e., 25 °C. Number of surviving shrimp was counted by stem of Pasteur tube with help of 3x magnifying glass after 24 and 48 hours intervals.

3.9 Phytotoxicity assay

Turker and Camper (2002) reported the radish seeds (*Raphanus sativa*) bioassay to assess the toxicity of all treated samples. Samples were subjected to toxicity test to observe seed germination, root and shoot length of reddish seedlings in the presence of untreated and treated dye samples.

a. ***Sample preparation***

Samples were picked from untreated and treated flask and were filtered to remove any suspended particles. Samples of different treatments were used for the assessment of toxicity.

b. ***Sterilization of radish seeds***

Surface sterilization of seeds was done by dipping the seeds into solution of 0.1% mercuric chloride for 5 minutes. Then radish seeds were rinsed with sterilized distilled H_2O three to four times and dried over sterilized filter paper.

c. ***Procedure***

An aliquot of 5ml of each treated sample and untreated dye were separately added to sterilized 10cm petriplates with sterilized filter paper (Whatman No. 1). Sterilized distilled H_20 was also poured into sterilized petriplates containing filter plates in equal volume in blank. Each plate was prepared in triplicate. Twenty sterilized seeds were placed over filter paper in each plate containing sample. Plates were then covered with lid and placed in dim light at 25°C. Number of seed germination, root and shoot length was measured at 3^{rd} and 5^{th} day of experiment.

Chapter 4

RESULTS

RESULTS

In the study biodegradation of two azo dyes (AR 151 & Or II) was done by *A. niger,* biologically synthesized and commercially available silver nanoparticles Ag^0NPs in separate and combined treatment and different parameters affecting the reaction were optimized for better control on degradation rate. Afterwards best optimized conditions were applied. The decolorization of AR 151 and Or II was regularly observed by visual observations, UV-Visible spectroscopy, FTIR and HPLC. Besides this preliminary study, phytotoxicity and cytotoxicity assays of biodegradation byproducts were also carried out.

4.1. Biodegradation of Acid red 151

4.1.1. Visual observation of degradation of AR 151:

A significant change in color was observed from dark red to grayish white in 120 hours of incubation time at pH 5 in shaker incubator (150 rpm) at 30^0 C (Fig. 4.1). Concentration of dye used in medium was 50 mg/l against 100 mg/l of Ag^0NPs that acted as photocatalyst. Gradual change in color of dye solution was observed during period of incubation. No change in color was observed in untreated dye sample (control) although same experimental conditions were applied.

Fig. 4.1: Change in color of AR 151 (50 ppm) by *A. niger, A. niger* + B Ag^0NP (100 ppm), B Ag^0NPS (100 ppm), C Ag^0NPS (100 ppm).

4.1.2. UV-Vis spectral analysis of AR 151

Optical density of the treated samples was taken at 512 nm after centrifugation at 10,000 rpm and filtration through pvdf filters. It decreased in all cases like treatement by *A. niger,* laboratory synthesized and commercial silver nanoparticles Ag^0NPs. But maximum and immediate decrease in optical density was seen in case of treatment of AR 151 by silver nanoparticles in combination with fungus in combined treatment. Reaction condition applied were constant in all experimental flasks i.e., 30 °C, pH 5, 50 mg/l dye and 100 mg/l B Ag^0NPs and C Ag^0NPs.

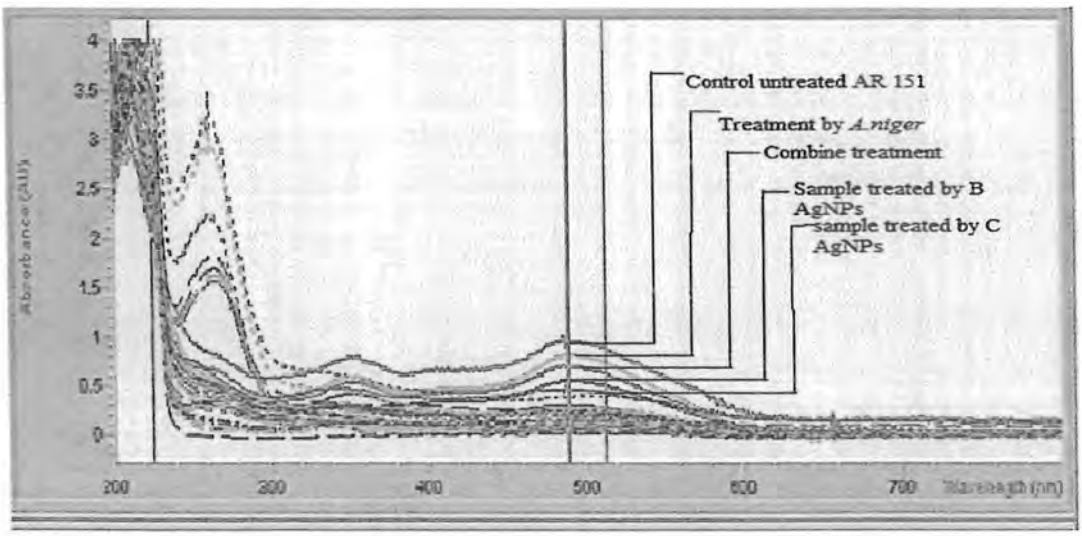


Fig. 4.2: UV-Vis absorption spectrum of AR 151 in presence of *A. niger*, *A. niger* + B Ag^0 NPs, B Ag^0 NPs and C Ag^0 NPs.

4.1.3. Optimization of perameters

4.1.3.1. Effect of temperature

Different temperatures applied for the decolorization of dye AR 151 by fungus and silver nanoparticles included 25, 30 and 35 °C. The decolorization of the dye AR 151 (50 mg/l) was observed initially at neutral pH i.e., 6 in shaker incubator for 7 days. Inverse effect of temperature rise (25 to 37 °C) was observed on degradation of dye by fungus *A.niger*. In first 24 hr color removal of dye by fungus was 70 % at 30 ^{0}C while it was 47 and 41 % at 25 and 37 °C % (Fig 4.3a). A considerable improvement in degradation was observed, when combined treatment was applied, decolorization of AR 151 for both 25 ^{0}C and 30 °C in first 24 hr. was 76 and 82 % (Fig 4.3b). For photocatalytic degradation by biologically synthesized and commercially available silver nanoparticles, maximum and rapid decolorization was observed at 30 °C in first 24 h. In first 24 hr 96 % degradation was observed at 30 °C while it was 81 % and 90 % for 25 °C and 37 ^{0}C respectively as shown in Fig 4.3c & Fig 4.3d. It was observed that 30^0 C is the most effective temperature for biologically synthesized B Ag^{0}NPs and commercial C Ag^{0}NPs which can degrade more than 80 % of dye within 24 hr.

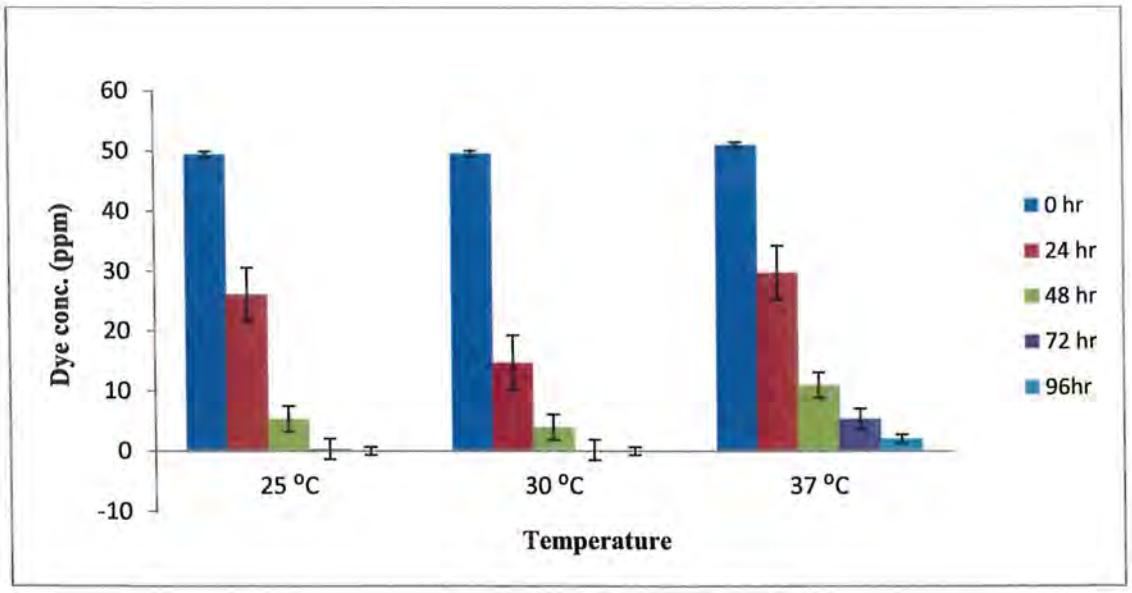


Fig. 4.3a: Effect of temperature on degradation of AR151 by *A. niger*.

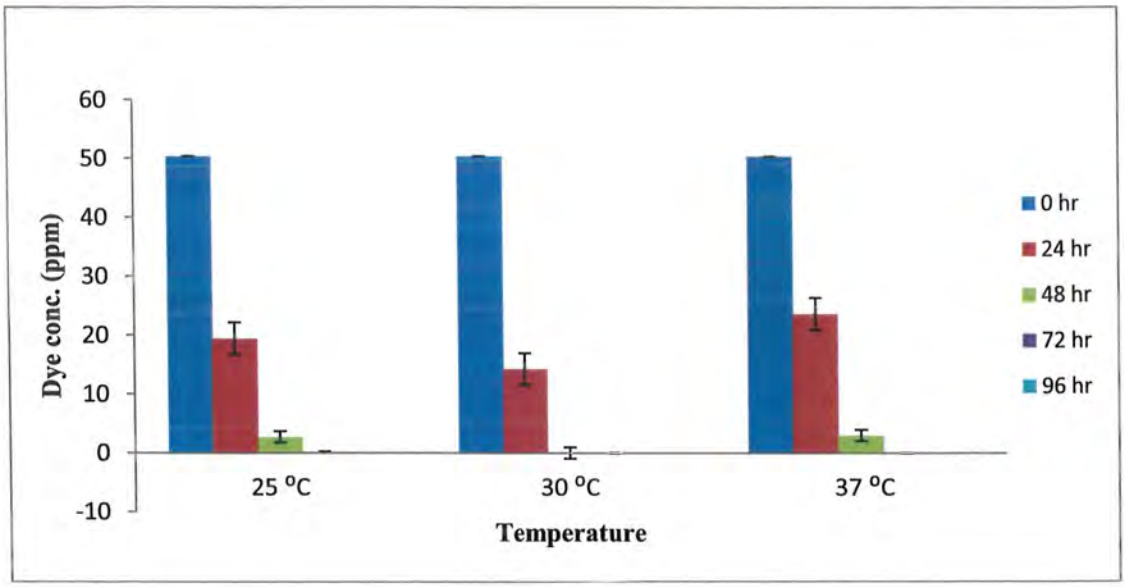


Fig. 4.3b: Effect of temperature on degradation of AR 151 by *A. niger* + B Ag^0Nps.

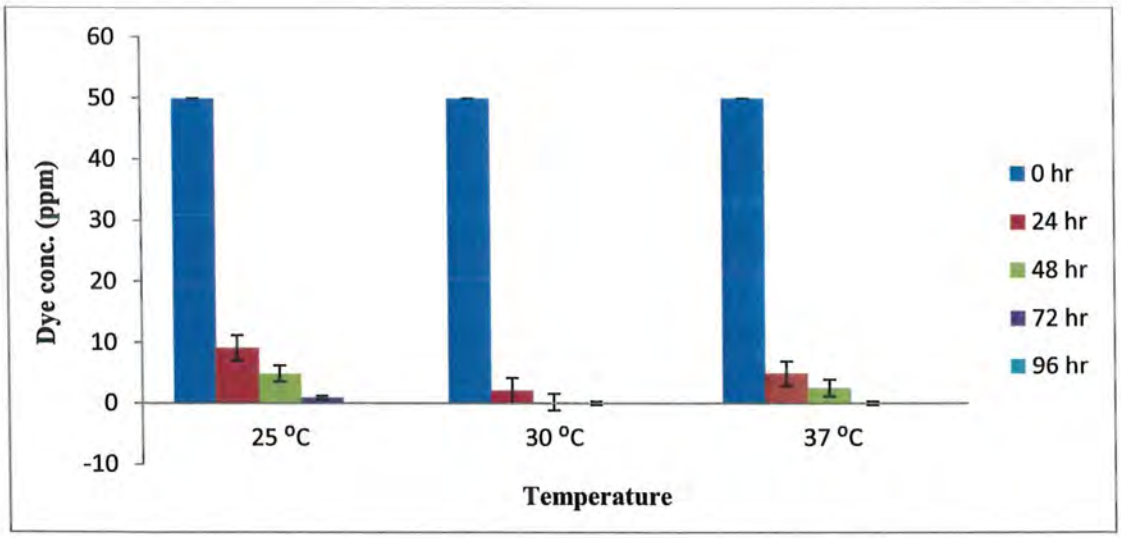


Fig. 4.3c: Effect of temperature on degradation of AR 151 by B Ag^0Nps (100 mgL^{-1}).

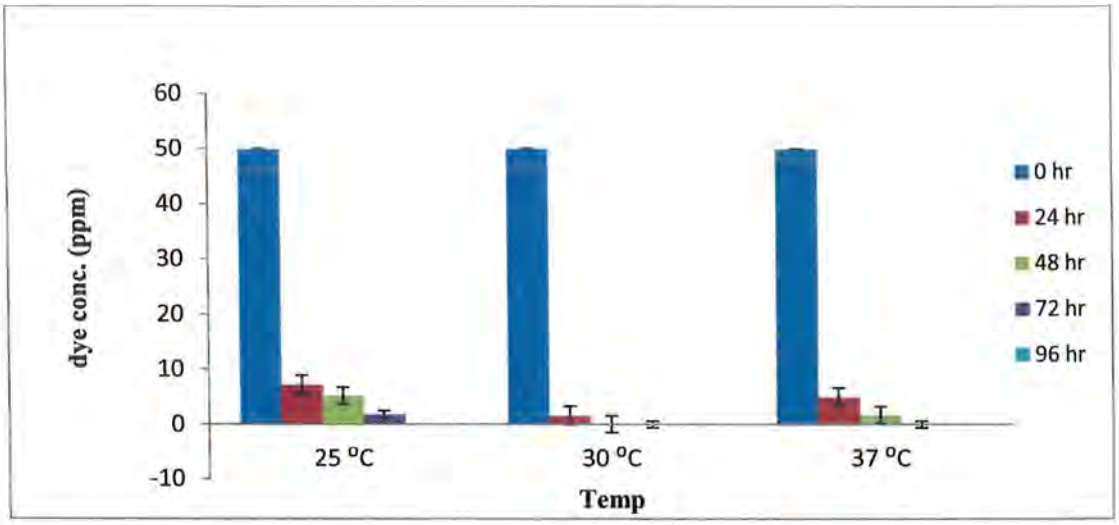


Fig. 4.3d: Effect of temperature on degradation of AR 151 by C Ag^0Nps (100 mgL^{-1}).

4.1.3.2. Effect of pH

Effect of pH on degradation of AR 151 by *A. niger* and silver nanoparticles was studied. Experiments were run at pH 3, 5, 7 and 9 to find optimum value for its degradation. The experiments were performed for 7 days in a shaker incubator (150 rpm) at 30 °C. Concentration of dye (50 mg/l) and Ag^0 nanoparticles ($100mgL^{-1}$) were kept constant in all experiments of pH optimization. After 24 hrs, % degradation was 26, 42, 71, and 40 % at pH 3 5, 7 and 9 respectively. After 96 hrs it was maximum i.e. 99 % at pH 5 while it was 81 %, 96 %, and 79 % at pH 3, 7 and 9 respectively. It can be seen that % degradation was very low at highly acidic and basic medium (Fig 4.4a). There was observed considerable improvement in percentage degradation in combined treatment at pH 3, 5, 7, and 9 and was found to be 90, 73, 49 and 85 % because of presence of Ag^0 nanoparticles which can help in degradation of organic compounds at any pH (Fig 4.4b).

Decolorization of AR 151 by biologically synthesized and commercial silver nanoparticles was more than 85 % at all pH conditions in 24 hrs. It was maximum i.e., 98 and 99 % at pH 3 and 9. While at pH 5 and 7 it was 91 and 89 %. Similar results were observed with commercial Ag^0 nanoparticles (Fig 4.4c & 4.4d). No significant

difference in % degradation was observed at different pH levels, as it was more than 85 % at all pH studied. So, B Ag^0NPs can be applied at any pH between rang of 3-9.

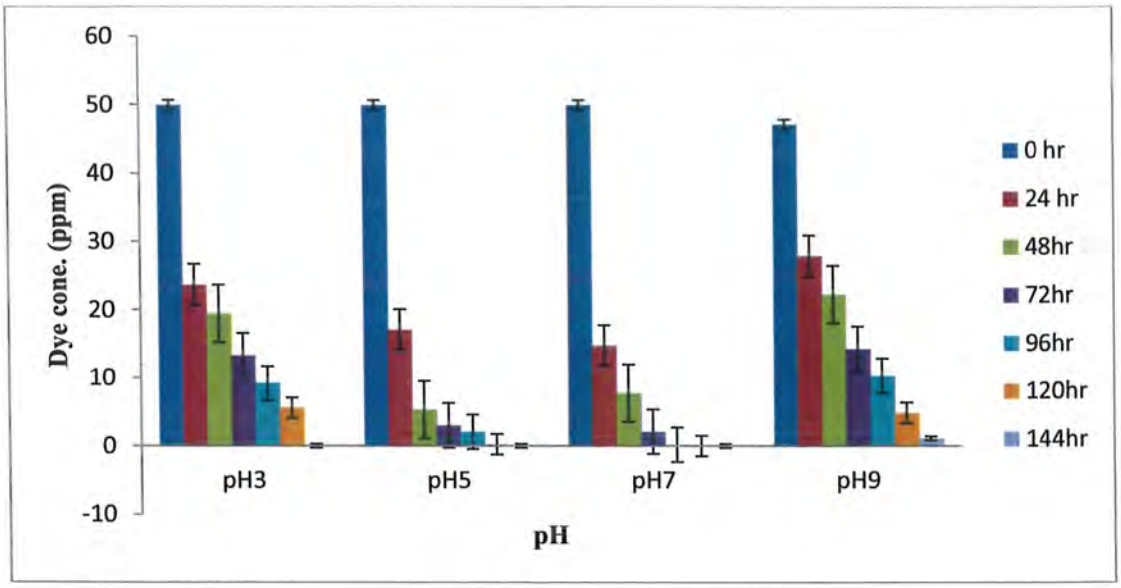


Fig. 4.4a: Effect of pH on degradation of AR 151 by *A. niger*.

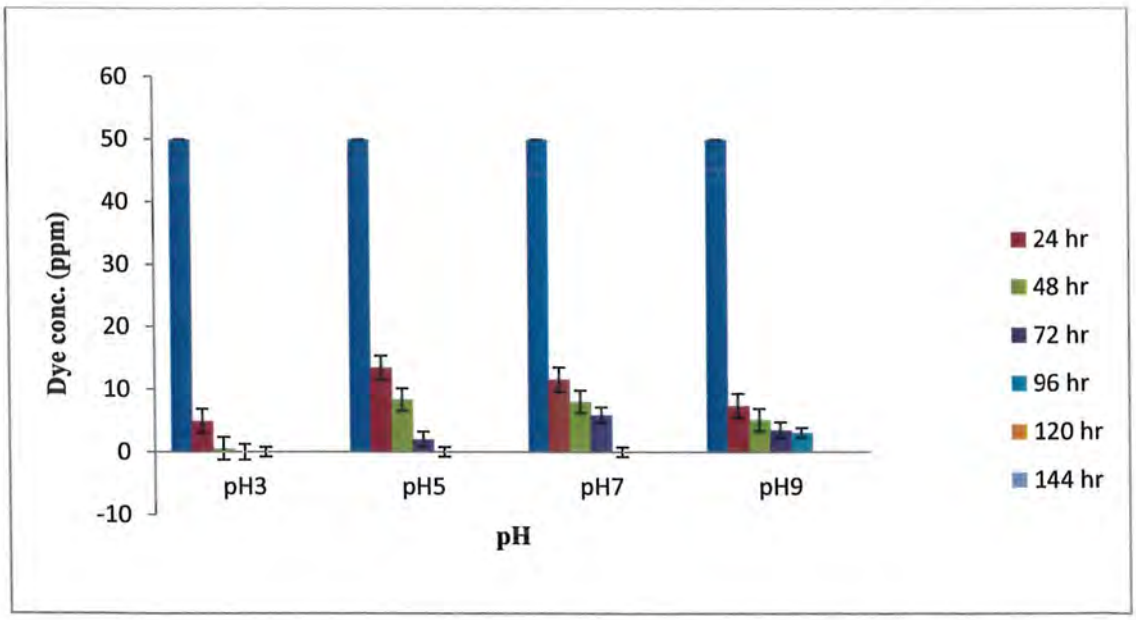


Fig. 4.4b: Effect of pH on degradation of AR 151 by *A. niger* + B Ag^0NPs.

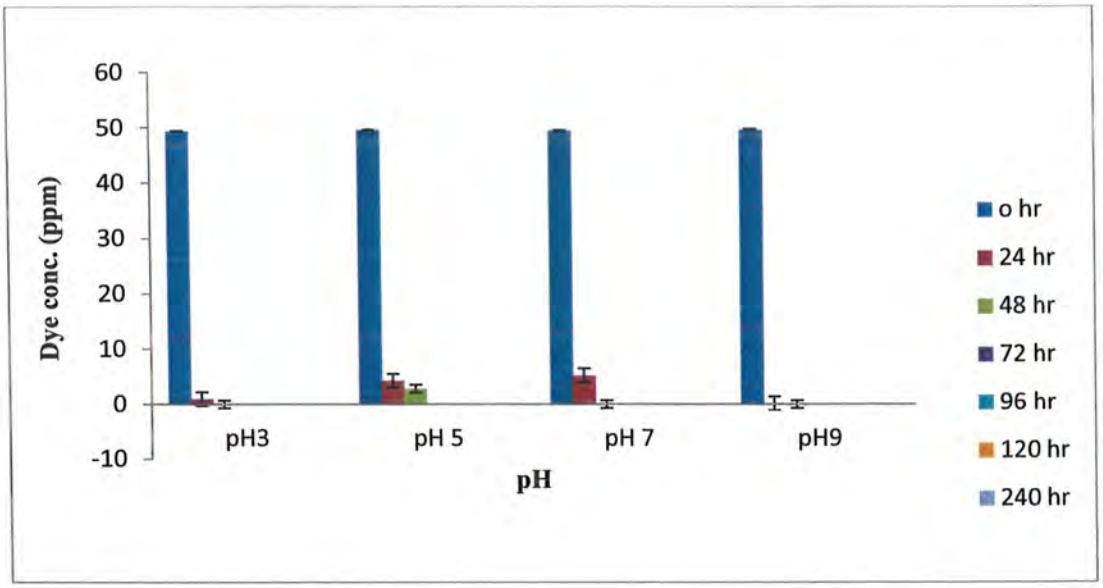


Fig. 4.4c: Effect of pH on degradation of AR 151 by B Ag^0Nps (100 mgL^{-1}).

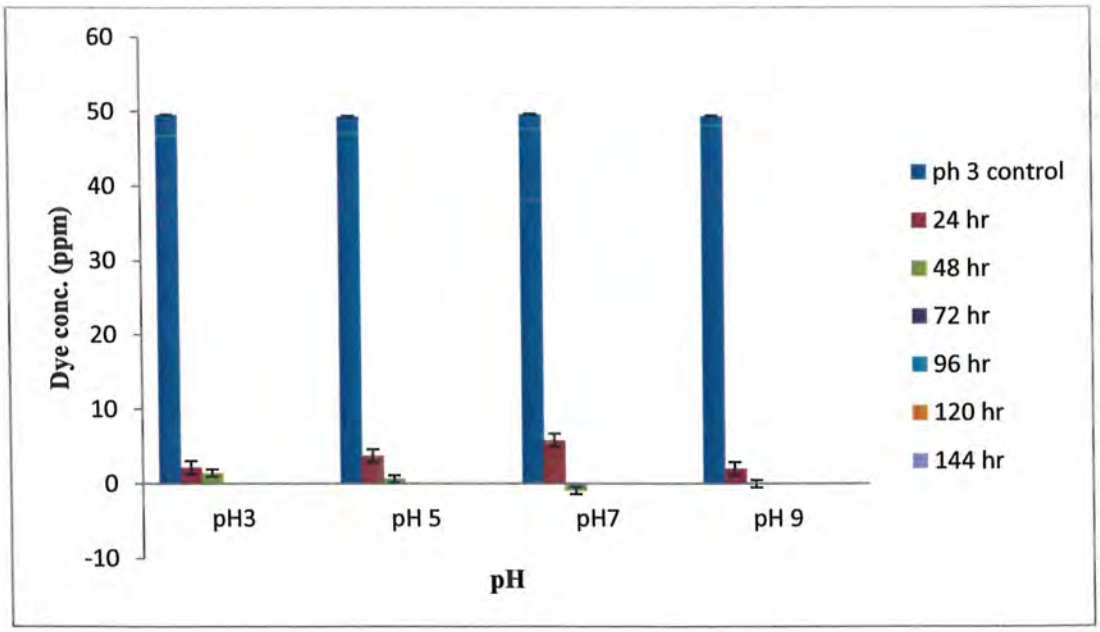


Fig. 4.4d: Effect of pH on degradation of AR 151 by C Ag^0Nps (100 mgL^{-1}).

4.1.3.3. Effect of varying dye concentration on % degradation of dye:

Experiments were performed to find out the effect of initial concentration of dye on % decolorization by using constant amount of catalyst i.e., Ag^0NPs (100 mgL^{-1}) and with constant quantity *A. niger* (0.001%) w/v. It is obvious from Fig (4.5a, b, c and d) that degradation efficiency of the dye slightly increased initially when dye concentration was increased from 10 to 40 ppm. However on further increasing dye concentration, % decolorization reduced to 40 % at 100 mg/l.

In first 24 h removal of dye by fungus was 97, 94, 88, 68, 58, and 31 % at 10, 20, 30, 40, 50 and 100 mgL^{-1} of AR 151 concentration (Fig. 4.5a). The color removal was considerably improved i.e., 100, 98, 91, 79, 68, and 54 % at 10, 20, 30, 40, 50, and 100mgL^{-1} of initial dye concentration in first 24 hrs, when Ag^0 nanoparticle in combination with fungus were used (Fig. 4.5b). Comparatively degradation was fond to be enhanced at higher concentration with commercially synthesized nanoparticles compared to biological synthesize particles. It was 98, 95, 94, 79, 58, 32 % with B Ag^0NPs (Fig. 4.5c) and 99, 96, 94, 81, 62, and 49 % at 10, 20, 30, 40, 50, and 100 mgL^{-1} in first 24 hrs with C Ag^0NPs (Fig 4.5d).

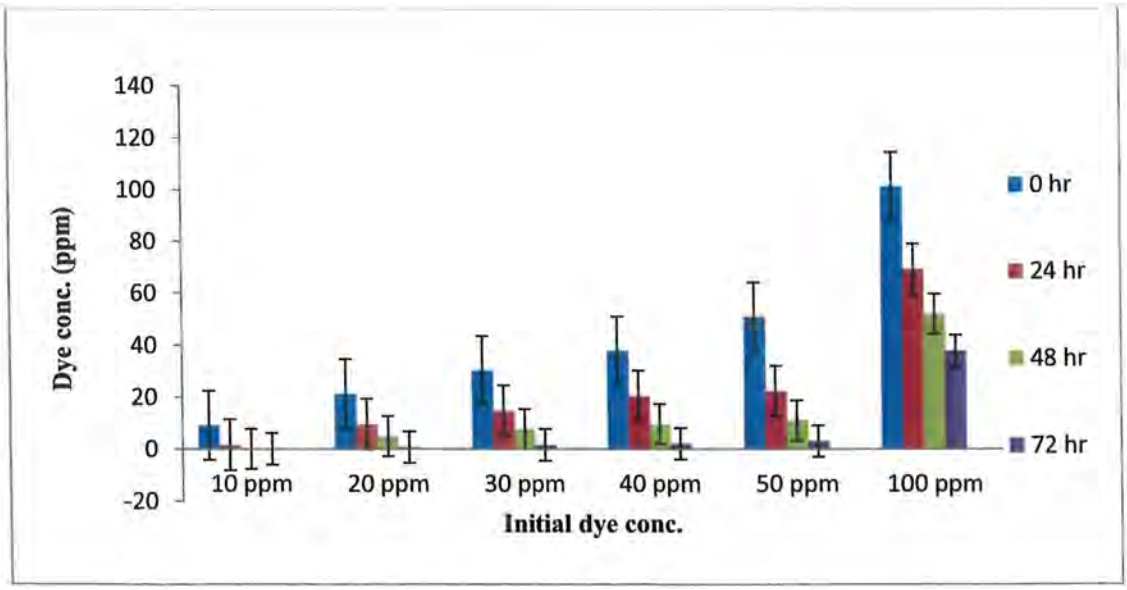


Fig. 4.5a: Effect of varying concentration of dye on degradation by *A. niger*

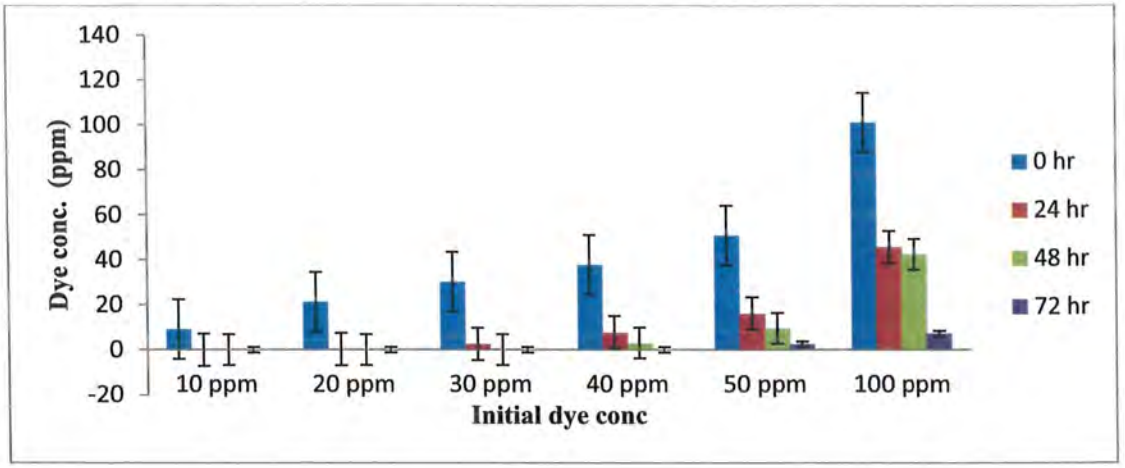


Fig. 4.5b: Effect of combined (*A. niger* + B Ag^0 NP (100 mg/l) treatment on varying concentration of dye.

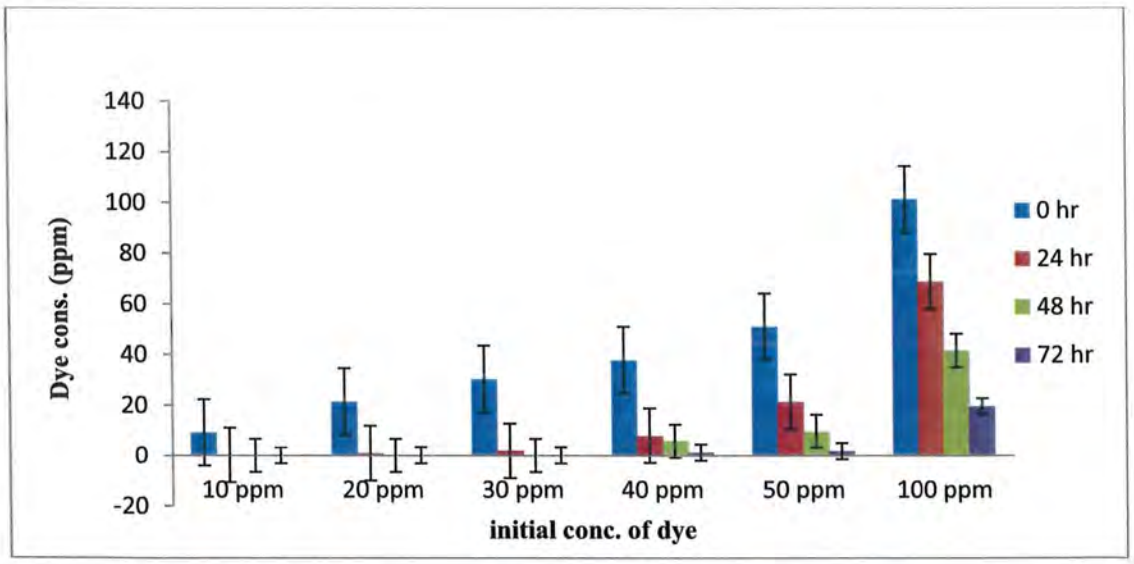


Fig. 4.5c: Effect of varying concentration of dye AR 151 on degradation by B Ag^0Nps (100 mg/l).

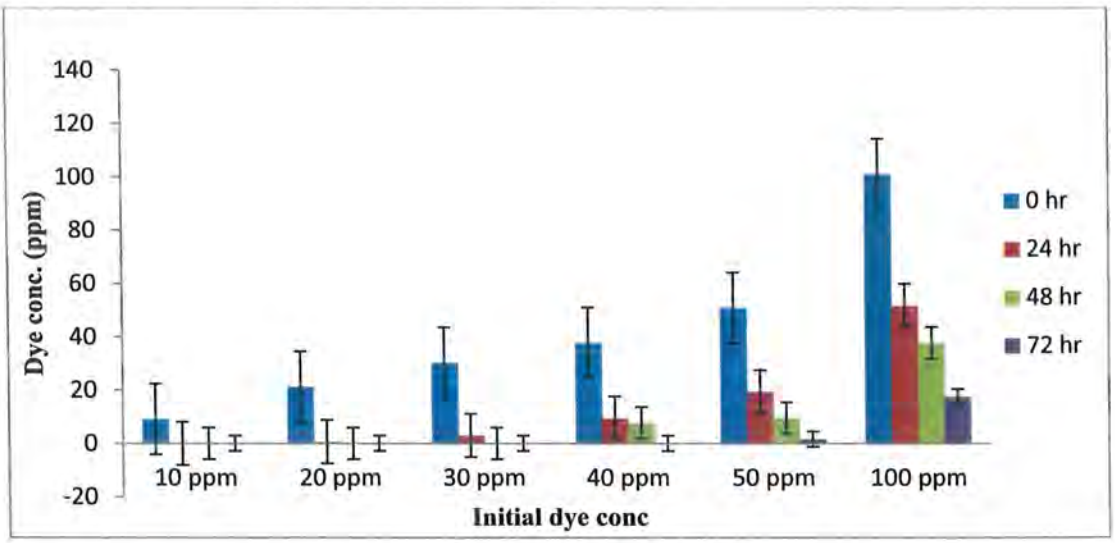


Fig. 4.5d: Effect of varying concentration of dye AR 151 on degradation by C Ag^0Nps (100 ppm).

4.1.3.4. Effect of different concentrations of biological and commercial silver nanoparticles and comparison with degradation by *A.niger*

The effect of quantity of photocatalyst (B Ag^0NPs, C Ag^0NPs) on degradation of AR 151 was studied at fixed concentration of dye i.e., (50 mgL^{-1}). It was observed that % degradation value increases with increase in concentration of photocatalyst up to 200mgL^{-1}. After 24 hr interval *A. niger* showed 52 % decolorization of dye at 50 mg/l (Fig. 4.6a). A significant improvement in % degradation was observed when combined treatment was applied in which dye solution was first sonicated with photocatalyst i.e., B Ag^0NPs at varying conc. i.e., 10, 50, 100, 150, 200 mg/l) for 30 min and then solution was inoculated with 0.001 % W/V *A. niger* degradation efficiency was 57, 73, 77, 83 and 91 % in 24 hrs interval (Fig. 4.6b). When dye was treated with B Ag^0 NPs separately at concentration of 10, 50, 100, 150, and 200 mgL^{-1} % color removal was found to be 52, 59, 75, 80 and 88 % and C Ag^0Nps showed similar % degradation of dye with similar conditions (Fig. 4.6c & 4.6d).

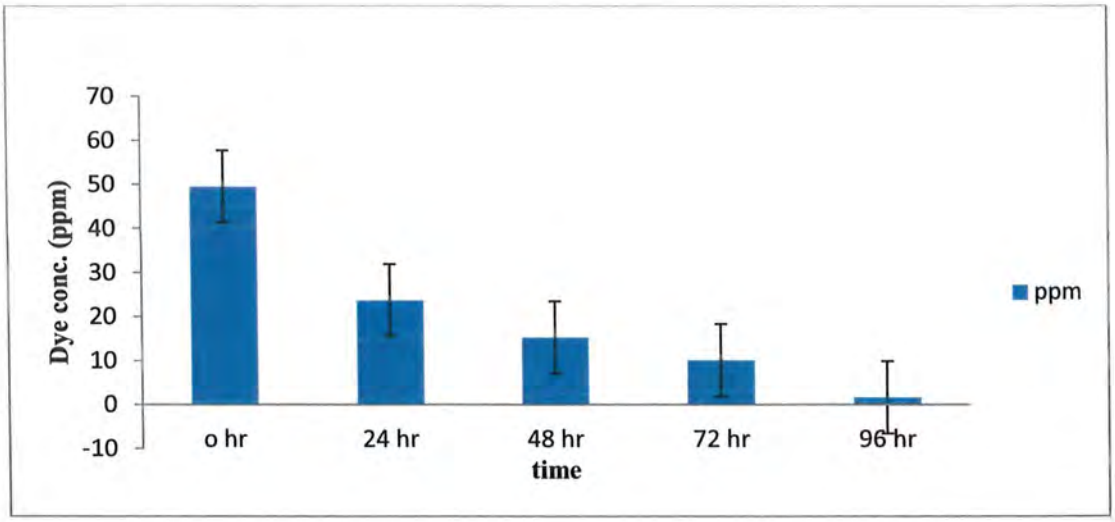


Fig. 4.6a: Degradation of 50 ppm AR 151 by *A. niger*.

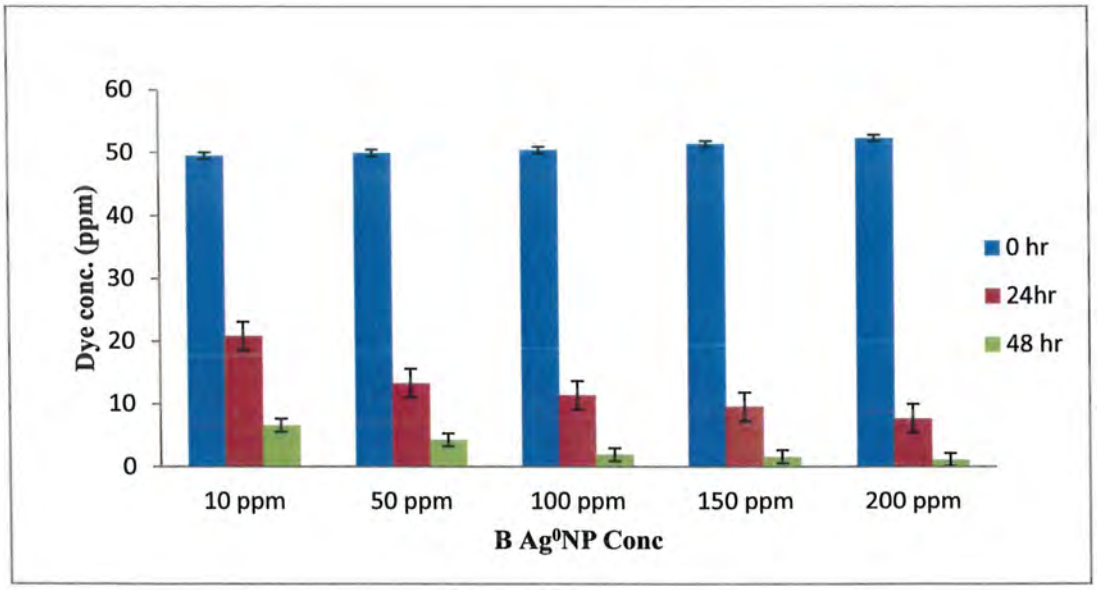


Fig. 4.6b: Degradation of AR 151 By fungal strain *A. niger* + B Ag^0Nps (100 mgL^{-1}).

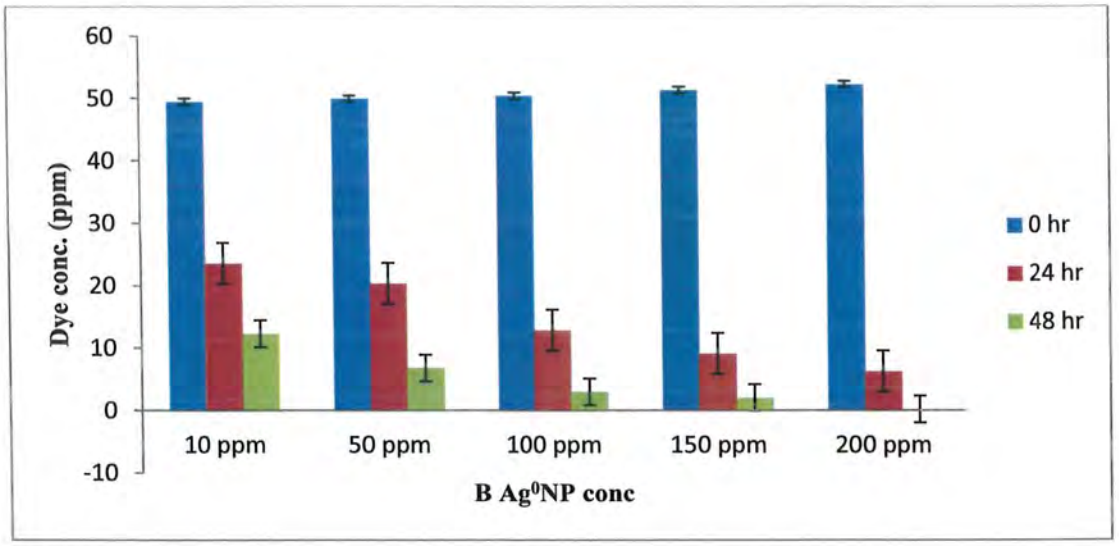


Fig. 4.6c: Effect of different concentration of B Ag^0Nps on 50 mgL^{-1} AR 151 dye degradation.

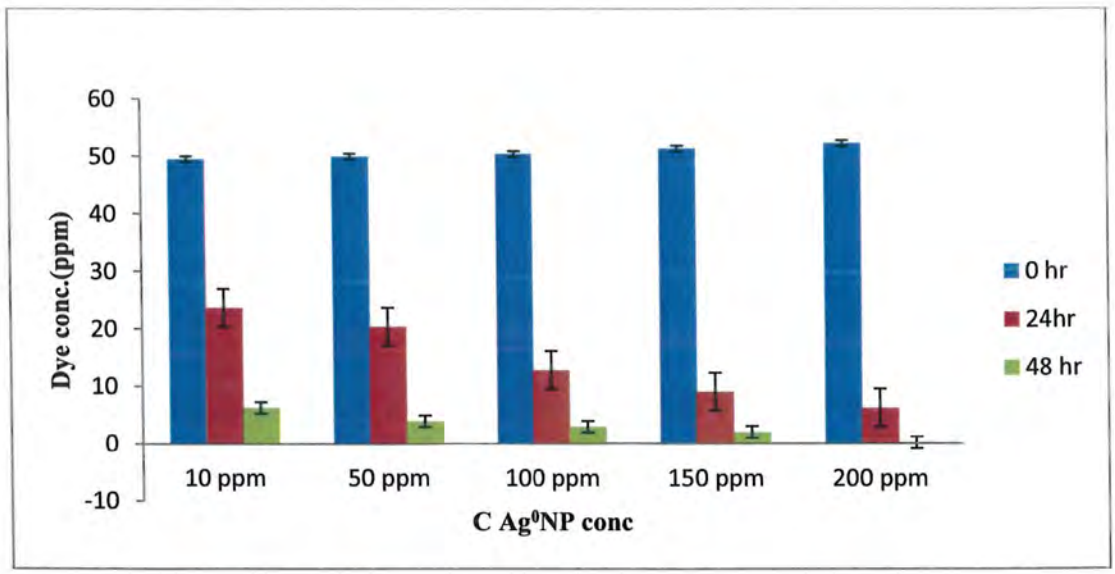


Fig 4.6d: Effect of different concentration of C Ag^0Nps on 50 mgL^{-1} AR 151dye degradation.

4.1.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of untreated and treated dye samples of different time intervals were formed. Then comparison was done by forming overlays which represented significant change in treated samples as compared to untreated dye.

FTIR spectra of control AR 151 showed C–H stretching at 2922 cm^{-1} , C–N stretching at 1535 cm^{-1} and peaks at 1206 and 1041 cm^{-1} showed S=O (Fig 4.7a). Band at 1514 cm^{-1} represents the presence of –N=N– bond vibrations similarly peaks at 1452, 1555 attributes to N=N in aromatic skeleton (Lucarelli *et al.*, 1999). Peaks at 1207.48 cm^{-1} represented S=O stretching of sulphur compound (Bandara *et al.*, 1999). Presence of peaks at 2922 $cm^{-1}$and 2858.60 cm^{-1} represents C–H stretching of alkanes (Jadhav *et al.*, 2009).

In 96, 120, 240 hrs extracted samples, changes in peak intensity at 2922, 2858, 1670, 1667cm^{-1}, 1458cm^{-1}, 1282.71cm^{-1}, 1078cm^{-1} was observed. Significant increase in peak intensity at 2972.87 cm^{-1}, 2922.25 cm^{-1} and 2858.60 cm^{-1}indicated fluctuations in C–H stretching of alkanes. Similar changes took place in regions of 1589 which indicates deformation of aromatic compounds, changes in peak intensity at 1458.23 cm^{-1} represented fluctuation of N=O stretching of nitrosamines, while changes in peaks intensity at 1282.71 cm^{-1} and at 1078.24 cm^{-1}represents fluctuation in C–N vibrations of aryl amines and S=O stretching of sulfoxids respectively expressed the breakdown of dye molecules (Kalme *et al.*, 2007; Jadhav *et al.*, 2009).

In figures 4.10, 4.11 & 4.12 untreated dye sample is compared with dye samples treated by *A. niger*, *A. niger* + B Ag^0Nps, B Ag^0Nps and C Ag^0Nps after 96 and 120 h time interval. Maximum increase in peak intensity has been shown by B Ag^0NPs after 72 hrs Increase in absorbance intensity can be seen at 2922 and 2858 cm^{-1} and in region of 1000-1500 cm^{-1}. After 120 hrs of incubation of dye with different treatments there was maximum difference in absorbance between control and treated dye solution, which showed that there was complete decolorization after 120 h of treatment time.

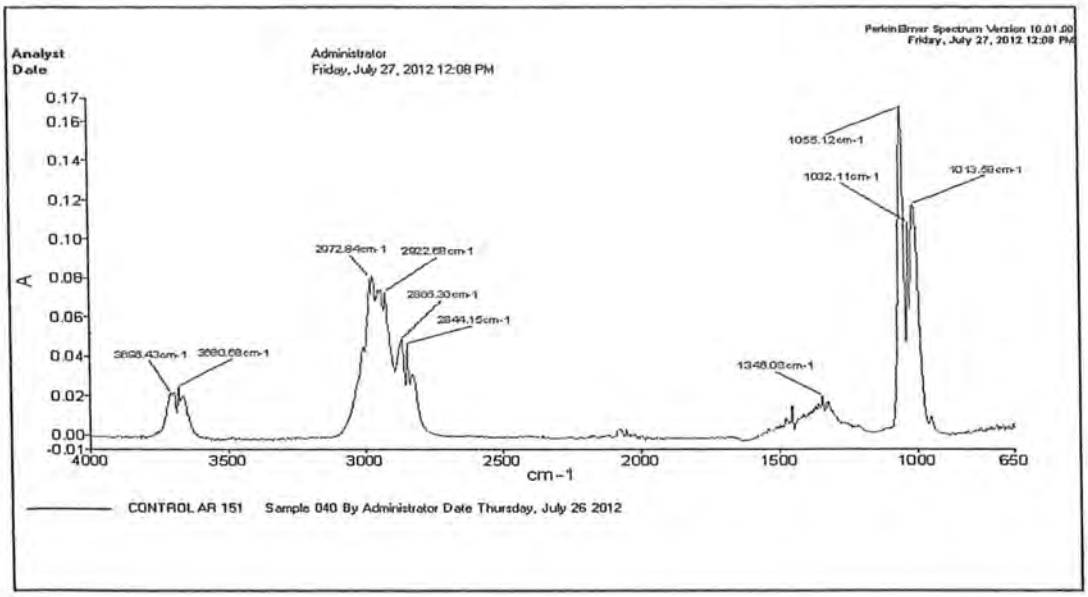


Fig. 4.7a FTIR spectrum of untreated AR 151(50mg/l)

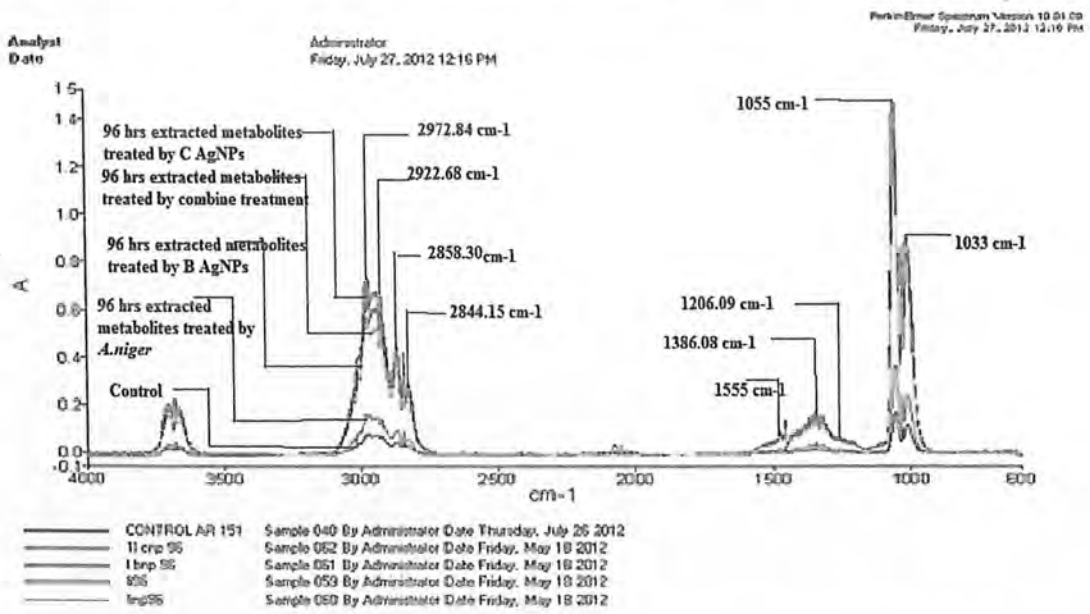


Fig. 4.10: Comparison of untreated and 96 hrs treated dye samples by *A. niger*, (*A. niger* + B Ag^0Nps), B Ag^0Nps, C Ag^0Nps.

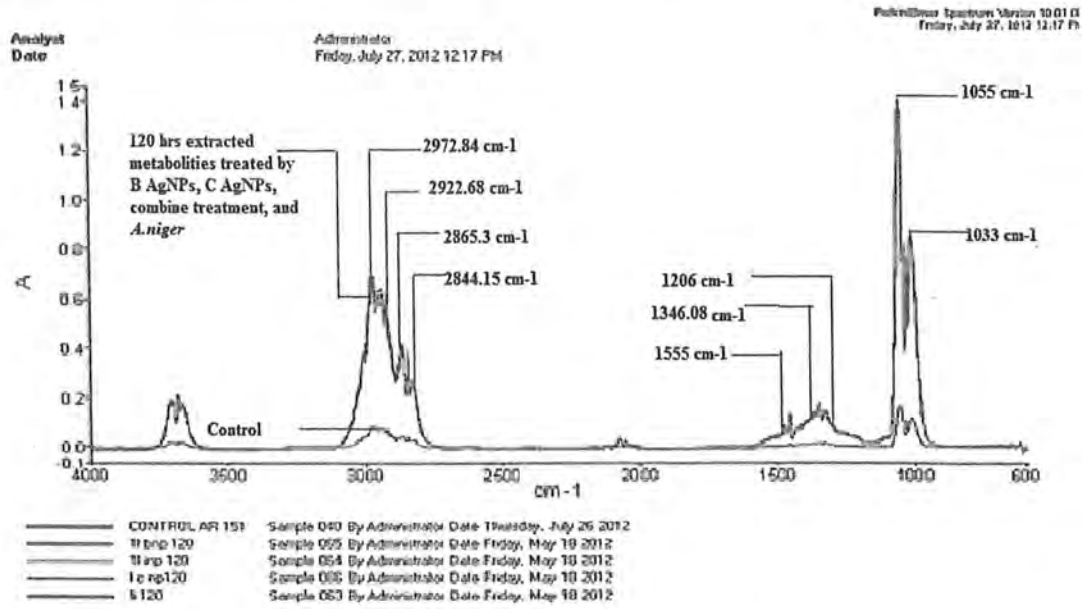


Fig. 4.11: Comparison of untreated and 120 hrs treated dye samples by *A. niger*, (*A. niger* + B Ag^0Nps), C Ag^0 NPs.

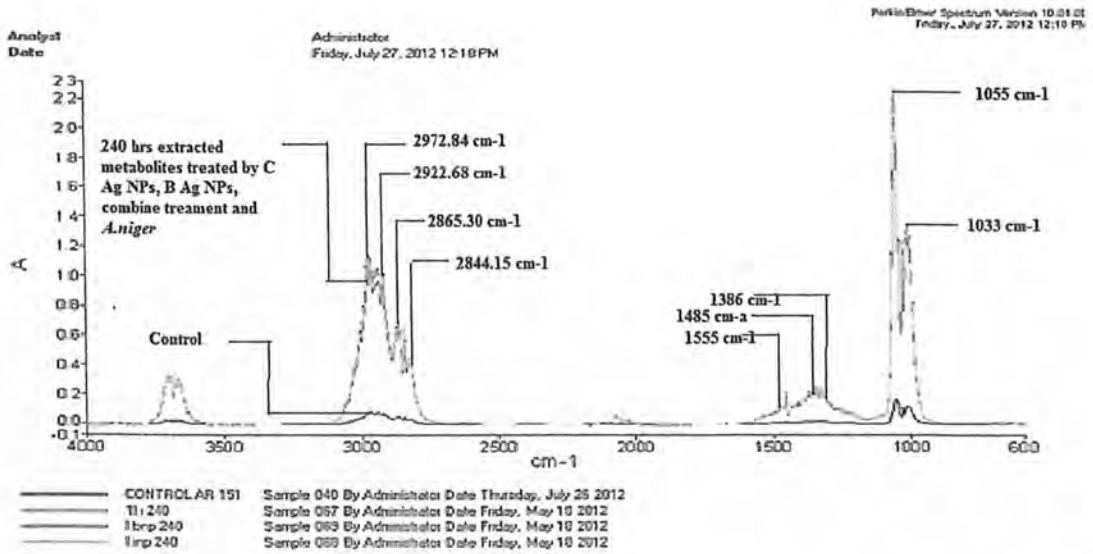


Fig. 4.12: Comparison of untreated and 240 hrs treated dye samples by *A. niger*, (*A. niger* + B Ag^0Nps), C Ag^0NPs.

4.1.5 High Performance Liquid Chromatography

Chromatographic analysis

HPLC analysis was done in order to detect byproducts after different treatments of dye. Two types of byproducts were expected Sulfanilic acid and aniline. These two pure compounds were used as standards for their detection in treated samples. Both sulfanilic acid and aniline at 10 ppm were analyzed through HPLC under previously described conditions. Sulfanilic acid showed retention time of 1.08 min while aniline showed 4.66 min of retention time in case of aniline the peak was broader in nature (Fig. 4.13(b) & 4.13(c)). Dye sample treated by laboratory synthesized Ag^0NPs showed two peaks at 1.08 min and 4.66min and similar peaks were also observed in sample treated by combine treatment and by biologically synthesized Ag NPs (Fig.4.13 (d) &4.13(e)). Large and obvious peak in similar area are seen in dye sample treated with commercial Ag NPs (Fig. 4.13(f)). While in control dye sample and dye sample treated by *A. niger* did not show any relevant peak after 48 hrs (Fig. 4.13(a)).

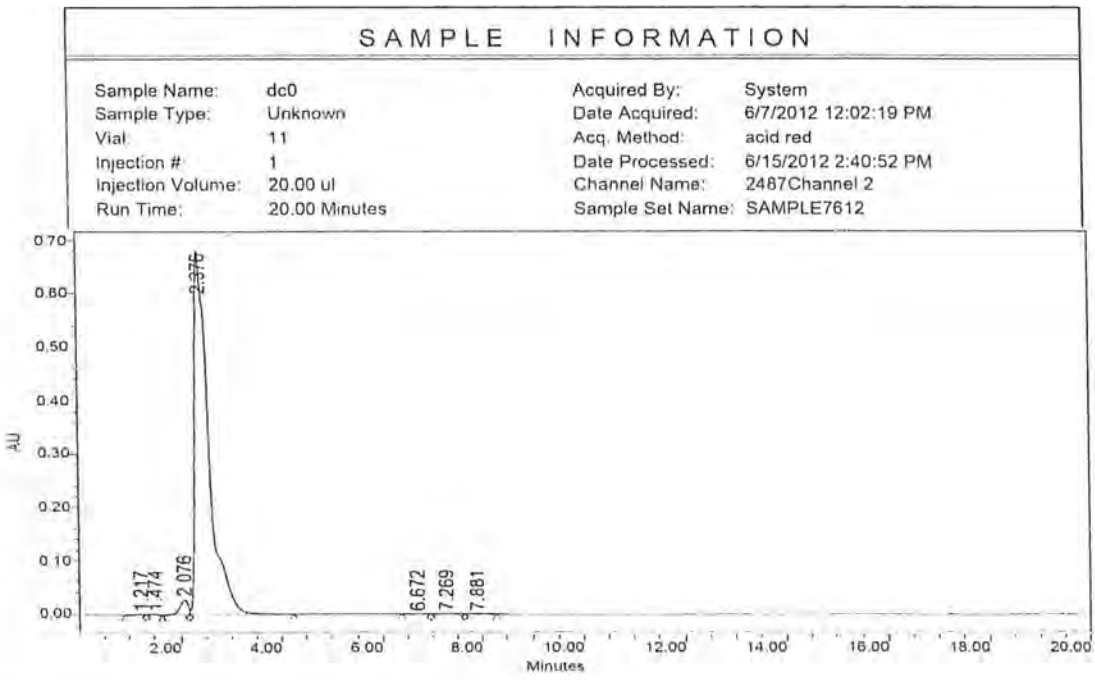


Fig. 4.13(a) : HPLC chromatogram of control (untreated) dye.

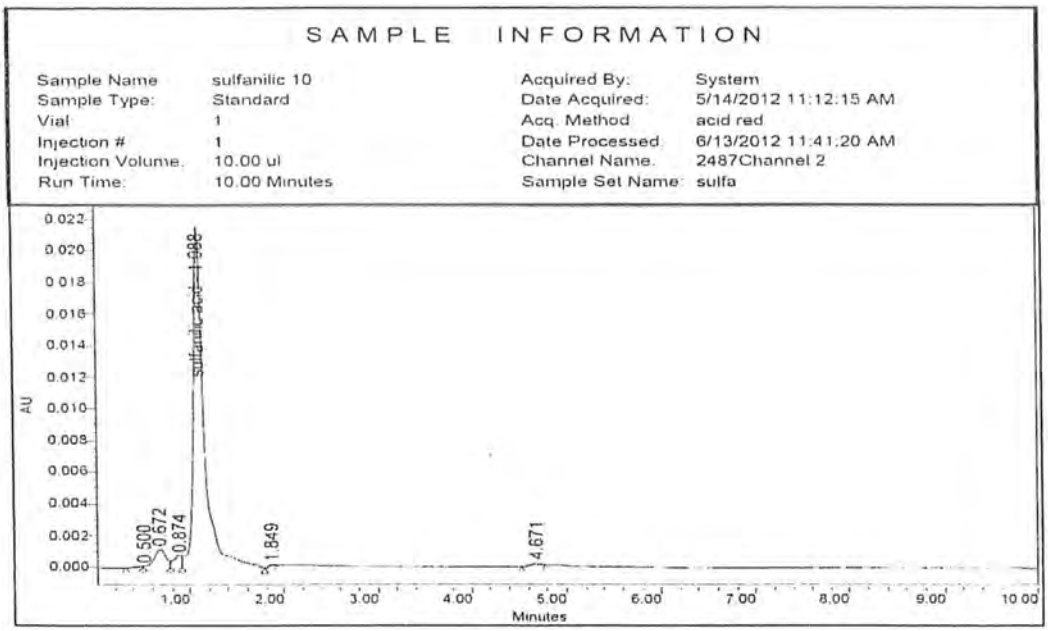


Fig. 4.13(b). HPLC chromatogram of standard Sulfanilic acid (10 ppm).

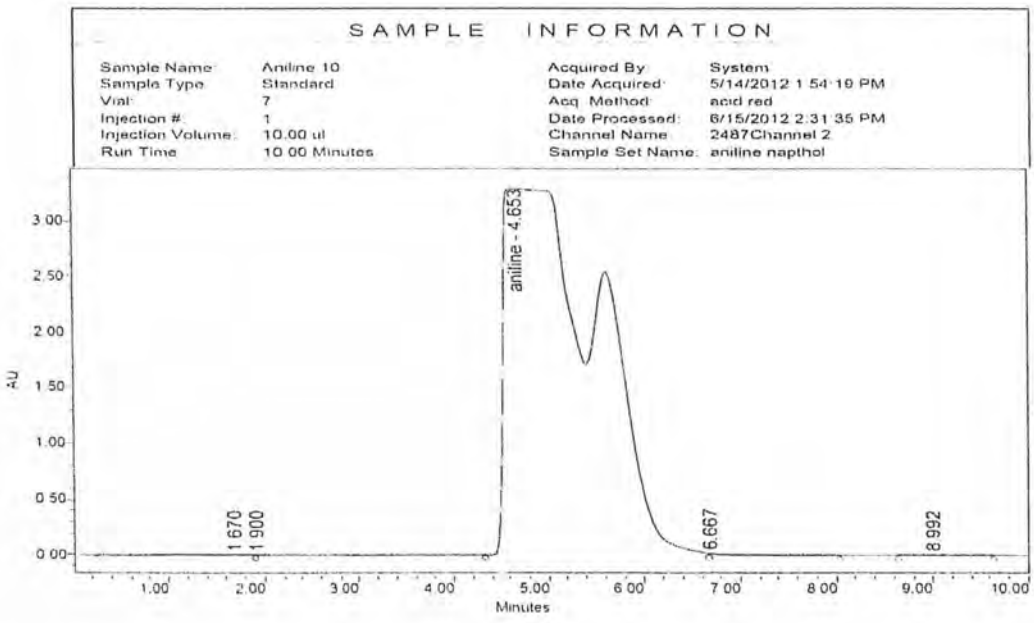


Fig.4.13(c):. HPLC chromatogram of standard aniline (10 ppm).

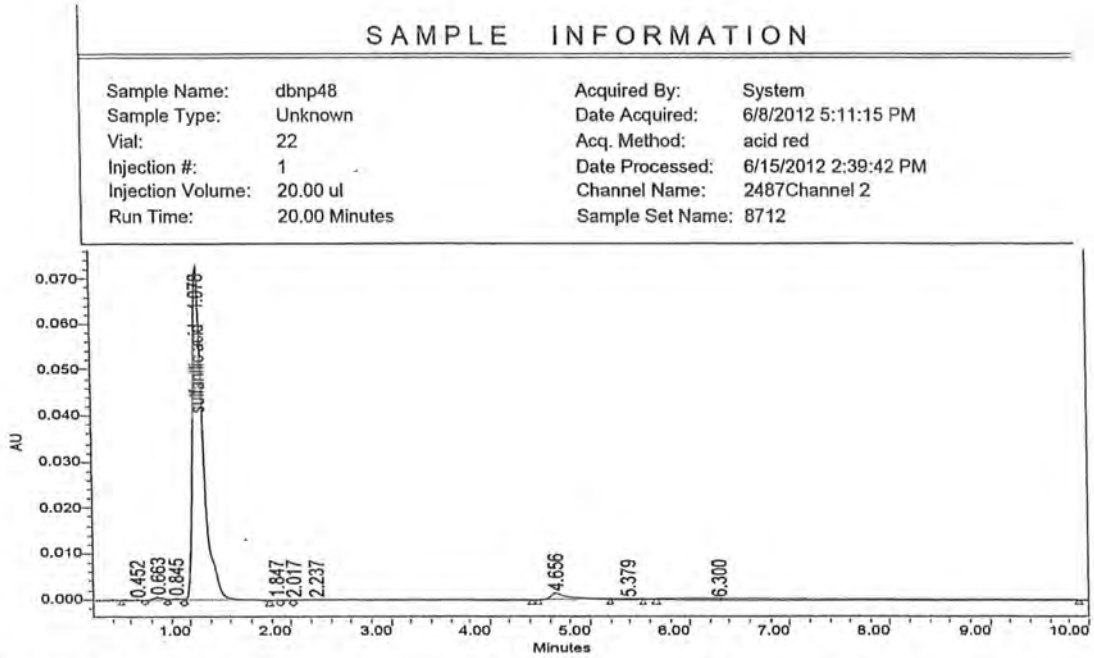


Fig. 4.13(d) HPLC chromatogram of sample treated by biologically synthesized Ag^0 NPs (dbnp).

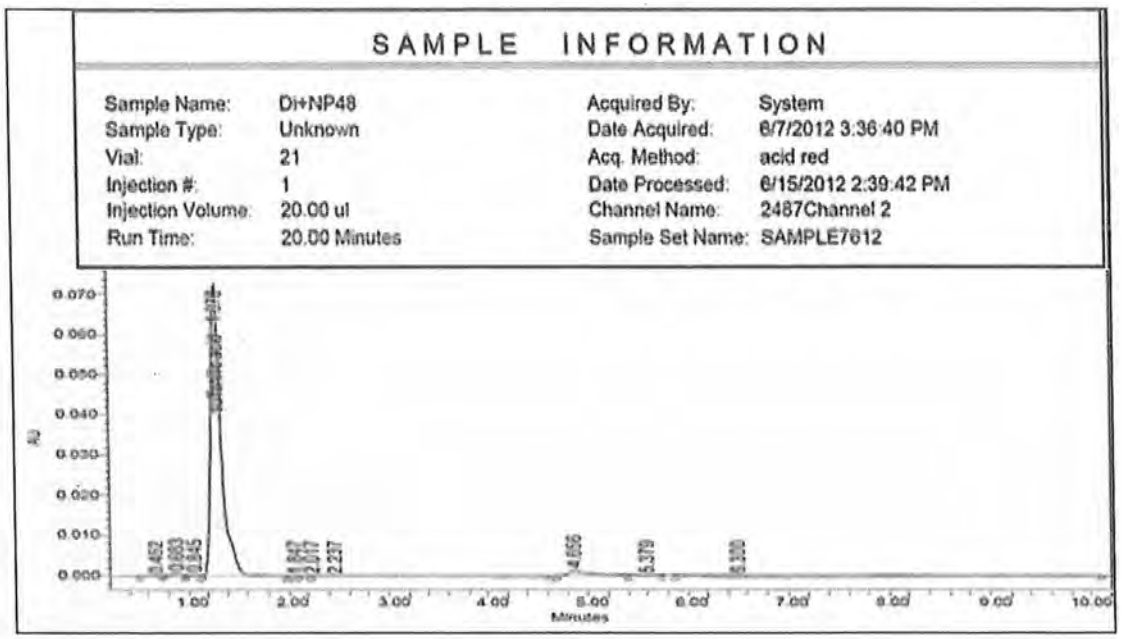


Fig. 4.13(e) HPLC chromatogram of sample treated after combine treatment (Di+NP).

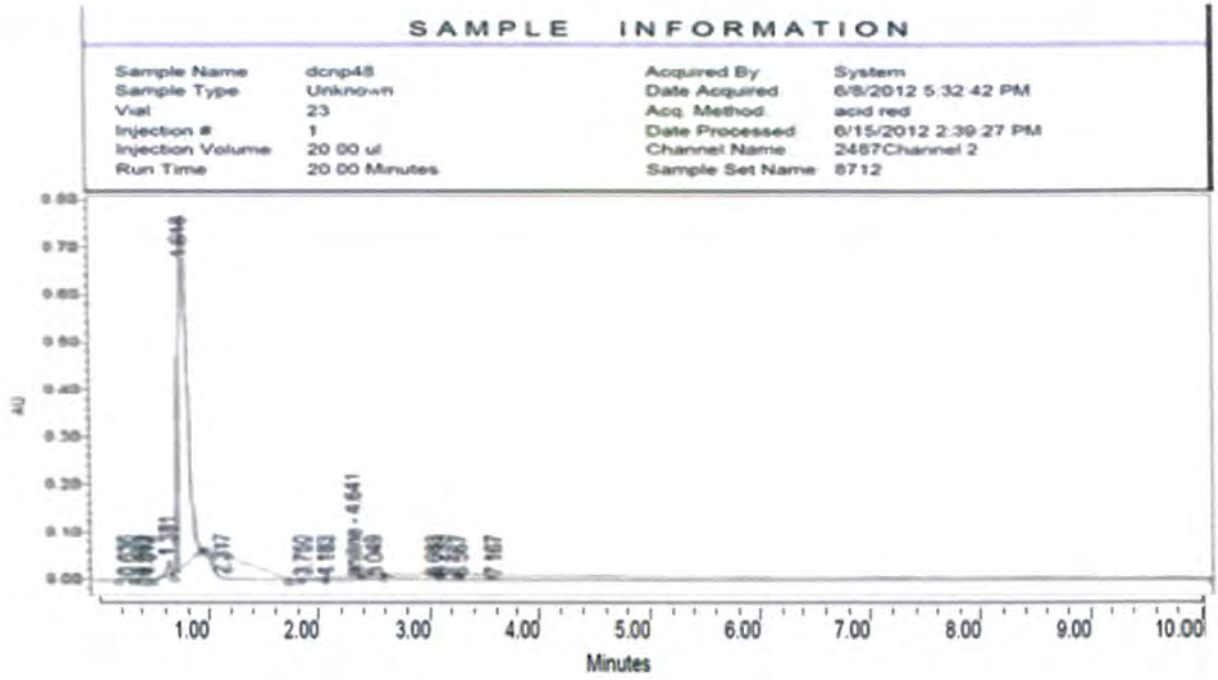


Fig. 4.13(f) sample treated by commercially available Ag NPs (dcnp).

4.2. Degradation of Orange II

4.2.1. Visual observation of degradation of Orange II

A continuous change in the color of dye from yellow to brown was observed after 96 hrs of incubation (Fig. 4.1.1) Reaction condition applied were 37 °C, pH 5, 150 rpm, 20 mg/l of dye and 100 mg/l of Ag^0NPs. There was no change in color of control flasks (without any treatment with fungi or nanoparticles) which was provided with same experimental conditions.

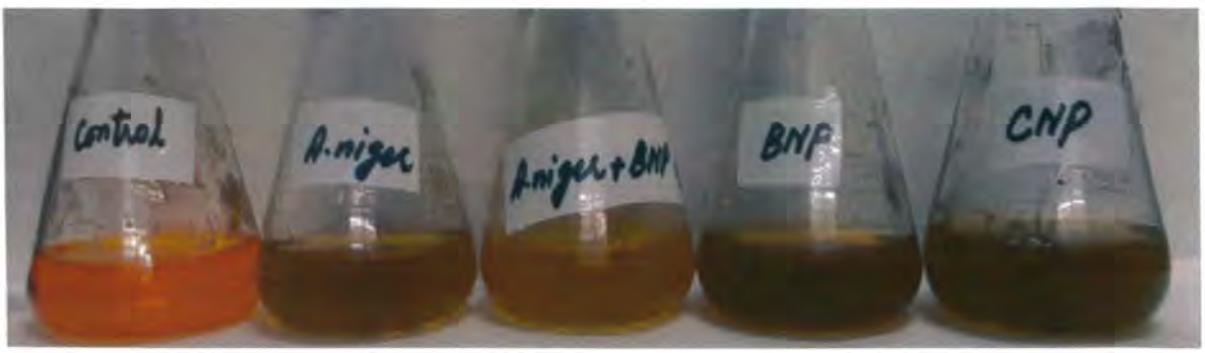


Fig. 4.14: Change in color of Or II (20ppm) by *A.niger*, *A.niger* + B Ag^0NP (100ppm), B Ag^0NP (100ppm), C Ag^0NPS (100 ppm).

4.2.2. UV-Vis spectral analysis of Orange II

Optical density of the treated samples was taken at 486 nm after centrifugation at 10,000 rpm and filtering the supernatant through pvdf filters(0.45µm) to remove all solid particles. It decreased in all cases like treatement by *A. niger. and* nanoparticles. But maximum and rapid decrease in optical density was seen in case of combined treatment of dye OrII by Ag^0 nanoparticles ($100mgL^{-1}$) and 0.001 % w/v *A. niger.* Reaction condition applied were constant in all experimental flasks i.e., 30 °C, pH 5, 50mg/l dye and 100mg/l B Ag^0NPs and C Ag^0NPs.

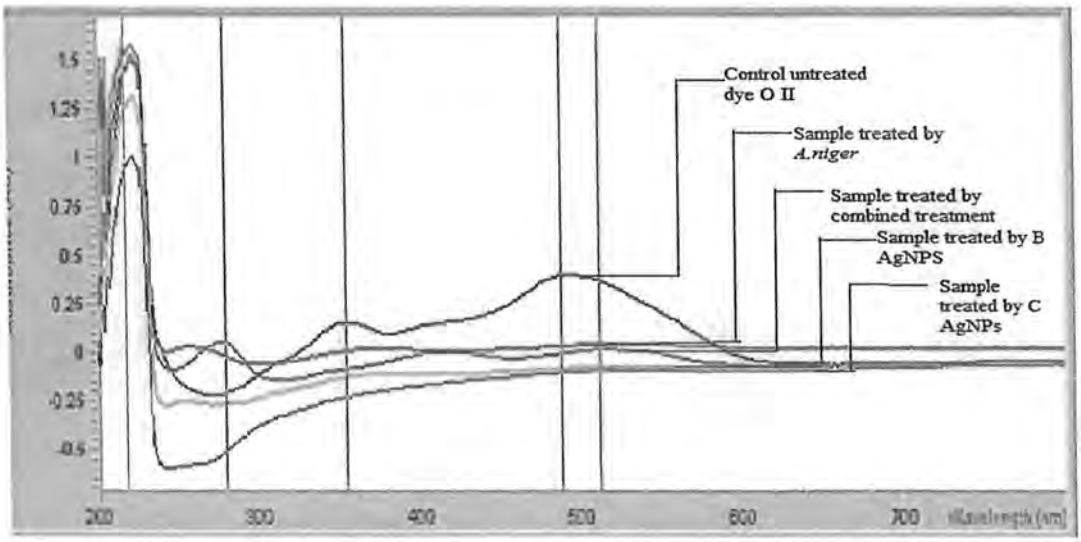


Fig. 4.15: UV-Vis absorption spectra of Or II in presence of *A. niger* , B A^0gNPs ($100mgL^{-1}$) and C Ag^0NPs ($100mgL^{-1}$).

4.2.3. Spectrophotometric results of Orange II

Treated dye samples were scanned through UV-Vis spectroscopy to find out the residual concentration of dye in STE medium and reaction mixture containing the particular dye solutions treated with *A. niger* and photocatalyst. Sample was withdrawn at continuous intervals, centrifuged at 10000 rpm for 10 min then filtered through pvdf filters and then optical density was determined through UV-visible spectrophotometer. The light absorption was examined in the range of 200–800 nm where major peak was found in region of 480-500 nm. Continuous decrease in absorption was observed from zero hr time to incubation period of 96 hrs (Fig.4.1.2).

4.2.3.1. Optimization of parameters

Degradation of azo dye Orange II was carried out by optimizing parameters including pH, temperature and concentration of dye and photocatalyst concentration. Analysis of the results was done on the as grounds as done with former.

4.2.3.2. Effect of temp variation

Temperatures applied for the decolorization of dye Or II by *A. niger* and photocatalyst include 25, 30, 37 °C. The decolorization of the dye was carried out at neutral pH i.e., 5 value in shaker incubator (150 rpm for 8 days). In initial 24 hrs interval % color removal by *A. niger* was 46, 39, and 77 % at 25, 30 and 37 °C (Fig 4.16a). Decolorization of Or II was observed maximum (96 %) at 37 °C by *A. niger* after 96 hour time interval at the same time degradation was 82 and 84 % at 25 and 30 ^{0}C. It was kept more than 80 % at a temperature range of 25-37 ^{0}C after 96 hrs of incubation. Increase in temperature proved to have positive effect on dye degradation of Or II. Color removal was significantly improved at 25 and 30 ^{0}C where both photocatalyst (100 mgL^{-1}) along with *A.niger* (0.001 % w/v) was applied, degradation was 87 %, 56 % and 66 % at 37 °C, 25 °C, and 30 °C respectively after 24 hrs interval (4.16b). Decolorization by biologically synthesized silver nanoparticles (100 mg/l) was observed maximum (71 %) at 37 C and at 25 and 30 °C,% degradation was 57 and 68 % (Fig. 4.16c). Similar results were observed when dye was treated with commercial silver nanoparticles (100 ppm) which was found to be 71, 65 and 80 % at 25, 30 and 37 °C (Fig.4.16d). *A. niger* took 96 hrs to degrade dye up to 96 % while B Ag^0NPs and C Ag^0 need only 24 hrs to degrade up to 87 %.

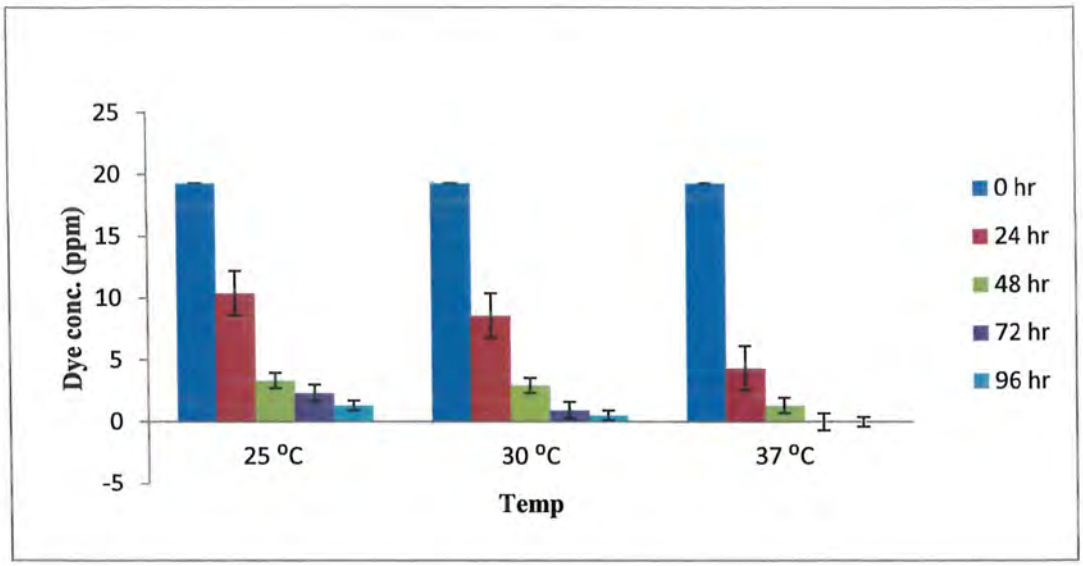


Fig 4.16a: Effect of temperature on degradation of Or II (20mgL^{-1}) by *A. niger*.

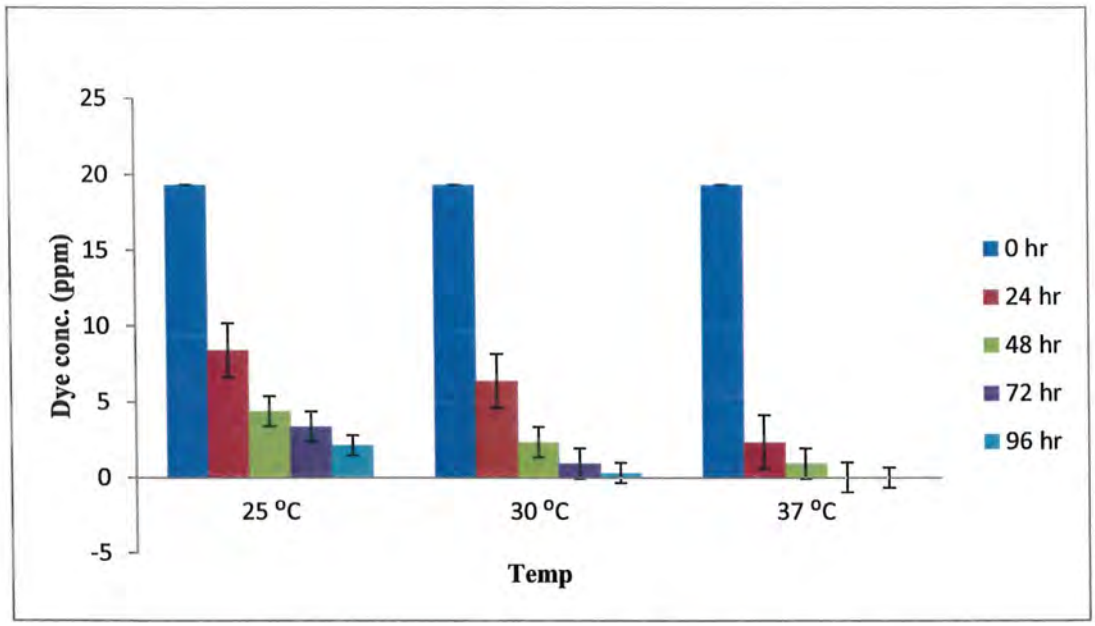


Fig. 4.16b: Effect of temperature on degradation of Or II (20mgL^{-1}) by *A. niger* + B Ag^{0}Nps.

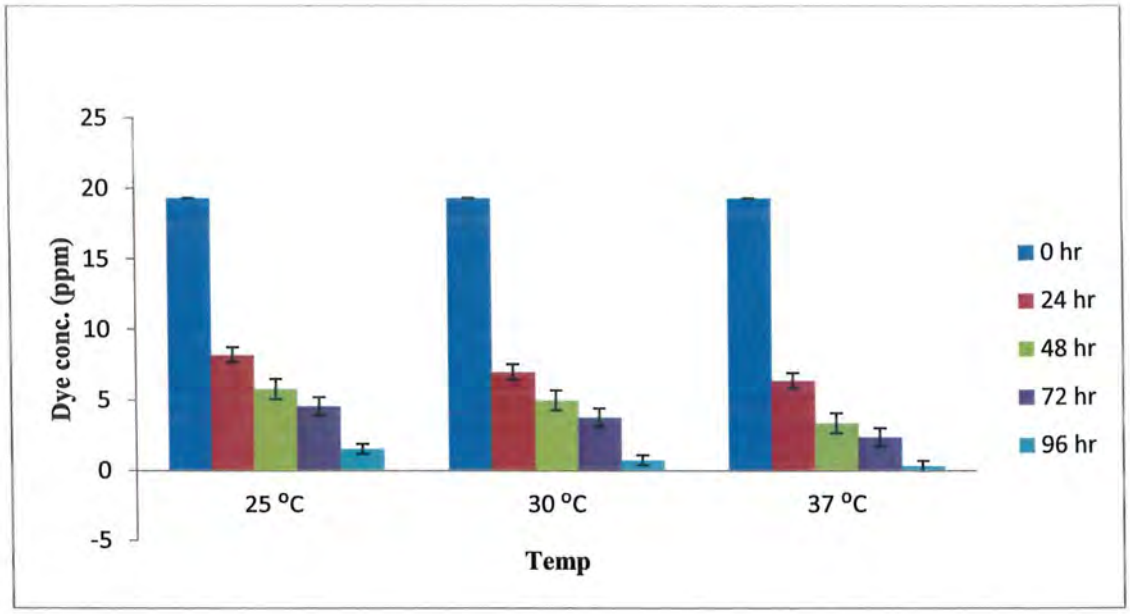


Fig. 4.16c: Effect of temperature on degradation of Or II ($20mgL^{-1}$) by B Ag^0NPs ($100mgL^{-1}$).

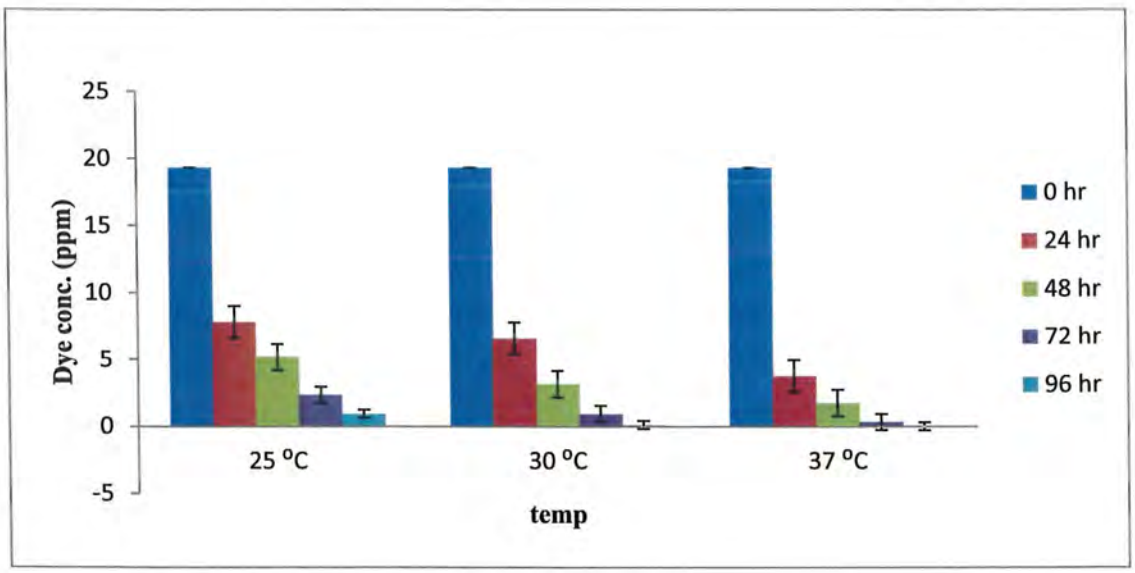


Fig. 4.16d: Effect of temperature on degradation of Or II ($20mgL^{-1}$) by C Ag^0Nps ($100mgL^{-1}$).

4.2.3.3. Effect of pH on fixed concentration of Or II and nanoparticles

The decolorization of the dye Or II was observed at a pH range of 3 to 9 in simulated textile effluent (STE) by *A. niger* and silver nanoparticles .The experiments were run for 8 days in a shaking incubator (150 rpm) at 30 ^{0}C.

In first 24 hrs % degradation was found to be 41, 71, 63 and 33% at pH 3, 5, 7, 9 respectively. After 96 hrs it was maximum i.e., 99 % at pH 5 while it was 81 %, 96 %, and 79 % at pH 3, 7 and 9 respectively. Increase in pH from 5 to 9 negatively effects the % degradation (Fig 4.17a). Combined treatment of dye resulted in increased decolorization which after 24 hrs of incubation was found to be 54, 61, 47 and 51 % (4.17 b).

Decolorization of Or II by biologically synthesized and commercial silver nanoparticles was more than 85 % at all pH conditions in first 48 hours of incubation. In 24 hour interval % degradation at pH 3, 5, 7, and 9 was found to be 71, 52, 41 and 81 % (4.17c). It was maximum i.e., 98 and 99 % in highly acidic and basic environment at pH 3 and 9. While at pH 5 and 7 it was 91 and 89 % after 48 hours of incubation. 48 hours of incubation time was found to be most effective in dye degradation by Ag^0NPs. Commercial Ag^0 nanoparticles also showed similar results. Maximum % degradation was observed at pH 3 (70 %) and 9 (84 %) after 24 hrs (Fig 4.17d).

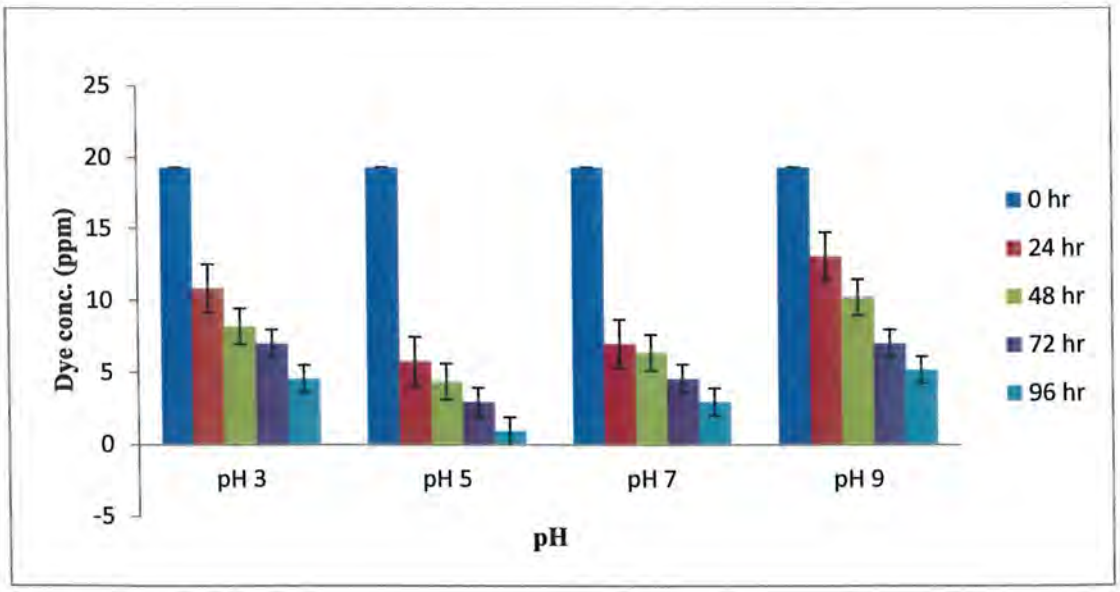


Fig. 4.17a: Effect of pH on Degradation of Or II (20mgL^{-1}) by *A. niger*.

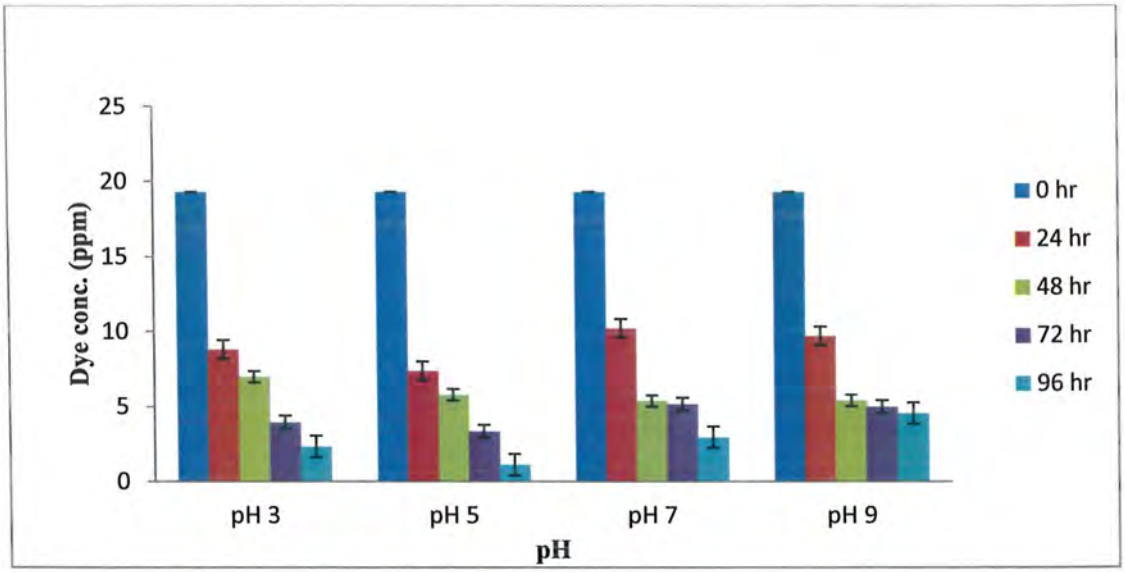


Fig. 4.17b: Effect of pH on Degradation of Or II (20mgL^{-1}) by *A. niger* + B Ag^{0}Nps (100 ppm).

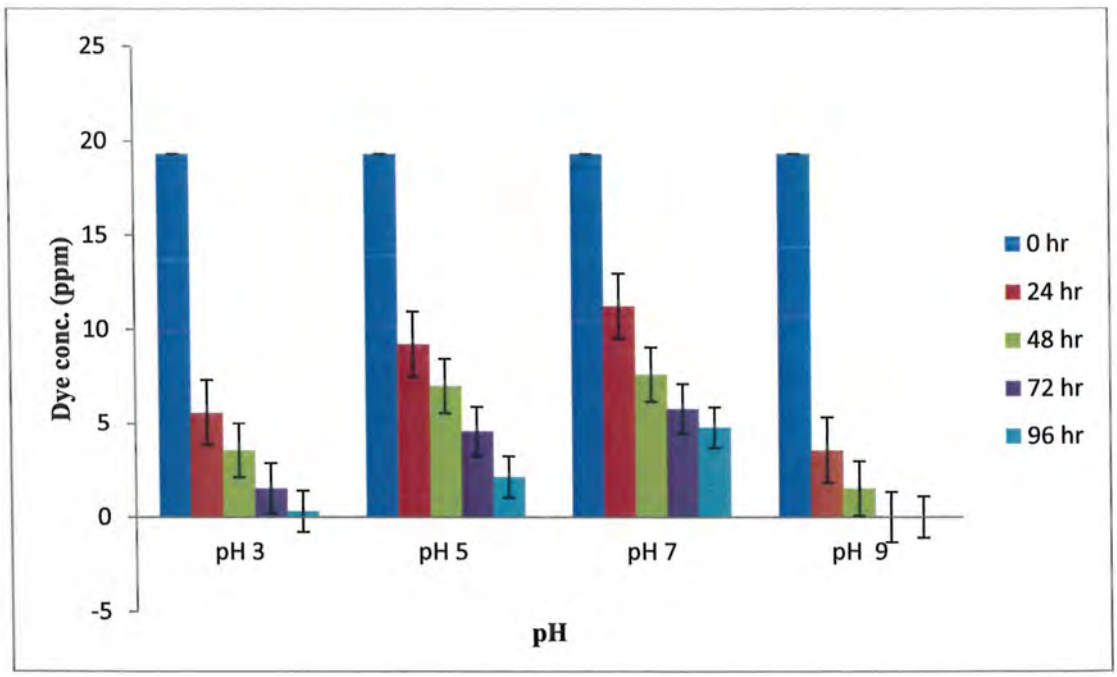


Fig. 4.17c: Effect of pH on Degradation of Or II (20 mgL^{-1}) by B Ag^{0}Nps (100mgL^{-1}).

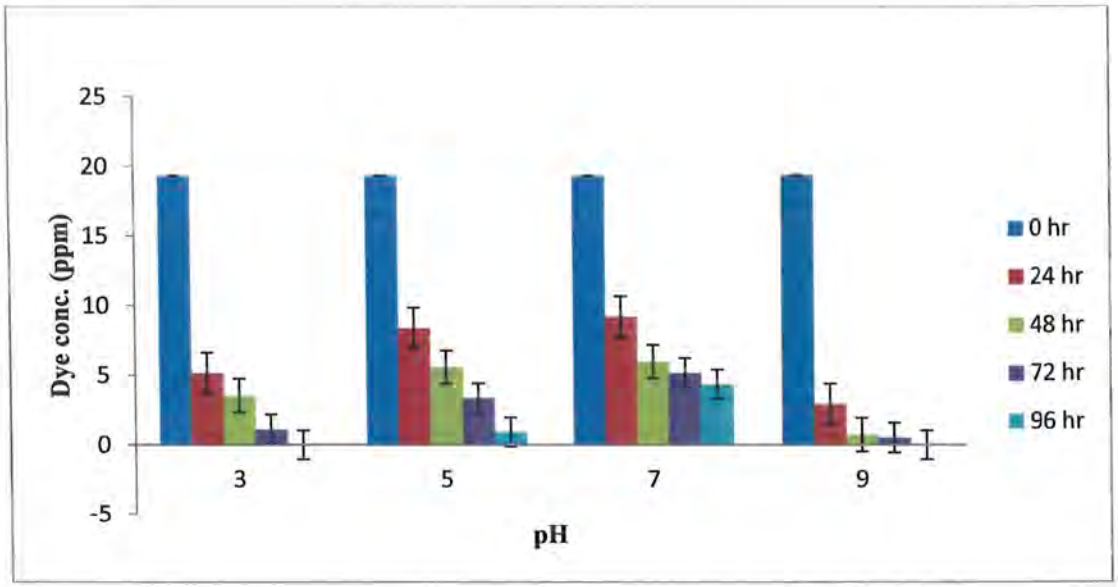


Fig 4.17d: Effect of pH on Degradation of Or II (20mgL^{-1}) by C Ag^{0}Nps (100mgL^{-1}).

4.2.3.4. Effect of different concentration of dye Orange II on degradation by fungi *A.niger* and photocatalyst Ag^{0}NPs:

The effect of initial concentration of dye Or II on decolorization was find out using constant amount of catalyst (100 mgL^{-1}) and *A. niger* (0.001% W/v) while varying the initial concentration of dye in experimental flasks(Fig. 4.18b-4.18d) show that degradation efficiency of the dye first increases with increasing concentration of dye from 10 to 20 ppm. After further increase in dye concentration, degradation and color removal was much reduced up to 40 % at 100mgL^{-1}. Temperature 37 °C and pH 5 were applied that was optimizes previously.

In first 24 hrs, removal of dye by fungus was 58, 59, 36, 33, 31 and 29 % at 10, 20, 30, 40, 50 and 100 mgL^{-1} of Or II (Fig. 4.18a). While in combine treatment involving laboratory synthesized Ag^0 nanoparticles and fungus (0.001% w/v) color removal in first 24 hours was 64, 68, 47, 39, 27 and 35 % at 10, 20, 30, 40, 50, and 100 mgL^{-1} of initial dye concentration (Fig. 4.18b). In experiment where different concentration of dye were treated with 100mgL^{-1} of laboratory synthesized B Ag^{0}Nps, % degradation of 10, 20, 30, 40, 50,and 100 mgL^{-1} in first 24 hrs was 69, 64, 32, 27, 22, 15 % (Fig.

4.18c). While % degradation was slightly greater when commercial Ag^0 nanoparticles were applied, it was observed to be 70, 72, 37, 32, 28, and 20 % when all conditions were kept constant(Fig. 4.18 d).

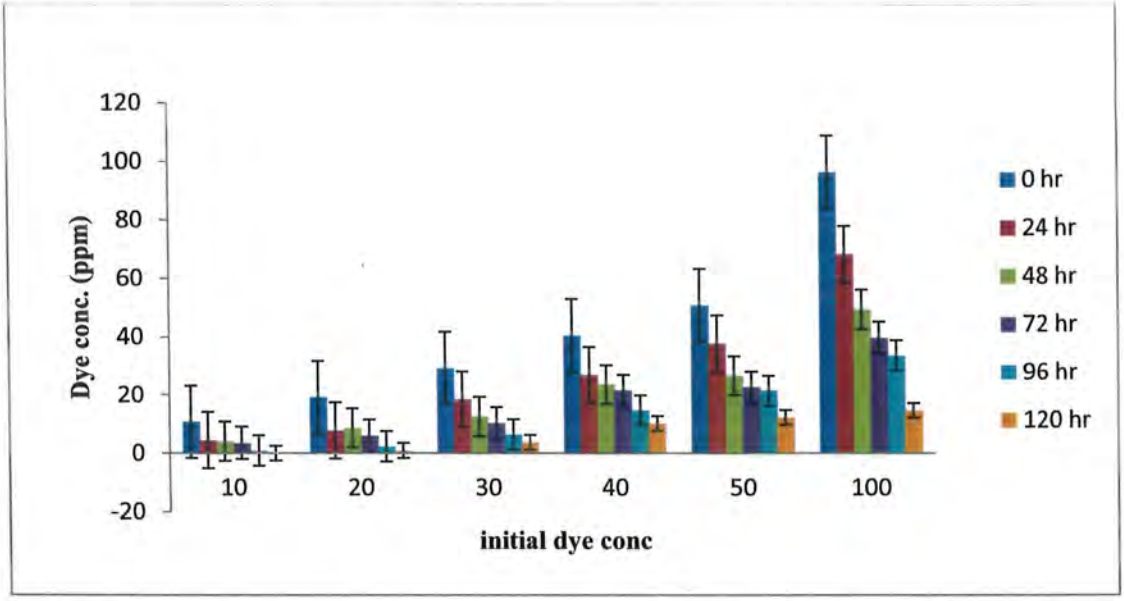


Fig 4.18a: Effect of varying concentration of dye Or II on degradation by *A. niger*.

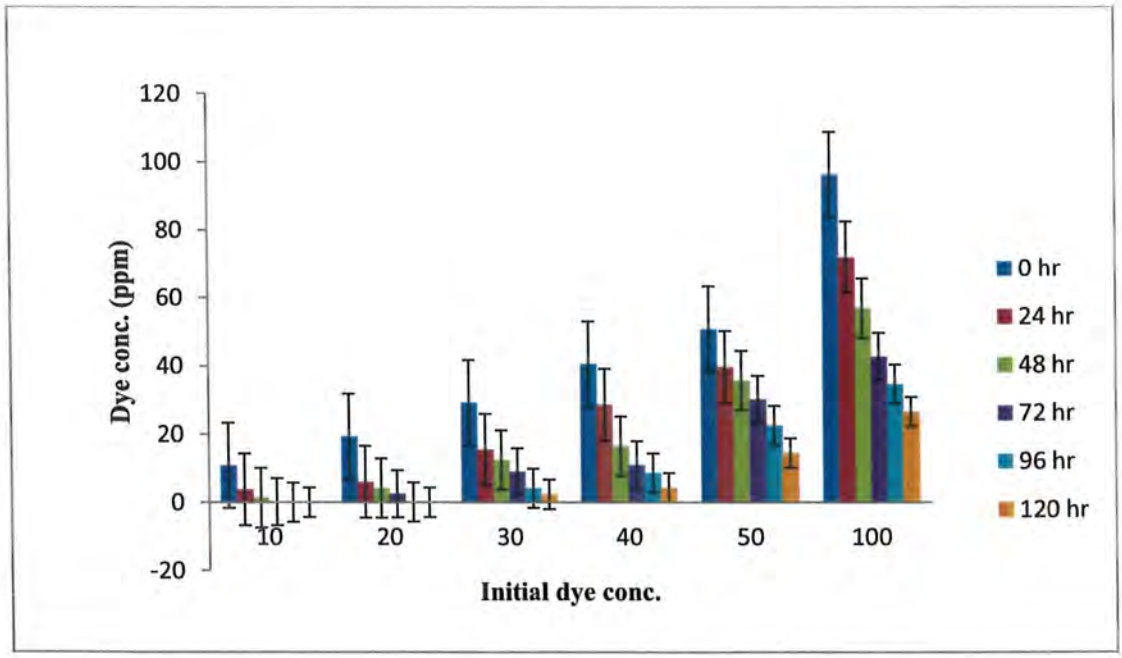


Fig 4.18 b: Effect of varying concentration of dye Or II on degradation by *A. niger* + B Ag^0NPs).

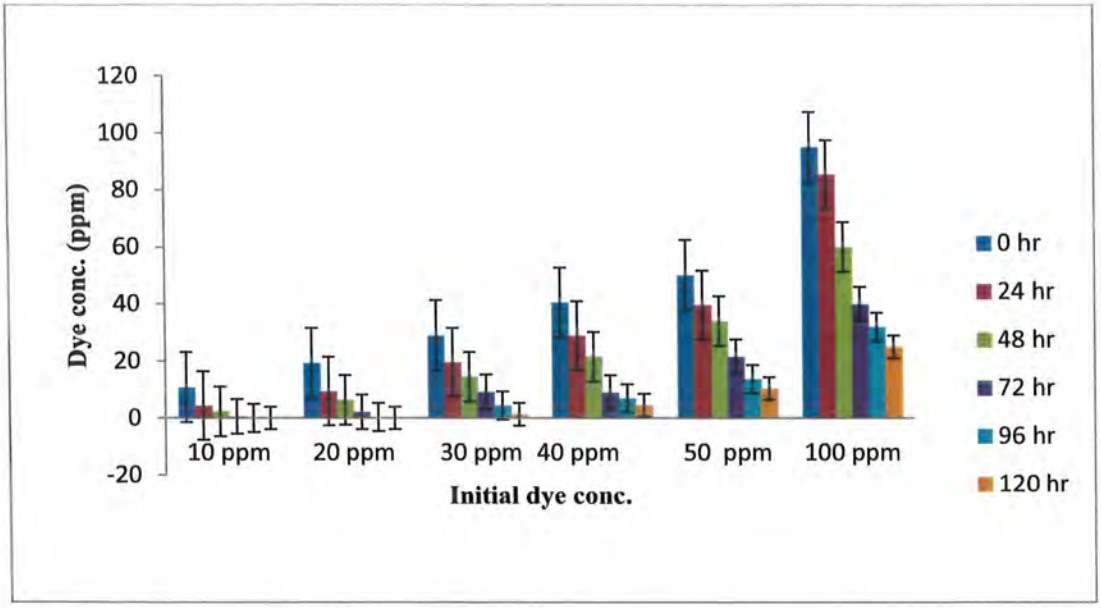


Fig. 4.18c: Effect of varying concentration of dye Or II on degradation by B Ag^0NPs ($100mgL^{-1}$).

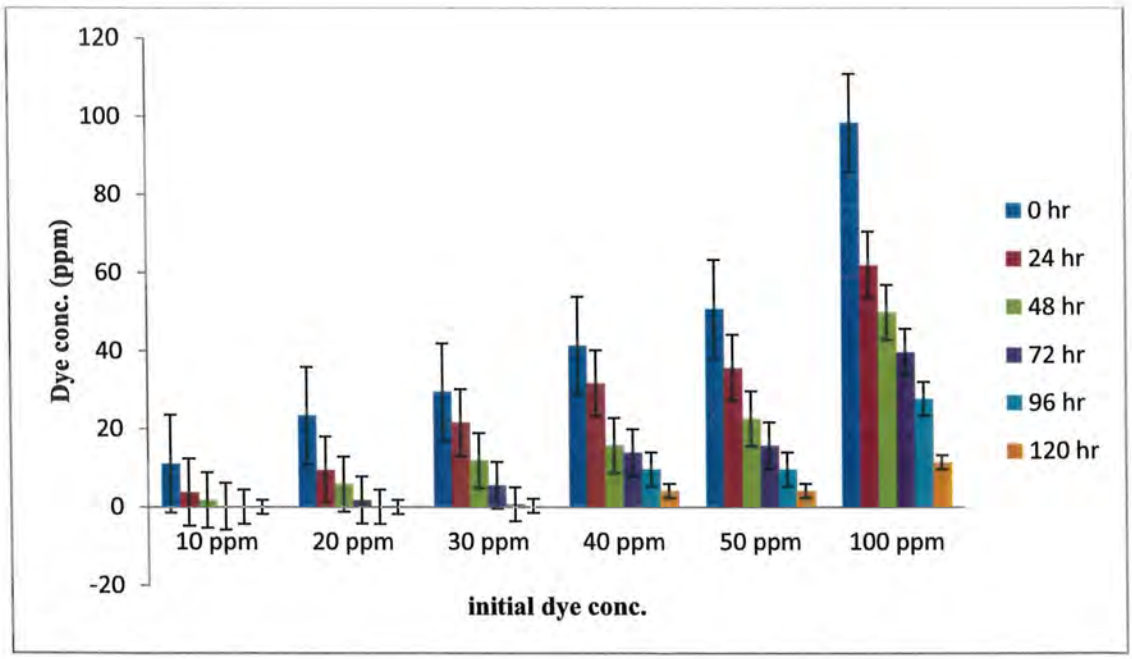


Fig. 4.18d: Effect of varying concentration of dye Or II on degradation by C Ag^0NPs ($100mgL^{-1}$).

4.2.3.5. Effect of different concentration of biological and commercial silver nanoparticles on fixed concentration of dye (20 ppm) and its comparison with *A.niger*

Effect of varying concentration of Ag^0NPs on degradation of Or II was observed using fixed concentration of dye Or II (20 mgL^{-1}). By increasing photocatalyst amount, % degradation of dye increased up to 200 mg/l (Fig. 4.19b-4.19d).

After 24 hrs *A. niger* showed 24 % decolorization efficiency, while considerable improvement in dye degradation was observed when dye solution was first sonicated with photocatalyst (100 mgL^{-1}) and then was inoculated with 0.001 % *A. niger*. Degradation efficiency was 57, 58, 61, 65, and 72 % in 24 hour interval with 10, 50, 100, 150, and 200 mg/l (Fig. 4.19b). When dye was treated with B Ag^0Nps separately at concentration of 10, 50, 100, 150, and 200 mgL^{-1} %, color removal was found to be 49, 59, 68, 75, 77 % (Fig. 4.19c). Similar results were observed when same concentration of dye was treated with different concentration of commercial Ag^0 nanoparticles (Fig. 4.19d)

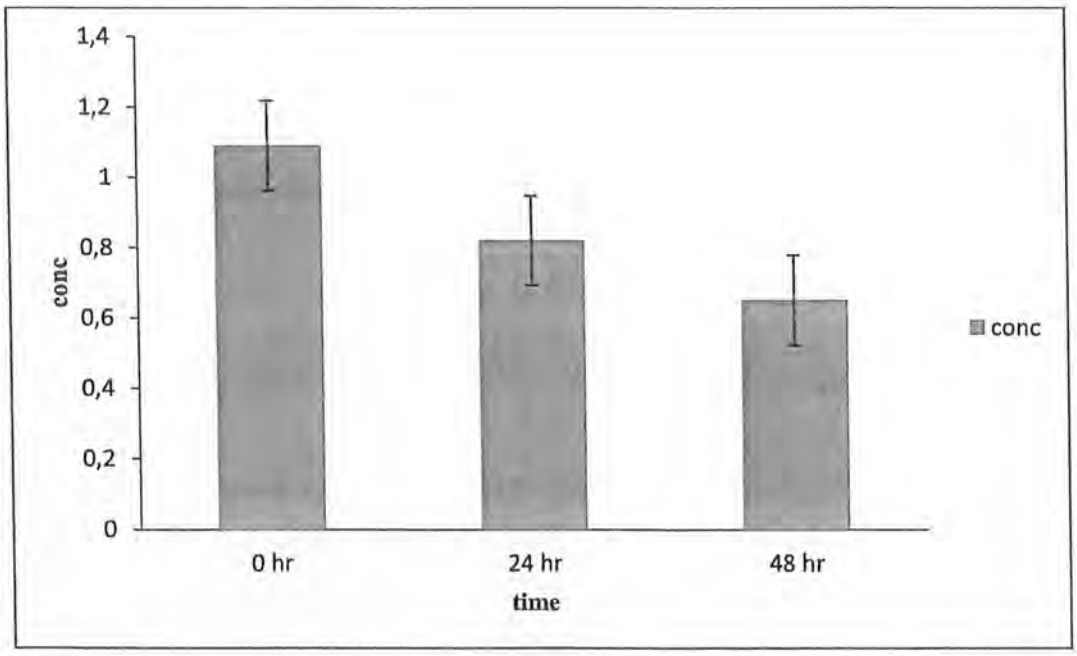


Fig. 4.19a: Effect of varying concentration of dye Or II on degradation by *A. niger*

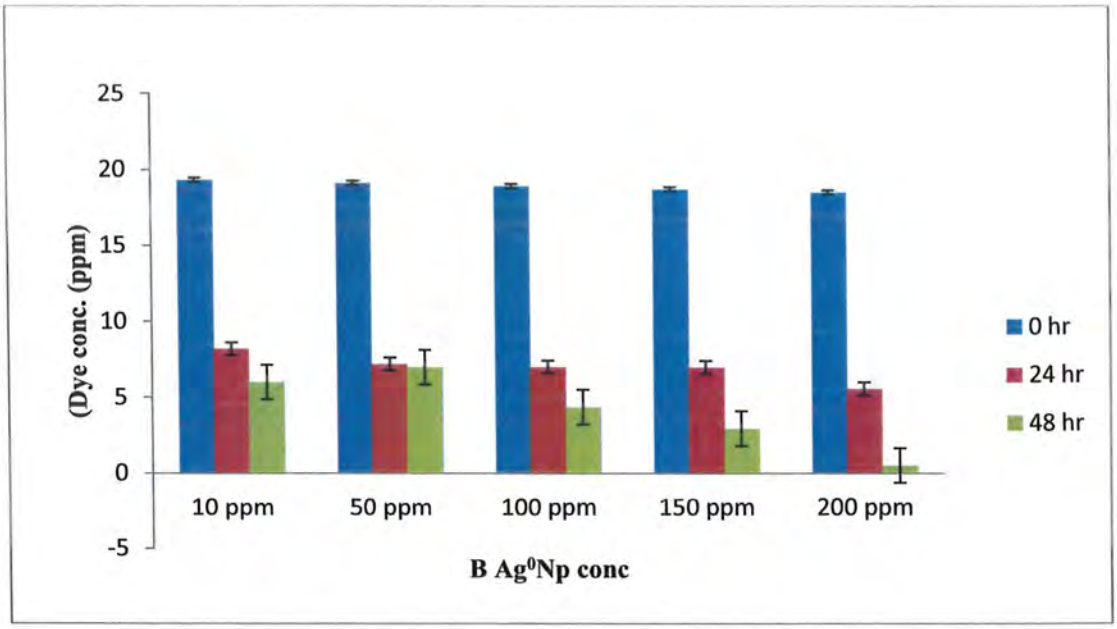


Fig. 4.19b: Effect of *A. niger* + varying concentration of B Ag^0Nps on degradation of Or II ($20mgL^{-1}$).

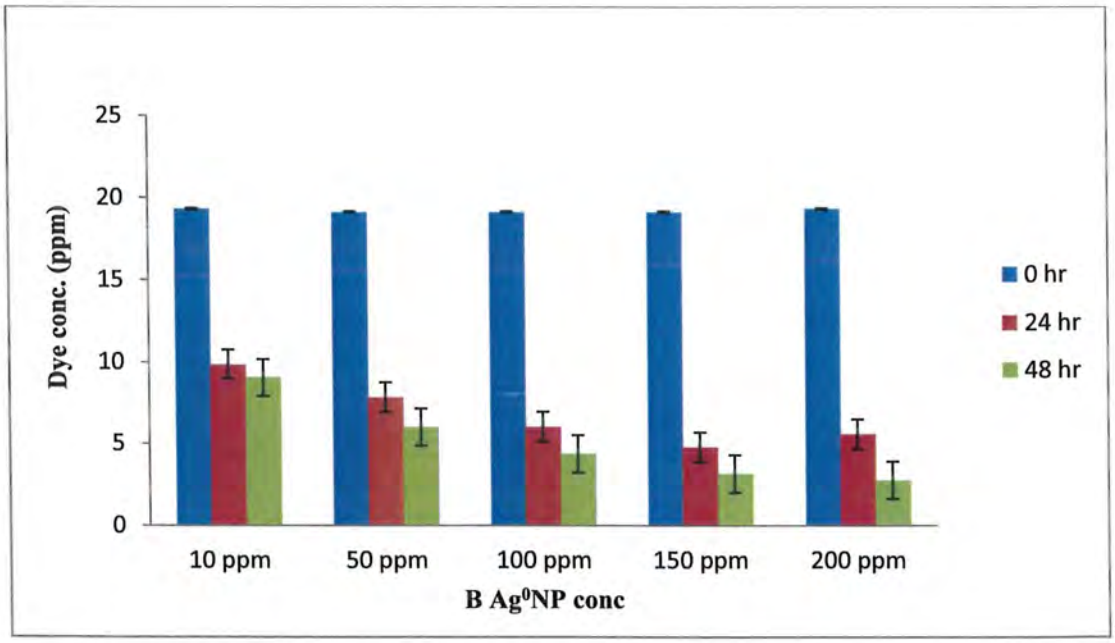


Fig 4.19c: Effect of varying concentration of B Ag^0Nps on degradation of Or II ($20mgL^{-1}$).

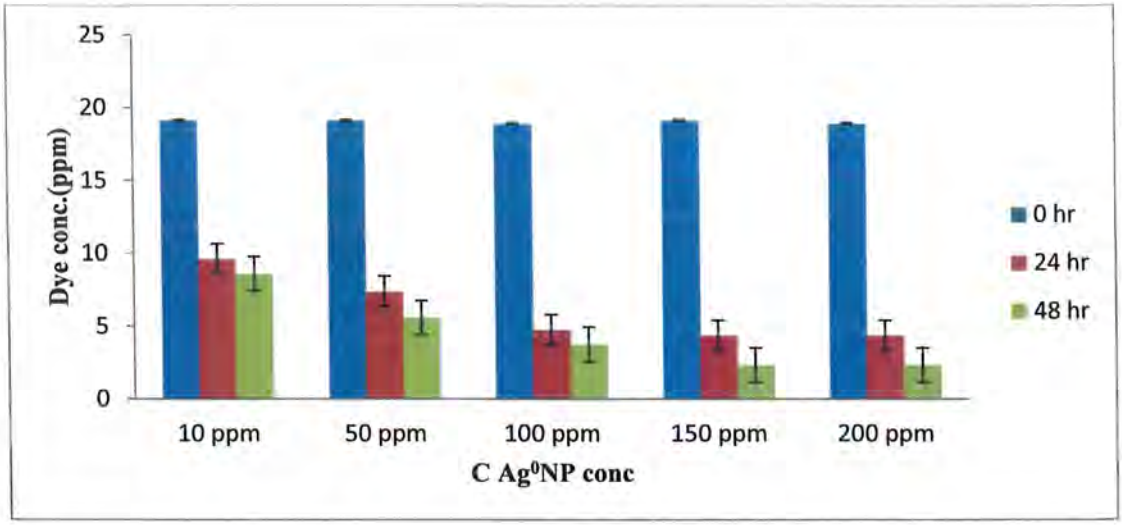


Fig. 4.19d: Effect of varying concentration of C Ag^0Nps on degradation of Or II ($20mgL^{-1}$).

4.2.4. FTIR results of Or II degradation

FTIR spectra of untreated and treated dye samples of different time intervals were formed. Then comparison was done by forming overlays which represented significant change in treated samples as compared to untreated dye.

FTIR spectra of control Or II showed C–H stretching at 2922 cm^{-1} , C–N stretching at 1535 cm^{-1} and peaks at 1206 and 1041 cm^{-1} showed S=O (Fig 4.7a). Band at 1514 cm^{-1}represents the presence of –N=N– bond vibrations similarly peaks at 1452, 1555 attributes to C=C in aromatic skeleton (Lucarelli *et al.,* 1999). Peaks at 1471.74cm^{-1} and 1207.48 cm^{-1} represent C–H bonds of alkanes and S=O stretching of sulphur compound (Bandara *et al.,* 1999). Presence of peaks at 2922 $cm^{-1}$and 2858.60 cm^{-1} represents C–H stretching of alkanes (Jadhav *et al.,* 2009).

In 48, 72, 96, and 120 hrs extracted samples, changes in peak intensity at 2922, 2858, 1670, 1667cm^{-1}, 1458cm^{-1}, 1282.71cm^{-1}, 1078cm^{-1} was observed. Significant increase in peak intensity at 2972.87 cm^{-1}, 2922.25 cm^{-1} and 2858.60 cm^{-1}indicated fluctuations in C–H stretching of alkanes. Similar changes took place in regions of 1589 which indicates deformation of aromatic compounds, changes in peak intensity at 1458.23 cm^{-1} represented fluctuation of N=O stretching of nitrosamines, while changes in peaks intensity at 1282.71 cm^{-1} and at 1078.24 cm^{-1}represents fluctuation

in C–N vibrations of aryl amines and S=O stretching of sulfoxids respectively expressed the breakdown of dye molecules (Kalme *et al.,* 2007; Jadhav *et al.,* 2009).

FTIR spectrum of untreated dye is compared with spectra of dye samples treated with *A. niger*, *A. niger* + B Ag^0Nps (100 mgL^{-1}), B Ag^0Nps (100 mgL^{-1}), C Ag^0Nps (100mgL^{-1}) after 48, 72, 96, hrs of treatment. Changes in absorbance intensity can be observed in regions of 1000-1600 cm^{-1} and 2700-3000 cm^{-1} which infers that some deformation is taking place in dye structure (Fig. 4.21: 4.26). In figure 4.22 formation of new peaks and increase in absorbance can be seen at 2922, 2858 cm^{-1} 1572 cm^{-1},1599 cm^{-1}, 1624 cm^{-1}. In treated sample after 48 hrs incubation time, maximum change in peak intensity is shown by B Ag^0NPs and C Ag^0NPs (Fig 4.22 & Fig 4.23).

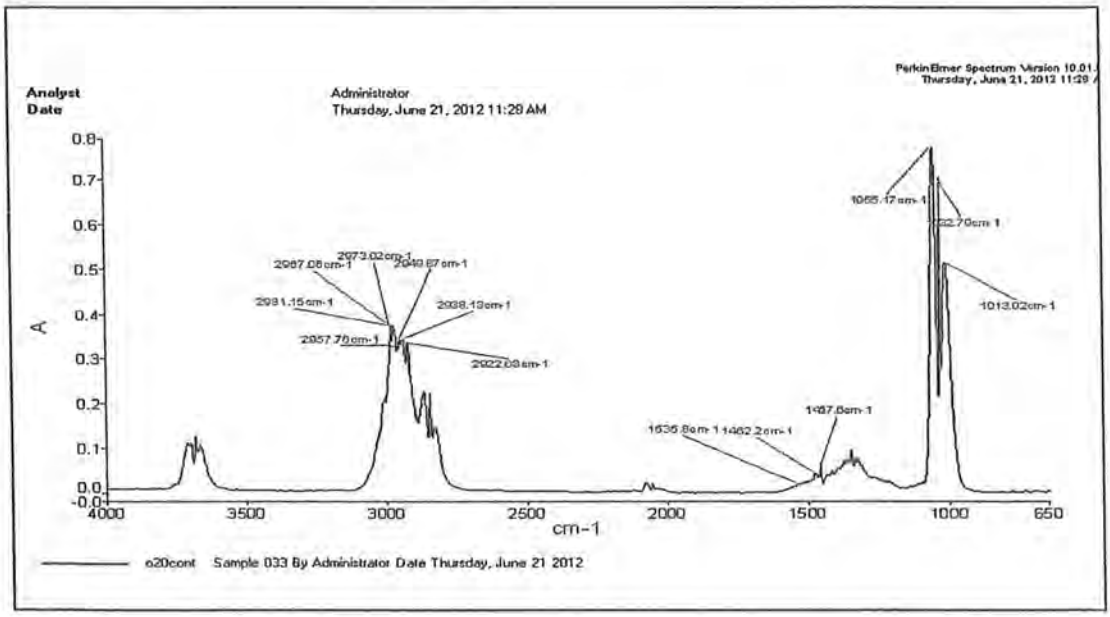


Fig. 4.20: FTIR spectrum of untreated Orange II.

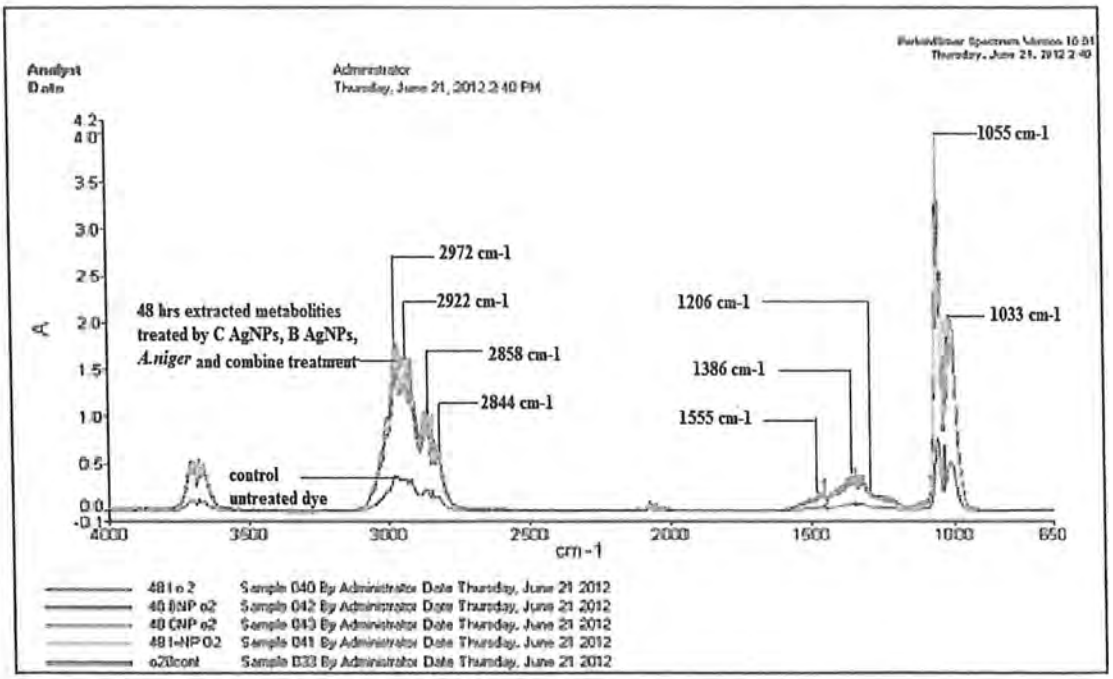


Fig. 4.22: Comparison of untreated and 48 hrs treated dye samples by *A. niger*, (*A. niger* + B Ag^0Nps), B Ag^0Nps, C Ag^0Nps.

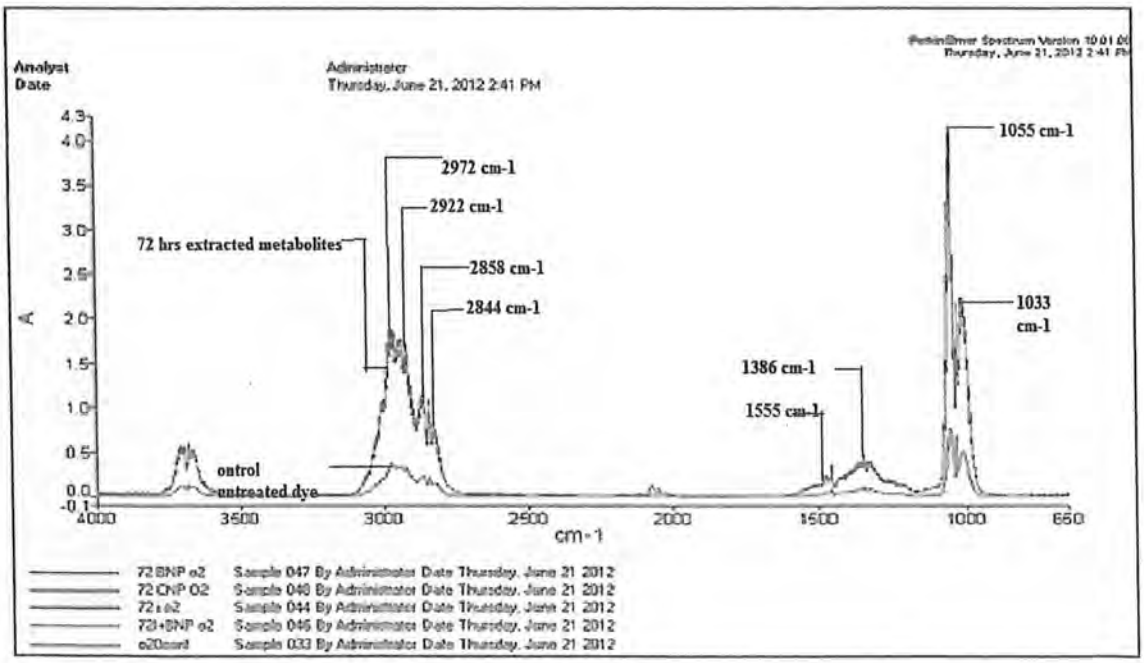


Fig. 4.23: Comparison of untreated and 72 hour treated dye samples by *A. niger*, (*A. niger* + B Ag^0Nps), B Ag^0Nps, C Ag^0Nps.

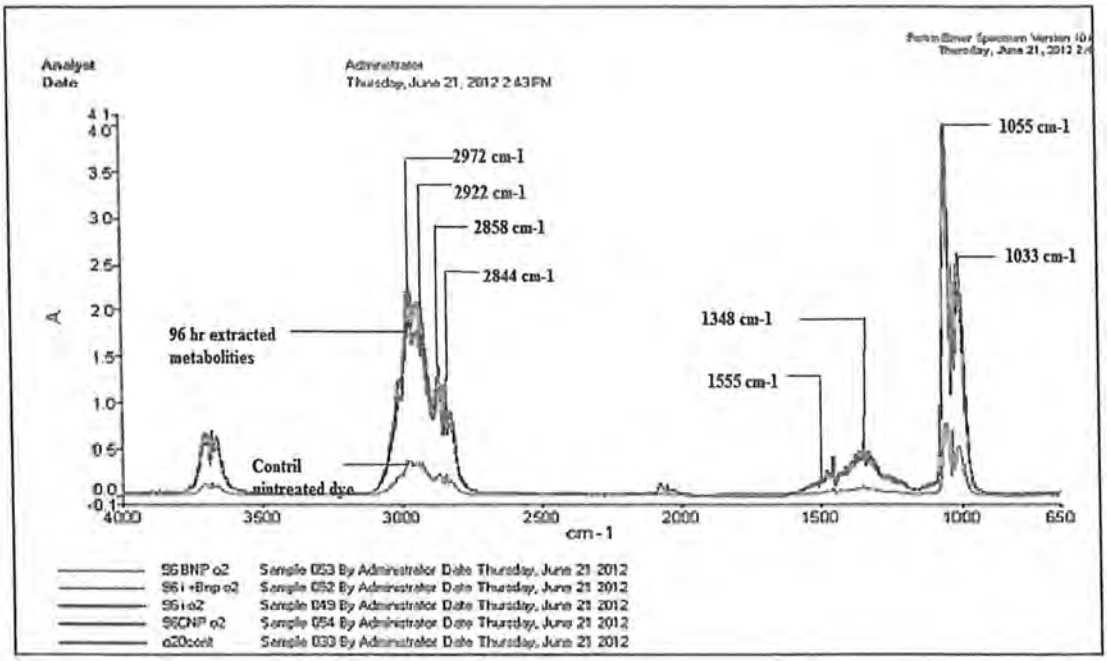


Fig. 4.24: Comparison of untreated and 96 hrs treated dye samples by *A. niger*, (*A. niger* + B Ag^0Nps), B Ag^0Nps, C Ag^0Nps

4.2.4.1 Comparison of all four types of treatment after 24, 48 72, 96, 120 hrs of incubation

A gradual change in peak intensities is seen in 24, 48, 72, 96, 120 hrs of treated dye samples (Fig. 4.26) which shows that gradual adsorption of dye takes place by *A.niger*. While a new peaks and sudden changes in peaks after 48 hrs of incubation time is observed in samples where combined treatment (*A.niger* + B Ag^0NPs), B Ag^0NPs, C Ag^0NPs were applied (Fig. 4.27 to Fig 4.29). After 120 hrs when degradation was completed, absorbance reduced which may represents formation of parent dye molecule by reaction between degraded metabolites.

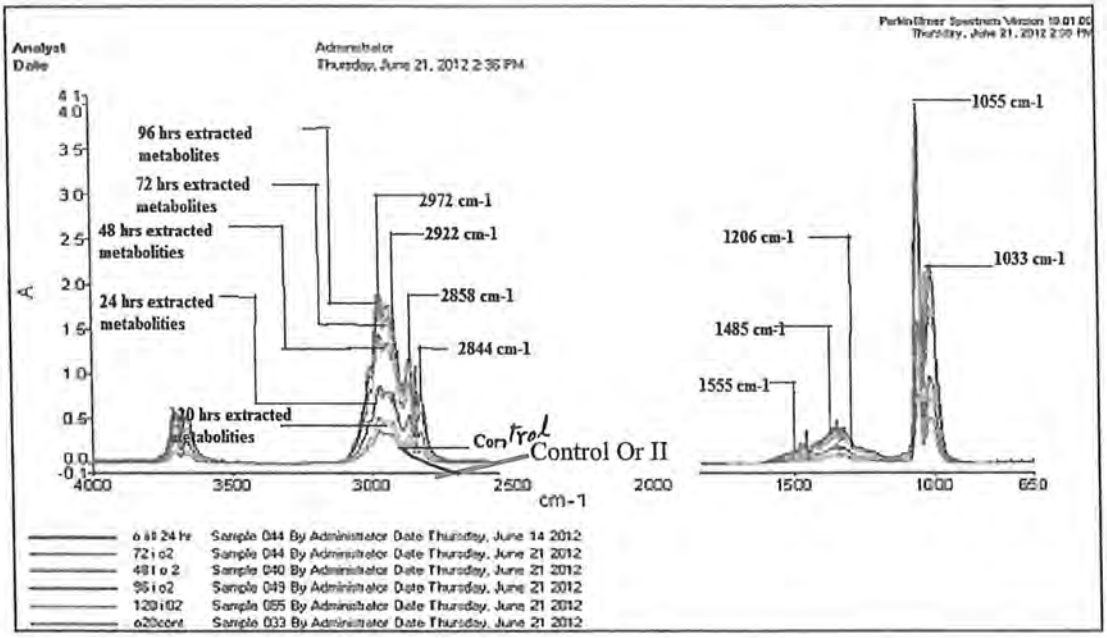


Fig. 4.26: Comparison of untreated dye with 24, 48, 72, 96 and 120 hrs extracted samples treated with *A. niger*

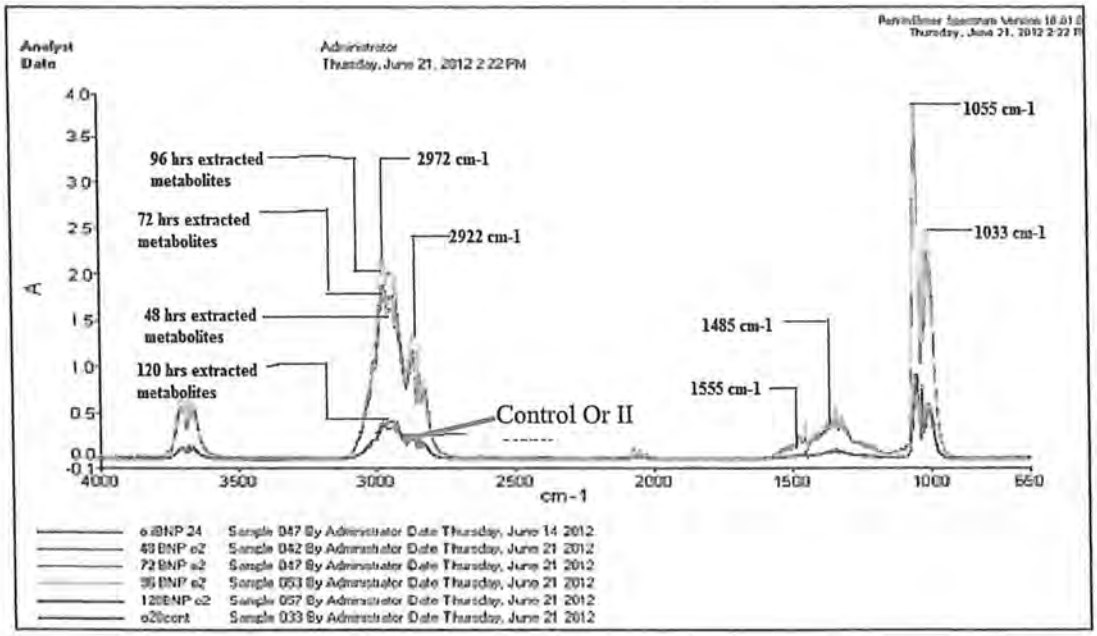


Fig. 4.27: Comparison of untreated dye with extracted samples treated with B Ag^0Nps (100 mgL^{-1}) after 24, 48, 72, 96 and 120 hrs.

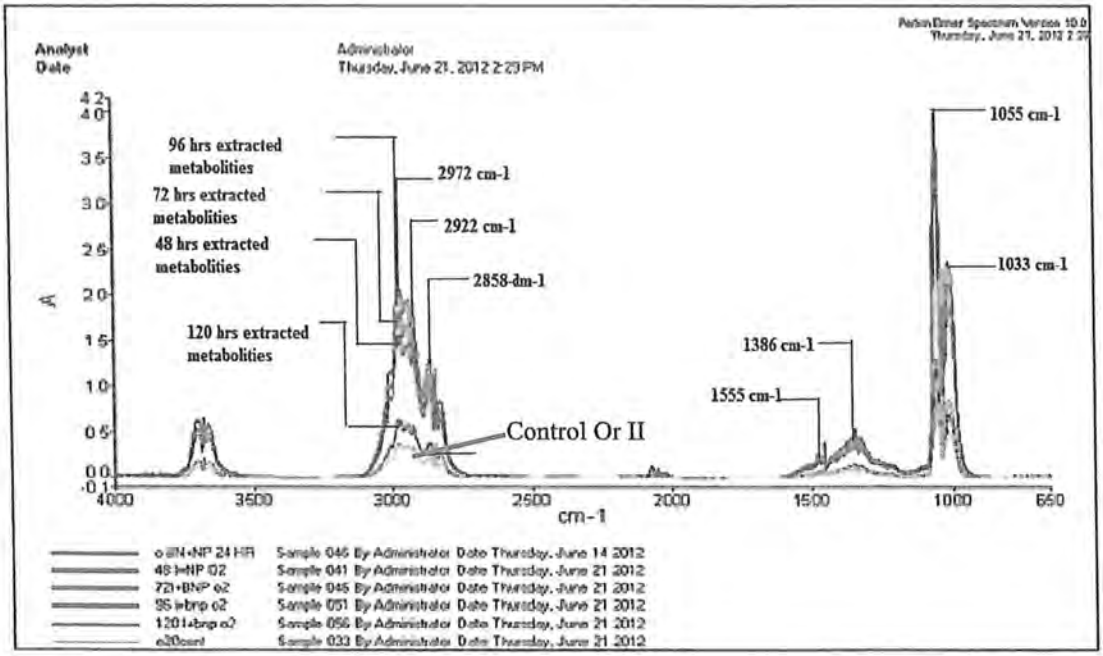


Fig. 4.28: Comparison of untreated dye with 24, 48, 72, 96 and 120 hrs extracted samples treated with *A. niger* + B Ag^0Nps ($100mgL^{-1}$)

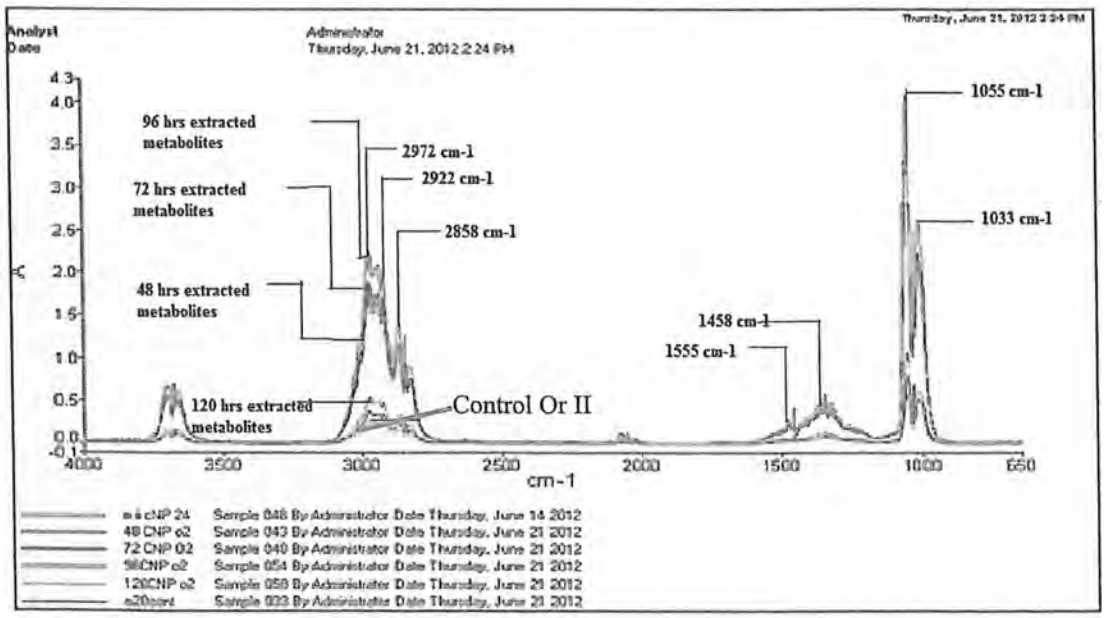


Fig. 4.29: Comparison of untreated dye with extracted samples treated with C Ag^0Nps ($100\ mgL^{-1}$) after 24, 48, 72, 96 and 120 hrs.

4.3. Phytotoxicity test of biodegradation byproducts of dyes

4.3.1. Effect of byproducts samples on radish seeds germination, root and shoot length after 3 and 5 days of experiment.

Byproducts formed during biodegradation of AR151 released in medium were evaluated for their toxicity by radish seed bioassay. Samples were taken after 240 hrs of incubation. Number of seeds germinated in each sample plate was counted after 3 and 5 days. Mean value of root and shoot length of each seedling was measured and percentage seed germination and inhibition was calculated. Use of radish seeds for assessment of phytotoxic effects was done by Turker *et al.*, 2008. Maximum seed inhibition was seen in case of untreated dye (50 mgL^{-1}). Decrease in root and shoot length in test samples of treated and untreated show inhibitory effect of dye and degradation metabolites on seed germination, and also on root and shoot growth. After 5 days of incubation untreated dye AR 151 (100 mg/l) showed 60 % seed germination inhibition. In contrast there was 15 % germination inhibition when metabolites produced after decolorization were applied. But growth in presence of metabolites was not normal as compared to distilled water. The root and shoot lengths were reduced by 17 % (Table 4.1).

Sample/Ag^0NPs	Seed germination (%)Total seeds 60)		Seed inhibition (%)	Mean value of seedling Root length (cm)	Mean value of Shoot length (cm)
	after 3 days	after 5 days	after days 5	after 5 days	after 5 days
MSM	60	75	25	6.1	6.05
AR151(20ppm)	45	55	45	4.1	4.8
AR151(50ppm)	30	40	60	2.7	3.5
BAg^0NPs (100ppm)	40	60	40	6.7	8.8
Dye(50ppm)+*A. niger*	40	55	45	2.5	3.5
Dye(50ppm)+*A. niger*+ BAg^0NPs (100ppm)	50	65	35	3.7	4.4
Dye(50ppm)+BAg^0 NPs (100ppm)	55	70	30	4.7	5.07
Dye(50ppm)+Cag^{0}N Ps (1000ppm)	55	70	30	5.9	7.2

Table: 4.1: Effect of byproducts samples on reddish seed germination, shoot and root length after 3 and 5 days of experiment.

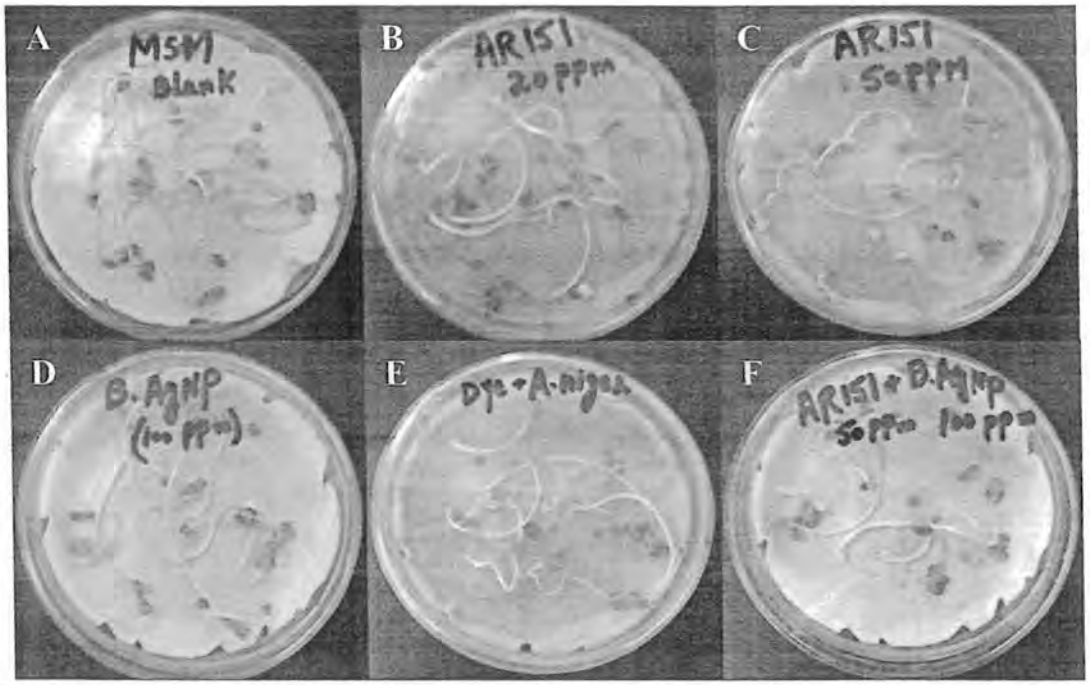


Fig 4.29: Effect of treated and untreated dye samples on raddish seed germination (A) Effect of msm on seed germination (B) Effect of 20 ppm untreated dye on seed germination (C) Effect of untreated AR 151 (50mgL^{-1}) on seeds germination (D) Effect of B Ag^0Nps (100mg/l) solution on seed germination (E) Effect of dye sample treated with *A. niger* (F) Effect of treated dye sample treated with B Ag^0Nps (100 mg/l)

4.4. Cytotoxicity assay of biodegradation byproducts

Brine shrimp assay:

Cytotoxic evaluation of byproducts formed during the process of degradation of AR 151 after 240 hours of incubation showed relative reduced mortality of nauplii as the treatment time of dye increased from zero hrs to 240 hrs. Death rate of naupli was 70 % when samples of 0 hr were examined. Death rate of brine shrimp nauplii showed that untreated dye was comparatively more toxic in nature while degradation byproducts were toxic to animal cell only up to 11% (Fig 4.30).

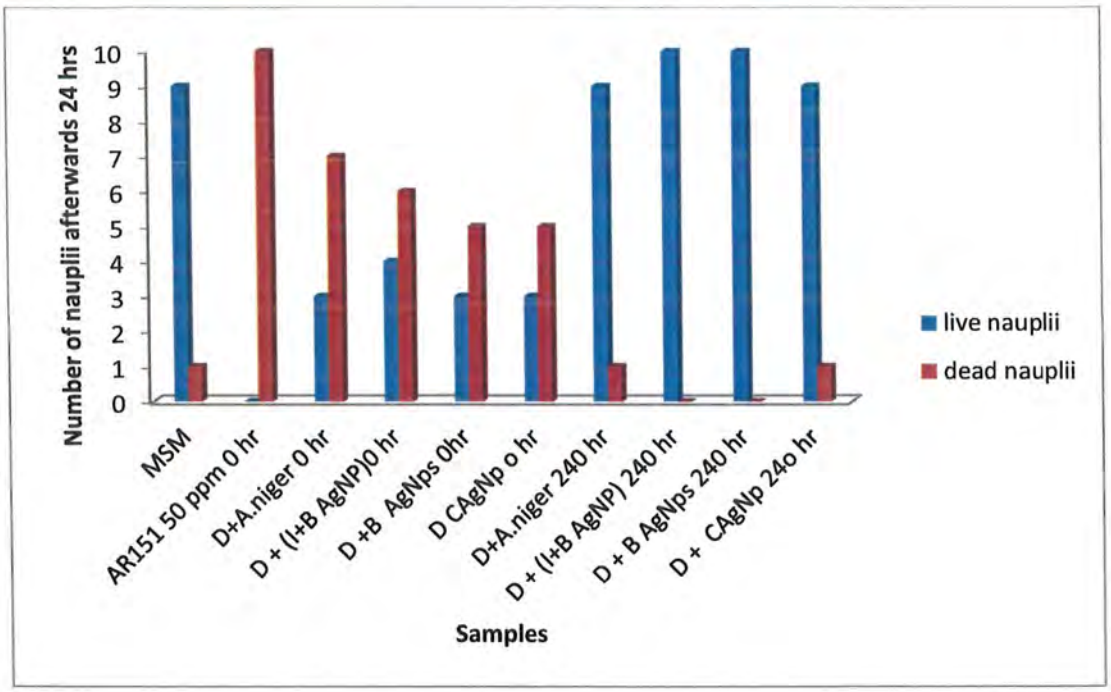


Fig. 4.30: Ratio of live and dead nauplii after 24 hours of experiment

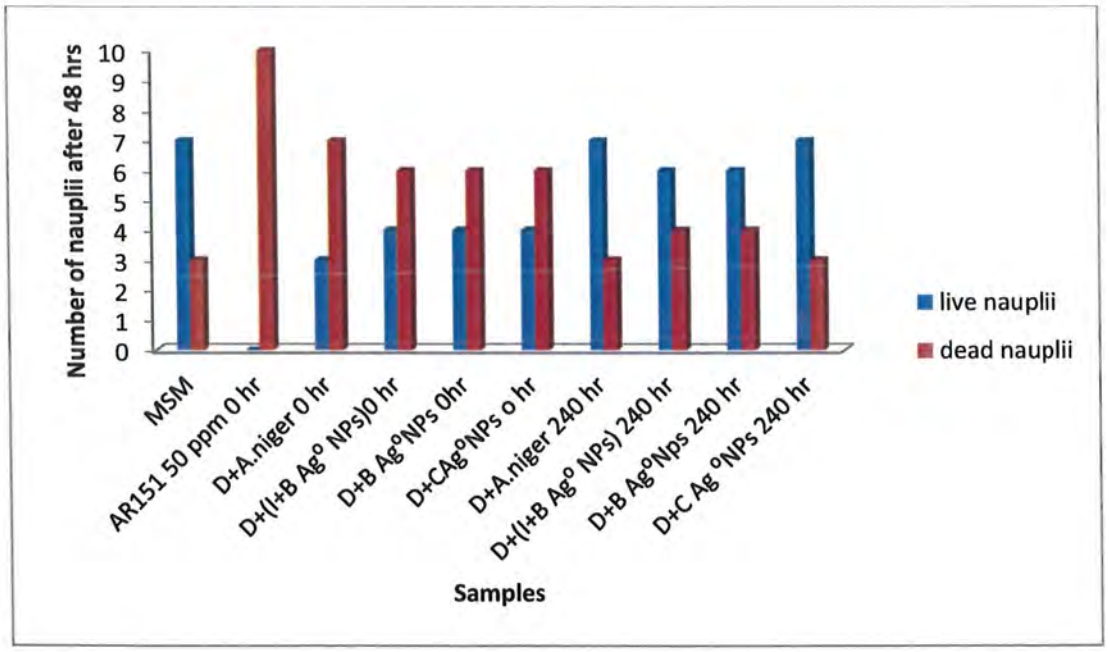


Fig 4.31: Ratio of live and dead nauplii after 48 hours of experiment

Chapter 5

DISCUSSION

Discussion

Dyes are extensively applied in the textiles, food, leather, paper, plastics, and cosmetic industry to color different materials. Organic dyes are commonly seen in many industrial discharges being chemically stable and recalcitrant in nature (Alzaydien *et al.*, 2009). These dyes being mutagenic and carcinogenic pose very serious threats to health and environment (Crini *et al.*, 2006). These colored effluents hamper photosynthetic processes as it decrease sunlight penetration and reduce oxygen level which results in suffocation and death of aquatic flora and fauna (Cheung *et al.*, 2009; Hu *et al.*, 2001). Waste water effluent from textile industry is considered as one of most important source of pollution because they are fast in color and are recalcitrant in nature. During dyeing processes, about 15-20 % of them are disposed of in wastewaters (Maynard *et al.*, 1983). Treatment of such waste water is very difficult because these dyes are resistant to oxidizing agents heat and light (Yee *et al.*, 2005). Azo dyes used in different industries are most important group of pollutants in waste water effluents (Hun and Wang, 1999; Kiwi *et al.*, 1993; Li *et al.*, 2004).

Most important criterion for using these dyes in textile industries is that they must be stable to heat, light and microbial attack. So these dyes are not rapidly degradable and cannot be easily removed from water by conventional biological and chemical treatment processes (Tang and An, 1995). The advantage of photocatalytic degradation by semiconductors and metal nanoparticles is their better efficiency over conventional processes such as rapid oxidation of pollutant and no formation of toxic polycyclic byproducts (Hoffman *et al.*, 1995; Sobana *et al.*, 2005).

Metal based nanoparticles are recently extensively used for removal of toxic materials due to their unique properties such as high ordered structure, high thermal and mechanical strength, large number of reactive surface sites and large surface area (Kaewprasit *et al.*, 1998). So in present study laboratory synthesized silver nanoparticles and commercial silver nanoparticles were used to degrade AR 151 and Or II in STE. Effect of several parameters like initial dye concentration, initial pH, temperature, and photocatalyst amount have been reported by several previous investigations (Körbahti and Rauf, 2009, Khataee *et al.*, 2010; Sakkas *et al.*, 2010; Zhang *et al.*, 2010;; Chong *et al.*, 2010).

In present study previously isolated fungi *A. niger* was used in decolorization experiments of both dyes in shaking condition (150 rpm). *A. niger* gave maximum color removal of AR 151 (68 %) and Orange II (45 %) in presence of STE after 24 hrs. It is very advantageous to use STE for studying bioremediation of dyes if some additional carbon and nitrogen source is supplied as a co substrate (Kumar *et* al., 1998; Fujita *et al.*, 2000). Mechanism behind removal of the dyes is found to be the biosorption by fungi instead of extracellular decolorization. Similar results have been reported by different researchers (Aksu and Tazer, 2000; Fu and Viraraghavan, 2000; Zheng *et al.*, 1999).

Culture stability is significant parameter related to initialization of specific metabolic system of the fungus for enzyme production and gene expression (Mester *et al.*, 2000; Dong *et al.*, 2005). Continuous stirring in shaker incubator makes possible the through mixing of nutrient in medium. While aeration is a very important factor that is essential for production and stability of enzyme that play important role in decolorization of specific dye (Manu and Chaudhary, 2002). At high oxygen concentration LiP and MnP are produced in larger amounts. Same is the case for laccase production which is enhanced by agitation (Kuwahara *et al.*, 1984). Decolorization of different azo dyes by *A. niger* is observed to be affected by some parameters which include pH, temperature, light and concentration of dyes in effluent (Jacob *et al.*, 1998; Mester and Tien, 2000).

Initial pH of the medium is an important parameter which can seriously effect growth and metabolic properties of *A.niger*. After 24 hrs interval color removal of dye AR 151 by *A. niger* was maximum (42 % and 71 %) at pH 5 and pH 7. while at pH 3 & 9 it was 26, 49 % (Fig 4.4a). Degradation of Or II observed after 24 hrs at pH 3, 5, 7, and 9 was 43, 71, 63, and 33%. So optimum pH was found to be 5 for degradation of azo dyes by *A. niger*. So it can be inferred that pH have significant effect on the production of fungal enzymes and their role in adsorption of dyes. Similar results showed that pH for maximum efficacy of laccases is also 3-7 (Levin *et al.*, 2002 and). It has been reported that maximum activity of LiP and MnP occurs at pH range of 3 to 7 (Hossain and Ananyharaman, 2006; Sam and Yesilada, 2001). Our results are quite complimentary to these observations as the optimum pH is found to be 5. Ali *et al.*, (2007) reported that decolorization was more than 50 % at different pH levels but it was significantly higher i.e., 97% at pH 5 by *A. niger* .

Initial pH of medium has some effect on photocatalytic degradation. Bokare *et al.*, (2007) demonstrated that pH of dye solution is central parameter because it effects the ionization state of nanoparticles surface and all chemicals present in dye solution such as acids and amines. In decolorization of Orange G by Fe-Ni bimetallic nanoparticles, maximum degradation efficiency (99 %) was observed at pH 2, but with increasing pH rate constant of the reaction decreased but in extremely alkaline medium % degradation was also significantly high (98%). Decolorization of AR 151 by biologically synthesized and commercial silver nanoparticles was more than 85 % at all pH conditions in first 48 hrs. It was maximum i.e. 98 and 99 % at pH 3 and 9. While at pH 5 and 7 it was 91 and 89 % (Fig. 4.17c & 4.17d). Similar results were observed with Or II (Fig 4.17c & 4.17 d). In alkaline medium where there is excess of hydroxyl ions, photogeneration of OH radicles increases, which play main role as oxidizing agent and results in enhancement of degradation rate (Madhusudhana *et al.*, 2011). This study supports the present study in case of optimum pH.

Mesophilic temperature range was examined in this study i.e., 25-37 °C for the decolorization of dyes by *A. niger*. An optimum temperature 30 °C was found most suitable for maximum decolorization (95 %) of AR 151 in 96 hrs (Fig. 4.3a) by *A .niger* and 37 °C was observed to be maximum (92 %) for Or II (Fig 4.16a). In literature it is also reported that mesophilic temperature (25–35 °C) proved to be most suitable (95.71–97.55%) for decolorization of AR 151 (Ali *et al.*, 2007). These results are quite evident from present study.

Changes in temperature can affect the chemical reaction to a larger extent and also provide some insight into reaction mechanism. It is reported that lower temperature caused incomplete decolorization of dye Methyl orange within 60 minutes. Increase in temperature has positive effect on dye degradation. As the temperature of textile effluent is commonly higher so application of nanoparticles is just a convenient technology (Liou *et al.*, 2005; Fan, 2009). Similar results are found in our study. A temperature range of 25 to 37°C was applied. Maximum decolorization (95 %) was observed at 30 ^{0}C for AR 151 (Fig 4.3 b & 4.3c) and for Or II best decolorization (82 %) was observed at 37°C in first 24 hours (Fig. 4.16b & 4.16c).

Overall by increasing the initial concentration of AR 151 resulted in decreased decolorization by *A. niger*. Using different concentration of dyes showed that with increasing concentration of dye, degradation efficiency goes on decreasing (Ali *et al.*,

2007). Arora and Chande, (2004) reported that increasing concentration of dye speeds up decolorization efficiency. It can be explained on the basis that higher concentration triggers metabolizing properties of the fungus or fungus might have started using dye as a carbon source. Such results are quite obvious from the present results which reported that decolorization slightly increased with increasing concentration of AR 151 from 10-20 mg/l (Fig. 4.18a) but after that % degradation reduced up to 40% at 100 mg/l of dye concentration. It can be justified as a considerable concentration of dye (substrate) sometimes is compulsory for stimulating metabolic activity in an organism.

Results of present study showed that degradation efficiency first increase slightly and then decreases by increasing concentration of dye when treated by photocatalytic nanoparticles (Fig. 4.18c). Several studies reported similar results that degradation of dye is directly proportional to formation of hydroxyl ions on surface of catalyst and their probability of reaction with dye molecules (Tang *et al.*, 1995; El-Kemary *et al.*, 2010). With increase in dye concentration, interaction between OH radicles and dye molecules increases. With further increase in dye concentration, generation of hydroxyl radicals gets reduced on photocatalyst surface, because the active sites of catalyst get completely shielded by dye molecules. At much higher concentration of dye, photons reaching at the surface of photocatalyst get reduced in number which results in slower generation of OH radicles (Kumar *et al.*, 2008; Tang *et al.*, 1995).

With increasing amount of catalyst load, active sites get increase in number which consequently leads to generation of larger number of hydroxyl radicles. This results in increased decolorization rate. Further increase in catalyst concentration results in reduction in decolorization because of reduction in light transmission if quantity exceeds a certain limit (Madhusudhana *et al.*, 2011). These results are quite similar to our results in present study. Optimum results were found at 100 mgL^{-1}concentration of silver nanoparticles (Fig. 4.5 c & 4.17 c).

Photodegradation of dye by catalyst is dependent upon illumination time. % degradation of AR 151 was observed to be 99 % in presence of light at pH 5 using 100 mg/l of B Ag^0NPs. While in dark same experiment was performed but the % degradation was found to be 38 %. A report demonstrated that at intermediate light intensity for short duration enabled complete degradation of methylene blue (El-Bahy *et al.*, 2009). It is obvious from results that photocatalytic activity increases with

increasing illumination time and 99% color removal is observed after 1 hr. Intensity and duration of light affects the extent of dye degradation (Daneshva *et al.,* 2003; El-Bahy *et al.,* 2009).At low intensity (0–20 mW/cm^2) rate of degradation increases linearly as the light intensity increases. When the light of intermediate intensity is used, rate of degradation depends upon square root of light intensity (Herrmann *et al.,* 2010). While using light of high intensity, rate of degradation becomes independent of it. At low light intensity, formation of electron hole pair is predominant and recombination of electron hole pair is negligible. At higher light intensity a competition starts between electron hole combination and generation.It has been proved in literature that dye degradation initially increase with increasing light intensity (Sauer *et al.*, 2002; So *et al.*, 2002)

In present study, FTIR analysis of treated samples of dye by *A.niger* and silver nanoparticles showed significant difference in absorbance at various regions and new peaks at regions of 2800-2900 cm^{-1} and 1200-1500 cm^{-1} indicated degradation of already present compounds. Peaks in the untreated dye spectra showed C–H stretching of alkanes at 1471.74 $cm^{-1}$and S=O stretching of sulphur compound at 1207.48 cm^{-1}. Specifically Azoic nature of complex dye was confirmed by N=N and N–H bond of azides and amides at 2077.40 cm^{-1} (Bandara *et al.,* 1999). Whereas obvious changes in peaks at 2922.25 and 2858.60 cm^{-1}, denoted fluctuations in C-H region of alkanes, similar changes took place in regions of 1589 which indicates deformation of aromatic compounds. Changes in peaks at 1458 cm^{-1} and 1281 cm^{-1} and 1077 cm^{-1} represented the deformation occurring in N=O stretching of nitrosamines, C–N bond of aryl amines and S=O stretching sulfoxids (Jadhav *et al.,* 2009). Similarly Stylidi *et al.*, (2004) reported presence of peak at $1506cm^{-1}$ that characterize azo bond of Acid orange 7 as well as peaks at $1454cm^{-1}$ for phenyl ring vibrations, and bands at $1123cm^{-1}$ and $1050cm^{-1}$ represents coupling between sulphonate group and benzene.

On analyzing the treated and untreated dye samples through HPLC, peaks in same area with almost same retention time with reference to two standards compounds i.e., aniline and Sulfanilic acid were observed. These two metabolites were identified by comparing retention time and spectrum of sample peaks with standards. So we can predict that these two peaks represent the metabolites produced after degradation. Similar peaks with retention time of 4.66 min and 1.08 were observed in dye sample

treated by B Ag^0NPs and in combine treatment under optimized set of parameters. Sample treated by C Ag^0NPs also showed peak at similar position but these peaks were quite large and obvious. No such peak appeared in untreated dye sample..

The performance of biological treatment of dye AR 151 was evaluated by cytotoxicity and phytotoxicity assays. In present study, percentage radish seed germination was observed on 3rd and 5th day of experiment. Germination of seeds was greater in control (STE) as compared to test samples. Root and shoot length were negatively affected when seeds were grown in untreated samples of dye. The untreated dye at 50 mgL^{-1} showed 60 % seed inhibition. While seed inhibition was 30% when they were grown in dye samples treated with laboratory synthesized and commercial silver nanoparticles after incubation of 240 hrs. Jadhav *et al.*, (2008) reported the effect of untreated dye and by products present in treated dye samples on germination and growth of two plant seeds. After treating with 300 mg/l of untreated dye Methyl red, seed germination inhibition in *Sorghum bicolor* and *Triticum aestivum* was found to be 88 and 72 %. While treated dye metabolites did not show germination inhibition. Growth of shoot and root was as normal as in distilled water. Use of radish seeds for assessment of phytotoxic effects was done by Turker *et al.*, (2008).

Cytotoxic assay of degradation byproducts showed that the untreated dye sample was very toxic for aquatic important organism i.e., shrimps nauplii. Decline in number of death of nauplii was observed when treated dye sample after 240 hrs of incubation were used. While no live nauplii was observed in untreated dye samples There are several published whole animal bioassays for assessment of chemical toxicity (Helliwell *et al.*, 1996). Brine shrimps were used as a benchtop bioassays' for purification of bioactive natural products and found that they can be used as excellent choice for toxicity assessment of consumer products. This made an inference that dye metabolites are slight toxic to aquatic organisms.

Conclusion

- Laboratory synthesized fungus mediated silver nanoparticles can decolorize textile azo dyes just like commercially available silver nanoparticles which are very expensive and are also able to perform much better than the plain culture. By applying optimized physico-chemical parameters such as temperature, initial pH, irradiation time, initial concentration of dye and silver nanoparticles, degradation efficiency increased up to 100 %.
- Biologically synthesized Ag^0NPs degraded textile dyes more than 85 % at pH range of 3-9 which is very convenient for very acidic or alkaline textile effluents containing different acids and amines.
- B Ag^0NPs could be used as visible light photocatalysts for degradation of organic pollutants in water and air, as Ag^0NPs exhibits strong absorption in visible light region because of plasmon resonance of Ag^0NPs and possess much higher photocatalytic activity.
- FTIR spectra of treated samples showed significant changes in peaks of all treated samples of dyes. This indicates break down of complex organic compounds. Similarly in HPLC chromatograms formation of peaks with same retention time and area confirmed the degradation of dyes by B Ag^0NPs both in combined and separate treatment.
- Phytotoxicity analysis showed that by products formed after degradation by B Ag^0NPs as well as C Ag^0NPs cause slight germination inhibition of radish seed. Similarly cytotoxic assessment of degraded metabolites indicated slight toxic nature of treated samples therefore some more extensive study and research is required for their complete degradation

Future prospects:

- Current study has just given a suggestion that biologically synthesized Ag^0NPs have a great potential in bioremediation of organic dyes. Further understanding of degradation efficiency and their photo catalytic properties is much needed for their large scale commercial application and treatments of pollutants. In this context, various analytical tools like LC/MS ,GC/MS and surface enhanced Raman spectroscopy (SERS) should be applied to obtain correlation between chromophoric moieties of dye molecules and characteristic Raman bands. Thus the structural changes that follow, as a result of photocatalytic degradation, would be monitored by fluctuation in SERS spectra.
- Textiles dyes due to their diverse nature and structures, pose a great demand in proper handing and manipulation in biological treatment technologies. Though exposure of different dyes to nanomaterials trigger new horizons in field of environment protection, but still single metallic nanoparticles may not treat properly a wide variety of organic dyes. Therefore it is very interesting to formulate stable, reusable, and efficient nanomaterials that may carry out comprehensive mineralization of a wide range of dyes.
- Efforts should be made to find out complete mechanism of degradation of these dyes with proper identification of each metabolite at every step. Better understanding of degradation mechanism by Ag^0NPs can help in better control over reaction process.
- This report will lead to development of rational photocatalytic degradation procedure for other metal nanomaterial to be used for treatment of various organic dyes present in textile effluent.

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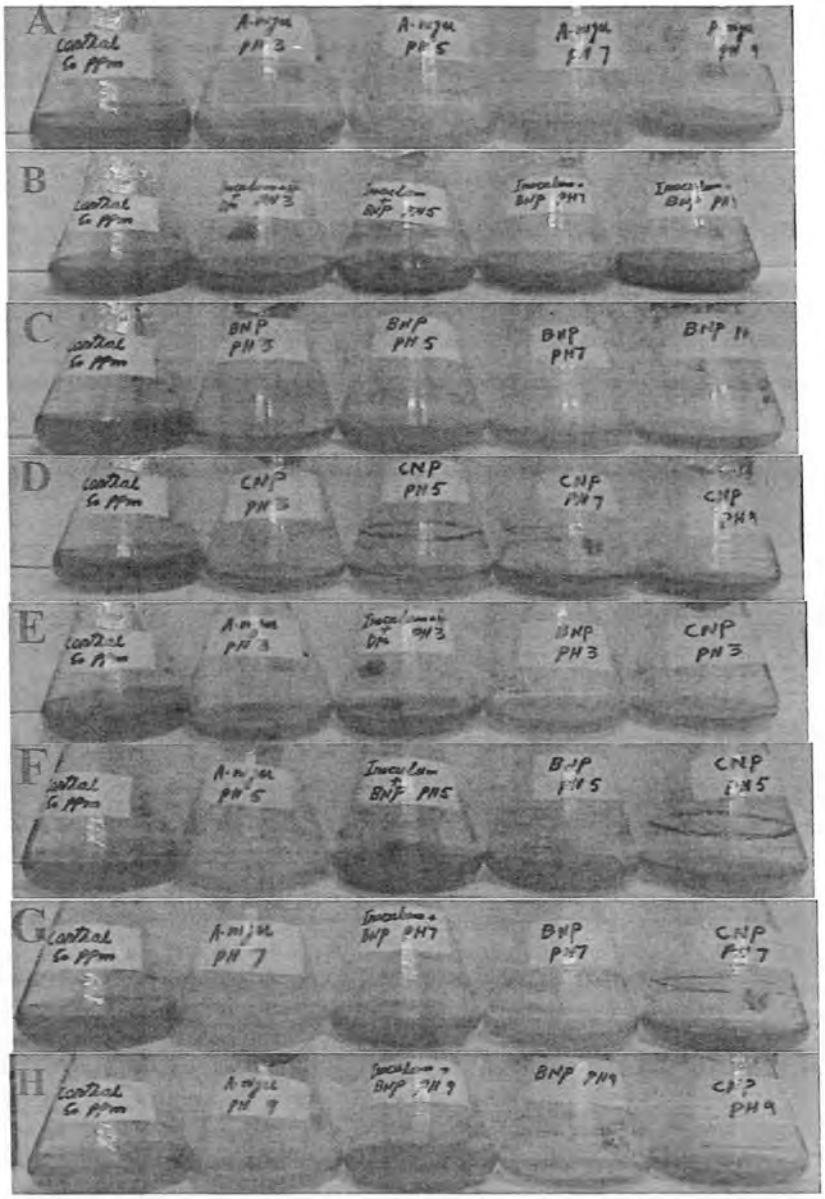
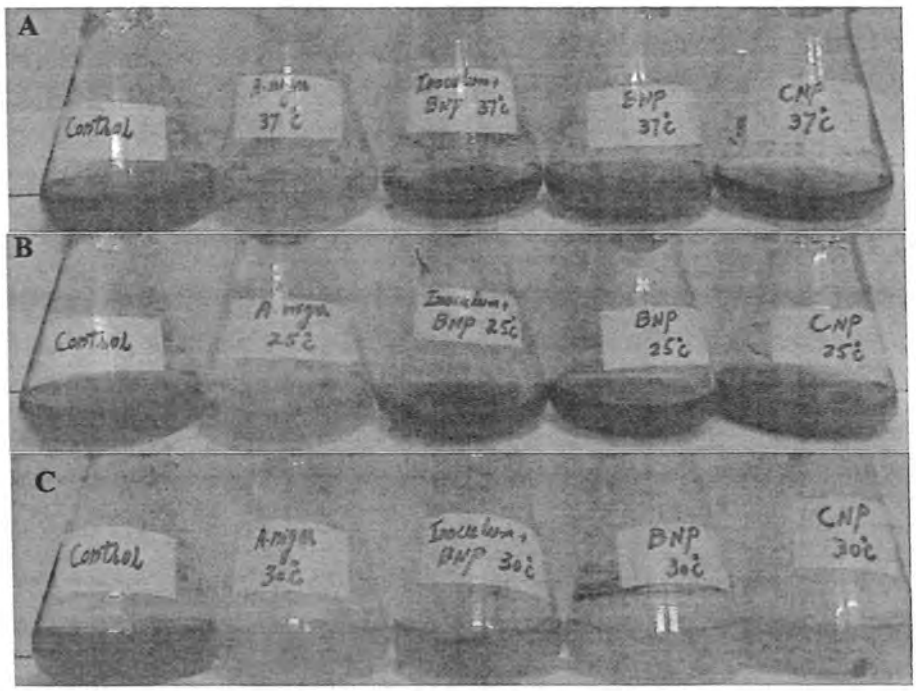


Appendix 1 (A): Effect of variation in pH on activity of *A. niger*, B Ag^0NPs , CAg^0Np (B) Effect of variation in pH on activity of *A. niger, A. niger* + B Ag^0NPs,B Ag^0NPs, CAg^0Np. (C) Effect of variation in pH on activity of *A. niger*,B Ag^0NPs , CAg^0Np,B Ag^0NPs(100ppm) (D) Effect of variation in pH on activity of C Ag^0NPs. (E) Comparison of AR 151 degradation by *A. niger*,B Ag^0N , CAg^0Np At pH3. (F) Comparison of AR 151 degradation *A. niger*,B Ag^0Np , CAg^0Np At pH 5.(G) Comparison of AR 151 degradation by *A. niger*, B Ag^0Np , CAg^0Np AT pH 7. (H) Comparison of AR 151 degradation by *A. niger*, B Ag^0Np, CAg^0Np At pH 9.



Appendix 2(A) Effect of variation in temperature on activity of *A. niger*, B Ag^0Np , CAg^0Np At 37 ^0C, (B) Effect of variation in temperature on activity of *A. niger* ,B Ag^0Np , CAg^0Nps At 25^0C, (C) Effect of variation in temperature on activity*A. niger* , B Ag^0Np , CAg^0Np At 30 ^0C

Appendix

AppendixA1: Effect of temp on activity of C Ag^0NPs on degradation of AR151

Time	25°C	30°C	37°C
0 hr	49.95	49.95	49.95
24 hrs	7.23	1.6	4.93
48 hrs	5.16	0	1.69
72 hrs	1.82	0	0
96 hrs	0	0	0

AppendixA2: Effect of temp on activity of B Ag^0NPs on degradation of AR151

Time	25°C	30°C	37°C
0 hr	49.95	49.95	49.95
24 hrs	9.11	2.07	4.88
48hrs	4.88	0.19	2.5
72hrs	0.9	0	0
96hrs	0	0	0

AppendixA3: Effect of temp on activity of (*A. niger* + BAg^0NPs)on degradation of AR151

Time	25°C	30°C	37°C
0 hr	50.4	50.4	50.42
24hrs	19.44	14.27	23.66
48hrs	2.72	0	3
72hrs	0.19	0	0
96hrs	0	0	0

AppendixA4: Effect of temp on activity of *A. niger* on degradation of AR151

Time	25°C	30°C	37°C
0 hr	49.4	49.5	50.94
24 hrs	26.01	14.7	29.7
48 hrs	5.35	3.94	10.9
72 hrs	0.35	0.18	5.35
96hrs	0	0	2.07

AppendixA5: Effect of pH on activity of *A. niger* on degradation of AR 151

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	49.95	49.95	49.95	47.31
24 hrs	23.66	17.9	14.74	27.89
48 hrs	19.44	5.3	7.7	22.25
72 hrs	13.33	3	2.07	14.27
96 hrs	4.56	2.09	0.19	10.25

AppendixA6: Effect of pH on degradation of AR 151 by combined activity of (*A. niger* + BAg^{0}NPs)

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	49.95	49.95	49.95	49.95
24 hrs	4.94	13.42	2.94	7.37
48 hrs	0.54	8.3	2.24	7.71
72 hrs	0	1.98	1.91	3.35
96 hrs	0	0	0	3

AppendixA7: Effect of pH on degradation of AR 151 by B Ag^{0}NP (100mg/l)

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	49.4	49.4	49.5	49.7
24 hrs	0.96	4.28	5.23	0
48 hrs	0	2.81	0	0
72 hrs	0	0	0	0
96 hrs	0	0	0	0

AppendixA8: Effect of pH on degradation of AR 151 by C Ag^0NP (100 mg/l)

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	49.4	49.4	49.41	49.41
24 hrs	2.19	3.72	5.8	2
48 hrs	1.42	0.63	0	0
72 hrs	0	0	0	0
96 hrs	0	0	0	0

AppendixA9: Effect of B Ag^0NP (100 ppm) on different.conc. of dye AR 151

Time	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	100 ppm
0 hr	9.12	21.33	30.25	37.76	50.91	101.14
24 hrs	0.2	1.14	3.49	11.94	21.33	68.75
48hrs	0	0	0	7.71	9.59	41.52
72 hrs	0	0	0	1.14	1.61	19.54

AppendixA10:Effect of C Ag^0NP (100 ppm) on different conc. of dye AR 151

Time	10ppm	20ppm	30ppm	40ppm	50ppm	100ppm
0 hr	9.12	21.33	30.25	37.76	50.91	101.14
24hrs	0	0.66	3	9.58	19.44	51.83
48hrs	0	0	0	7.71	9.59	37.76
72hrs	0	0	0	0	1.61	17.57

AppendixA11: Effect of (*A.niger* + BAg^0NPs)on degradation of different conc. of dye AR 151

Time	10ppm	20ppm	30ppm	40ppm	50ppm	100ppm
0 hr	9.12	21.33	30.25	37.76	50.91	101.14
24 hrs	0	0.19	2.53	7.74	16.15	45.73
48 hrs	0	0	0	3.02	9.59	42.46
72 hrs	0	0	0	0	2.55	7.24

AppendixA12: Effect of Fungion degradation of different conc. of dye AR 151

Time	10ppm	20ppm	30ppm	40ppm	50ppm	100ppm
0 hr	9.11	21.3	30.23	37.75	50.89	101.13
24 hrs	1.6	9.58	14.74	20.38	22.25	69.2
48 hrs	0	4.88	7.7	9.58	10.99	51.83
72hrs	0	0.66	1.6	2.07	3	37.75

AppendixA13: Effect of varying conc. of B Ag^0 NPs in combined treatment for degradation of AR 151

Time	10 mg/l	50 mg/l	100 mg/l	150 mg/l	200 mg/l
0 hr	49.5	49.5	50	50.2	51.3
24 hrs	23.84	13.38	11.4	9.58	7.7
48 hrs	6.6	4.27	1.92	1.6	1.13

AppendixA14: Effect of varying conc. of B Ag^0NPs on degradationof AR 151

Time	10 mg/l	50 mg/l	100 mg/l	150 mg/l	200 mg/l
0 hr	49.5	49.5	50	50.2	51.3
24 hrs	23.06	20.38	10.86	9.11	6.29
48 hrs	6.29	3.94	3	2.07	0.19

AppendixA15: Effect of varying conc. of C Ag^0NPs on degradationof AR 151

Time	10 mg/l	50 mg/l	100 mg/l	150 mg/l	200 mg/l
0 hrs	19.11	19.11	18.91	19.11	18.91
24 hrs	23.66	19.38	11.4	9.58	7.7
48 hrs	6.92	4.27	1.92	1.6	1.13

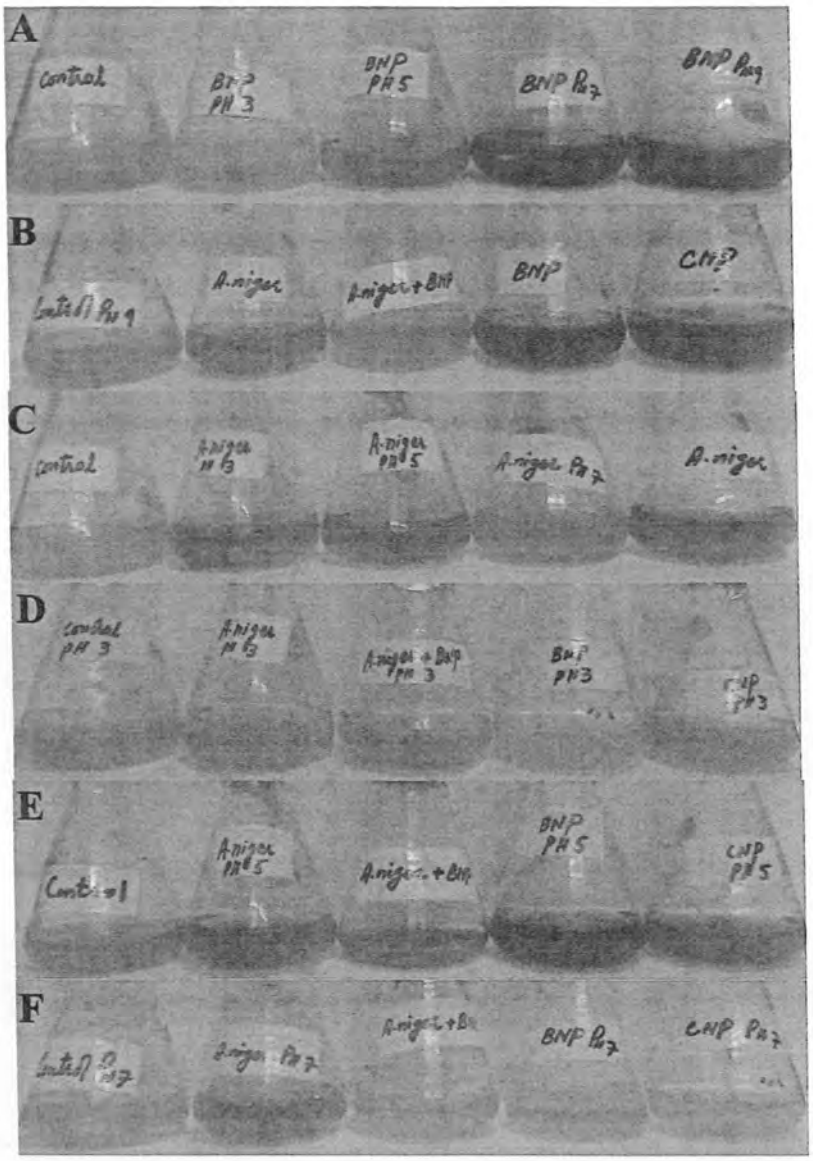


Fig A3 (A)Degradation of Or II pHEffect of variation in pH on activity of ,B Ag^0NPs . (B)Comparison of AR 151 degradation by*A. niger*, B Ag^0N , CAg^0Np At pH 9. (C) Effect of variation in pH on activity of *A. niger*.(D)Comparison of AR 151 degradation by *A. niger*,B Ag^0N , CAg^0Np At pH 3 .(E) Comparison of AR 151 degradation by*A. niger*,B Ag^0N , CAg^0Np At pH 5. (F) Comparison of AR 151 degradation *A.niger*,B Ag^0Np , CAg^0Np At pH 7.

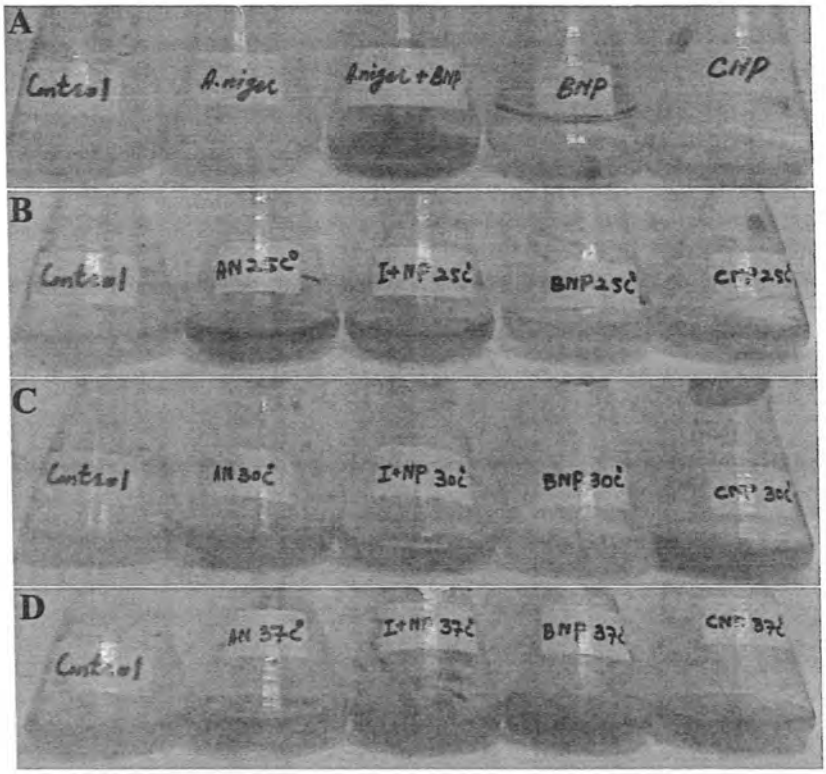


Fig. A4 Temperature dependent degradation of Or II.(B) Effect of 25 ^{0}C on degradation activity of *A. niger*,B Ag^{0}Np , CAg^{0}Np at 25^{0}C, (C) Effect 30 ^{0}C of on degradation activity of *A. niger* ,B Ag^{0}NP , CAg^{0}NPs at, (D) Effect of 37 ^{0}C on degradation activity*A. niger* ,B Ag^{0}NPs , CAg^{0}NPs.

AppendixA16: Effect of temp on degradation of Or II by *A.niger*

Time	25^{0}C	30^{0}C	37^{0}C
0 hr	19.31	19.31	19.31
24 hrs	10.42	8.61	4.36
48 hrs	3.35	2.95	1.33
72 hrs	2.34	0.93	0
96 hrs	1.33	0.52	0

AppendixA17: Effect of temperature on degradation of Or II by combined activity of (*A.niger* +Ag ^{0}NPs).

Time	25^{0}C	30^{0}C	37^{0}C
0 hr	19.31	19.31	19.31
24 hrs	8.4	6.38	2.34
48 hrs	6.38	2.34	0.933
72 hrs	4.36	0.933	0
96 hrs	2.14	0.32	0

AppendixA18: Effect of temp on degradation of Or II by B Ag^{0}NP.

Time	25^{0}C	30^{0}C	37^{0}C
0 hr	19.31	19.31	19.31
24 hrs	8.2	6.99	5.57
48 hrs	5.78	4.97	3.55
72 hrs	4.56	3.76	0.93
96 hrs	1.53	0.73	0.32

AppendixA19: Effect of temp on degradation of Or II by C Ag^0NPs (100 mg/l).

Time	25^0C	30^0C	37^0C
0 hr	19.31	19.31	19.31
24 hr	7.8	6.58	3.76
48 hrs	5.17	3.15	1.74
72 hrs	2.34	0.93	0.32
96 hrs	0.93	0.12	0

AppendixA20: Effect of pH on fungal degradation of Or II

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	19.31	19.31	19.31	19.31
24 hrs	10.83	5.78	6.99	13.05
48 hrs	8.2	4.36	6.38	10.22
72 hrs	6.99	2.95	4.56	6.99
96 hrs	4.56	0.93	2.95	5.17

AppendixA21: Effect of pH on degradation of Or II by combined activity of (*A.niger* +Ag ^{0}NPs).

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	19.31	19.31	19.31	19.31
24 hrs	8.81	7.39	10.22	13.05
48 hrs	6.99	5.78	5.37	9.4
72 hrs	3.96	3.35	5.17	6.99
96 hrs	2.34	1.13	2.95	4.56

AppendixA22: Effect of pH on degradation of Or II by B Ag^0NP (100mg/l).

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	19.31	19.31	19.31	19.31
24 hrs	5.57	9.21	11.23	3.55
48 hrs	3.55	6.99	7.6	1.53
72 hrs	1.53	4.56	5.78	0
96 hrs	0.32	2.14	4.77	0

AppendixA23: Effect of temp on degradation of Or II by C Ag^0NP activity (100 mg/l)

Time	pH 3.0	pH 5.0	pH 7.0	pH 9.0
0 hr	19.31	19.31	19.31	19.37
24 hrs	5.17	8.4	9.21	2.95
48 hrs	3.55	5.57	5.98	0.73
72 hrs	1.13	3.35	5.17	0.52
96 hrs	0	0.93	4.36	0

AppendixA24: Effect of varying conc. of dye Or II on degradation by *A. niger*.

Time	10mg/l	20mg/l	30mg/l	40mg/l	50mg/l	100mg/l
0 hr	10.83	19.31	29.21	40.52	50.83	96.32
24 hrs	4.5	9.8	23.55	35.68	43.76	68.32
48 hrs	4.1	8.6	21.53	29.62	37.7	49.41
72 hrs	3.55	5.98	16.48	21.53	29.62	39.72
96 hrs	0.93	2.34	6.38	14.67	21.53	33.66
120 hrs	0	0.93	3.76	10.22	12.24	14.67

AppendixA25: Effect of varying conc. of dye Or II on degradation by combined activity of (*A.niger*+B Ag^0NPs).

Time	10 mg/l	20 mg/l	30 mg/l	40 mg/l	50 mg/l	100 mg/l
0 hr	10.83	19.31	29.21	40.52	50.83	96.32
24 hrs	3.76	8.4	15.47	33.66	39.72	82
48 hrs	1.33	4.16	12.44	16.48	35.68	56.89
72 hrs	0.125	2.54	9.01	11.03	30.22	42.75
96 hrs	0	0.12	4.16	8.61	22.54	34.67
120 hrs	0	0	2.34	4.36	14.46	26.58

AppendixA26: Effect of varying conc of Dye on degradation by B Ag^0NPs (100 mg/l).

Time	10 mg/l	20 mg/l	30 mg/l	40 mg/l	50 mg/l	100 mg/l
0 h	10.83	19.31	29.01	40.52	50.22	95.1
24 hrs	4.36	10.42	19.56	34.67	39.72	85.4
48 hrs	2.34	6.38	14.46	21.53	34.06	60
72 hrs	0.52	2.14	9.21	9.01	21.53	40
96 hrs	0	0.32	4.36	6.99	13.66	32
120 hrs	0	0	1.33	4.56	10.42	25

AppendixA27: Effect of varying conc. of dye on degradation by C Ag^0NPs (100 mg/l).

Time	10 mg/l	20 mg/l	30 mg/l	40 mg/l	50 mg/l	100 mg/l
0 hr	11.03	21.35	29.41	41.33	50.83	98.3
24 hrs	3.76	9.41	21.53	31.64	35.68	61.94
48 hrs	1.74	5.78	11.84	15.68	22.54	49.822
72 hrs	0.12	1.74	5.57	13.86	15.68	39.72
96 hrs	0	0	0.73	9.62	9.62	27.6
120 hrs	0	0	0.32	4.16	4.16	11.43

AppendixA28: Effect of varying conc. of B Ag^0 NPs in combined treatment for degradation of Or II

Time	10 mg/l	50 mg/l	100 mg/l	150 mg/l	200 mg/l
0	19.32	19.11	18.91	18.71	18.5
24 hrs	13.06	10.42	8.2	6.99	5.57
48 hrs	10.43	6.99	4.36	2.95	0.52

AppendixA29: Effect of varying conc. of B Ag^0NP on Or II degradation

Time	10 mg/l	50 mg/l	100 mg/l	150 mg/l	200 mg/l
0 hrs	19.31	19.11	19.11	19.11	19.31
24 hrs	9.82	7.8	5.57	4.77	5.57
48 hrs	9.01	5.98	4.36	3.15	2.75

AppendixA30: Effect of different C Ag^0NP on Or II degradation

Time	10 mg/l	50 mg/l	100 mg/l	150 mg/l	200 mg/l
0 hrs	19.11	19.11	18.91	19.11	18.91
24 hrs	9.61	7.39	4.76	4.36	4.36
48 hrs	8.6	5.57	3.75	2.34	2.34