FUNGAL MEDIATED BIOTRANSFORMATIONS OF ORGANIC (CARBONYL GROUP) AND INORGANIC COMPOUNDS

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(IN CHEMISTRY)

BY

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JULY 2003

.....dedicated to my beloved parents

CERTIFICATE

Certified that the work incorporated in the thesis, **"Fungal mediated biotransformations of organic (carbonyl group) and inorganic compounds"** submitted by **Mr. Deendayal Mandal,** for the Degree of **Doctor of Philosophy,** was carried out by the candidate under my supervision in the Catalysis Division, National Chemical Laboratory, Pune 411 008, India. Material that has been obtained from other sources is duly acknowledged in the thesis.

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ACKNOWLEDGEMENTS

I take this opportunity to express my reverence to my research supervisor ***Dr. Rajiv Kumar*** *who introduced me to a fascinating realm of chemistry. His inspiring guidance and constant motivation have helped me to understand better and remain optimistic. I am grateful to him for giving me liberty in the work undertaken and for continuous encouragement during the course of the present study. Although this eulogy is insufficient, I preserve an everlasting gratitude for him.*

My heartfelt thanks to Dr. M. I. Khan, Dr. A. Ahmad, Biochemical sciences Division and Dr. M. Sastry, Materials Chemistry Division, for their motivation, subtle guidance, constant help, technical support in exploring the new fields like biotransformation and nanotechnology.

I am thankful to Dr. S. Sivasanker, Head of Catalysis Division, Dr. A. V. Ramaswamy, former Head of Catalysis Division, and Head of Biochemical Sciences Division, NCL, for providing divisional facilities.

I am grateful to all scientific and non-scientific staff members of the Catalysis and Biochemical Sciences Division for all their help.

The invaluable help I received from Dr. M. Bhadbhade, Mrs. R. Parischa, Mr. R. Gonnade for TEM and Dr. S. R. Sainkar for SEM is also appreciated.

I always had a hearty inspiration from my labmates: Priyada, Subhash, Chitta, Anirban, Senapati, Mahesh, Raina, Upadhyay and Amit. This work remained incomplete without their help, cooperation and discussions.

I am also thankful to my numerous friends: Aditya, Annyt, Amrita, Ambarish, Anjanda, Anamitra, Anand, Anil, Anuradha, Arindam, Ashavani, Atul, Abhaya, Bhaskar, Bibhas, Bikash, Bennur, Biju, Chanchal, Chidambaram, Debasis, Dibyendu, Debdut, Dhana, Dhanashree, Dilip, Feroz, Gourda and Nandita boudi, Halder, Kavitha, Kala, Karuna, Kartick, Kausik, Kusum, Lahada, Late Golok, Mantri, Mukul, Mahuadi, Mannada, Boudi and Nitu, Mr. and Mrs. Murugan, Muthukumar, Nirmalya, Pai, Pallavi, Pandeyji, Prabal, Prabhas, Pradeep, Parthada, Pratap, Priti, Raja, Rahman, Rita, Sachin, Shiju, Saikat, Sailesh, Samanta, Sankar, Saptarshida, Soumitra, Shabana, Shamina, Shahida, Sharda, Shainaz, Siddhartha, Smriti, Somnath, Sujata, Subarnada, Sumanda, Sushamadi, Sukhen, Subho, Surendran, Subhendu, Tarasankar, Thomas, Tressa, Tikla, Tarun, Utpal, Vandana and Venkatesan for their help and charming company.

I am obliged to my parents, sisters, Tushar and other family members for their love, unfailing support, tremendous patience, sacrifice and encouragement they have shown in their own way during my long period of studies. They have been a constant source of strength and inspiration for me.

I am grateful to my teachers who inspired my interest in chemistry and ushered me towards a career in science.

Finally, my thanks are due to Council of Scientific and Industrial Research, New Delhi, India for financial support and Dr. P. Ratnasamy, former Director and Dr. S. Sivaram, Director, NCL, Pune for allowing me to carry out my research and extending all possible infrastructural facilities at NCL, and permitting me to submit the present work in the form of thesis.

(Deendayal Mandal)

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Abbreviations

ATCC	American Type Culture Collection
BV	Baeyer-Villiger
BY	Baker's yeast
BVMO	Baeyer-Villiger monooxygenase
CHMO	Cyclohexanone monooxygenase
Confg	Configuration
Conv.	Conversion
EDX	Energy dispersive X-ray
ee	Enantiomeric excess
FAD	Flavine adenine dinuecleotide
Fig.	Figure
FT-IR	Fourier-transform infrared
g	Gram
GC	Gas chromatography
GCIR	Gas chromatography infrared spectroscopy
GCMS	Gas chromatography mass spectrometry
h	Hour
MGYP	Malt extract-glucose-yeast extract-peptone
min	Minute
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotidephosphate
NADPH	Nicotinamide adenine dinucleotidephosphate (reduced)
NMR	Nuclear magnetic resonance
NCIM	National collections of industrial microorganism
NCIMB	National Collections of Industrial and Marine Bacteria
NRRL	Northern Regional Research Laboratory
XRD	X-ray diffraction
PDA	Potato-dextrose-agar
Sel.	Selectivity

SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TPABr	Tetrapropyl ammonium bromide
UV-Vis	Ultraviolet-visible
XRD	X-ray diffraction
ZSM-5	Zeolite with MFI structure

1.1 GENERAL BACKGROUND AND INTRODUCTION

Microbial transformations in general, and yeast-mediated transformations in particular, have been extensively used since the early days of mankind for the production of bread, dairy products and alcoholic beverages. All of these early applications used mixed cultures of microorganisms and all of these biotechnological operations have primarily been directed in the areas of agriculture and human nutrition. However, in 1862 Pasteur [1] laid a scientific foundation of one of these early applications, namely the oxidation of alcohol to acetic acid by using a pure culture of *Bacterium xylinum* [2]. Investigations of the oxidation of glucose to gluconic acid [3] by *Acetobacter aceti* and of sorbitol to sorbose by *Acetobacter* sp. [4] followed. The reducing action of fermenting yeast, *Saccharomyces cerevisiae*, was first observed by Dumas in 1874 [5]. He reported that, on addition of finely powdered sulfur to a suspension of fresh yeast in a sugar solution, hydrogen sulfide was liberated. The reduction of furfural to furfuryl alcohol under the anaerobic conditions of fermentation by means of living yeast [6,7] was the first "phytochemical reduction" [8] of an organic molecule described in literature. Numerous further enzymatic or microbial biotransformations, bioconversions, biodegradations and fermentations followed, and as Chaleff [9] pointed out, in the initial excess of enthusiasm [10] that invariably accompanies the birth of a new field [11], biotransformations were hailed as a solution that would ultimately displace traditional organic chemistry [12,13].

The uses of enzymes for the biotransformation of man-made organic compounds have been used for more than hundred years employing whole cells, organelles or isolated enzymes [14]. Biochemical methods represent a powerful synthetic tool to complement other methodology in modern synthetic organic chemistry. Biotransformations have a number of advantages when compared to the corresponding chemical methods. Economically, some

biotransformations can be cheaper and more direct than their chemical analogues and the conversion normally proceeds under conditions that are regarded as ecologically acceptable [15].

Microorganisms have been used with considerable success in biotechnological applications such as remediation of toxic metals [16], but reports on their use in the synthesis of nanomaterials have been rather limited.

1.1.1 Nature's Catalyst

At the molecular level, Nature's catalyst, the enzymes provide a remarkable enhancement in reaction rates and selectivity over the corresponding unanalyzed reactions [17]. A major selectivity advantage of biocatalysts over traditional systems includes formers ability to exhibit high stereoselectivity. Enzymes provide a means to utilize alternate feedstocks which cannot be selectively activated by conventional catalysts [18]. While enzymes represent the most efficient catalytic systems known, their impact on the chemicals industry relative to traditional catalysts is still small. However, biocatalysts are biodegradable and environmentally benign. These are important advantages for carrying out reactions from "Green Chemistry" point of view.

1.2 ADVANTAGES AND DISADVANTAGES OF BIOCATALYSTS

Advantages:

- Enzymes are very efficient catalysts and are environmentally acceptable.
- Enzymes act under mild conditions of temperature [20-40 °C] and in the pH range of 5-8. This also helps to minimize undesired side reactions.

- As enzymes are compatible, several biocatalytic reactions can be performed in one flask. This is advantageous for sequential reactions using multienzyme systems, as the isolation of unstable intermediates can be omitted.
- Enzymes are not bound to their natural role.
- Enzymes exhibit high selectivity. The three major selectivities they show are chemoselectivity, regioselectivity and enantioselectivity.

Disadvantages:

- Enzymes are provided by nature in one enantiomeric form.
- Enzymes require narrow operation parameters. If a reaction proceeds only slowly, under given parameters, there is only a narrow scope of alteration as it leads to deactivation of the protein.
- Enzymes display their highest catalytic activity in water, which is the least desired solvent for most of organic compound. But shifting of enzymatic reaction from an aqueous to an organic medium reduces its activity.
- Many enzymatic reactions are prone to substrate or product inhibition, which causes the enzymes to cease to work at higher substrate or product concentrations.
- Enzymes may cause allergies.

1.3 CLASSIFICATION OF BIOCATALYSTS

Biocatalysis presents a cross-section of recent advances in catalytic science and biotechnology. Enzymes are known to catalyze a myriad of different chemical reaction types. Table 1.1 summarizes the classification of enzymes based on function.

Table 1.1

Function based classification of some enzyme groups

Enzyme group	Reaction types	Potential products
1. Oxidoreductase	Oxidation	Alcohol, Epoxide, Lactone, Amino Acid, Sulphoxide
	Reduction	Alcohol, Lactone
2. Transferase	Hydroxy methyl transfer	Hydroxyamino acid
	Amino group transfer	Amino acid, Amine
3. Hydrolase	Ester hydrolysis	Alcohol, Carboxylic acid, Carboxylic ester
	Trans-esterification	Alcohol, Carboxylic acid, Carboxylic ester
	Nitrile/amide hydrolysis	Carboxylic acid
	Hydantoin hydrolysis	Amino acid
	Alkylhalide hydrolysis	Haloalkanoic acid, Alcohol, Epoxide
4. Lyase	C-C bond formation	Amino acid, Acyloin, Cyanohydrin
	C-O bond formation	Alcohol, Amino acid
	C-N bond formation	Amino acid
5. Isomerase	Lactone formation	Lactone

1.4 ISOLATED ENZYMES VERSUS WHOLE CELL SYSTEMS

The physical state of biocatalysts that are used for biotransformations can be very diverse. Isolated enzyme systems or intact whole organisms may be used for biotransformation depending on the factors such as:

a) The type of reaction.

b) If there are cofactors to be recycled.

c) The scale in which the biotransformation has to be performed.

Each approach has its own advantages and disadvantages. Many isolated enzyme systems are now commercially available or are relatively easy to isolate and they can be stable and easy to use, often giving single products. However, for some reactions in which a cofactor is used, the need to regenerate the cofactor can be an added complication. Whole organisms do not have this disadvantage and, although, they do tend to give more than one product, they are often cheaper to use than isolated enzyme systems [15]. The advantages and disadvantages for both the methods are given in the Table 1.2.

Table 1.2

Comparison of isolated enzyme and whole cell

Biocatalyst	Form	Advantages	Disadvantages
Isolated enzymes	Any	Simple apparatus, simple work-up, better yield due to higher concentration tolerance	Cofactor recycling necessary
	Dissolved in water	High enzyme activities	Side reactions possible, lipophilic substrates insoluble, work-up requires extraction.
	Suspended in organic solvents	Easy to perform, easy work-up, lipophilic substrates soluble, enzyme recovery easy	Low activities
	Immobilized	Enzyme recovery easy	Loss of activity during immobilization
Whole cells	Any	No cofactor recycling necessary	Expensive equipment, tedious work-up due to large volumes, low productivity due to lower concentration tolerance, low tolerance of organic solvents, side reactions likely due to uncontrolled metabolism
	Growing culture	Higher activities	Large biomass, more byproducts, process control difficult
	Resting cells	Work-up easier, fewer byproducts	Lower activities
	Immobilized cells	Cell reuse possible	Lower activities

1.5 OXIDATION REACTIONS

Oxidation constitutes one of the key steps for the introduction of functional groups into the raw material of organic synthesis. Commonly used raw materials include mainly an alkane, an alkene or an aromatic molecule. Traditional methodology is plagued by several drawbacks, such as:

- Many oxidants are based on toxic metal ions such as Cr, which are environmentally incompatible.
- Undesired side reactions are common due to a lack of specificity.
- Molecular oxygen as the cheapest and the most innocuous oxidant cannot be used efficiently.
- It is rather difficult to perform oxidation reactions with high regio- and stereoselectivity.

1.5.1 Oxygenases

Enzymes which catalyze the direct incorporation of molecular oxygen into an organic molecule are called 'oxygenases' [19-22]. Enzymatic oxygenation reactions are particularly intriguing since direct oxyfunctionalization of unactivated organic substrates remains a largely unresolved challenge to synthetic chemistry. This is particularly true for those cases where regio- or enantioselectivity is desired. On the other hand, highly selective oxygenation reactions can be achieved by means of biocatalysts.

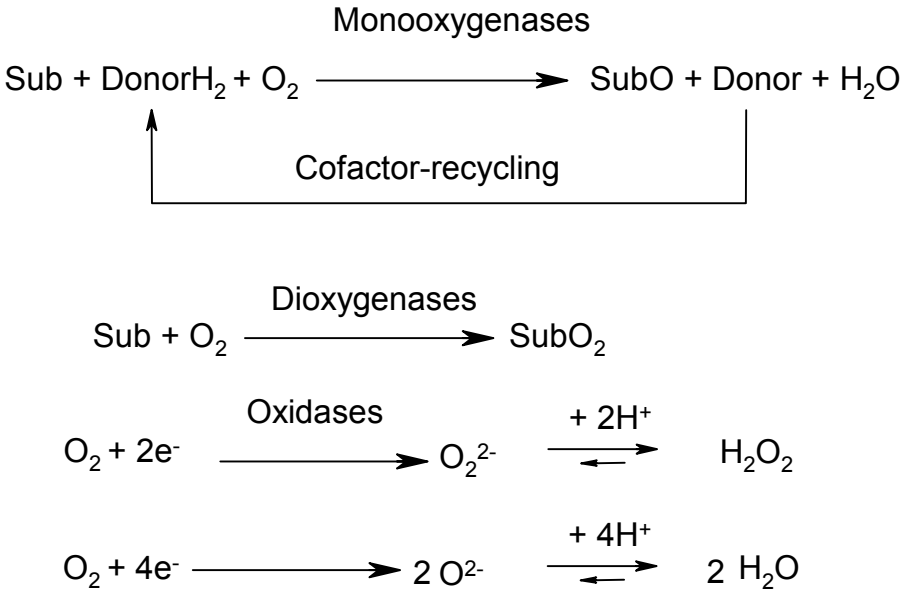
Oxygen transfer into organic acceptor molecules may proceed through three different mechanisms (Scheme 1.1).

i) **Monooxygenases** incorporate one oxygen atom from molecular oxygen into the substrate; the other is reduced at the expense of a donor (usually NADH or NADPH) to form water [23-

25]. The net reaction and a number of synthetically useful oxygenation reactions that may be performed by monooxygenases are shown in Scheme 1.2.

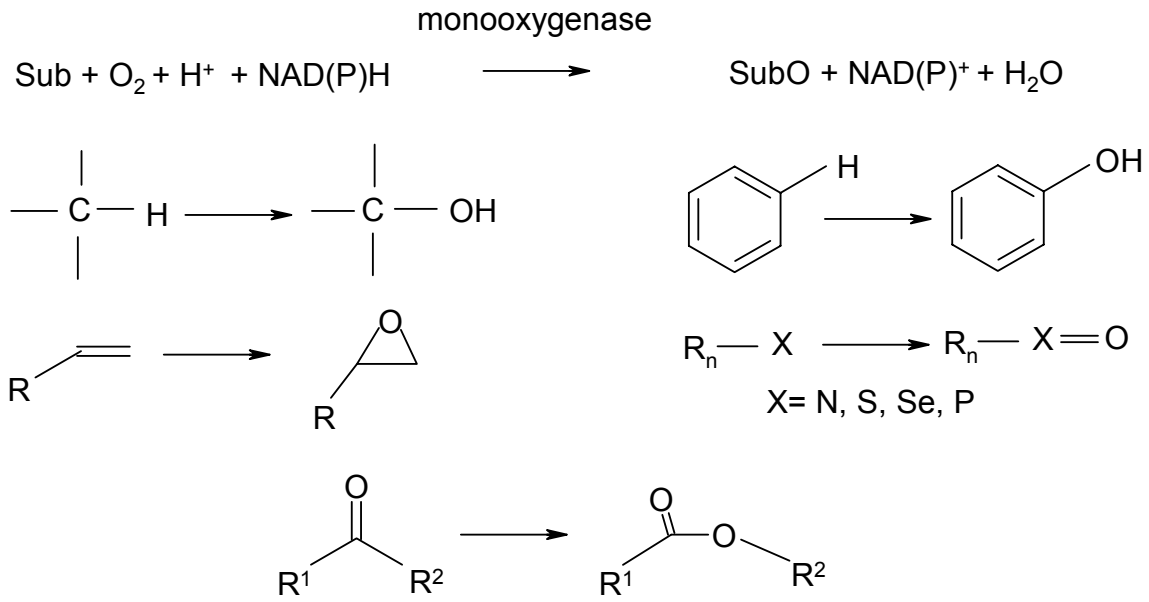
ii) **Dioxygenases** simultaneously incorporate both oxygen atoms of O_2 into the substrate, thus they are sometimes misleadingly called oxygen-transferases (although they are redox enzymes).

iii) **Oxidases**, on the other hand, mainly catalyze the electron transfer onto molecular oxygen. Oxidases include flavoprotein oxidases (amino acid oxidases, glucose oxidase), metalloflavin oxidases (aldehyde oxidase) and hemeprotein oxidases (catalase, H_2O_2-specific peroxidases). Some of the enzymes have been found to be very useful. For instance, D-glucose oxidase in combination with catalase is largely used as a food antioxidant [26]. However, from a synthetic viewpoint, oxidases have not been utilized extensively [27].



Sub: Substrate

Scheme 1.1 Enzymatic oxygenation reactions

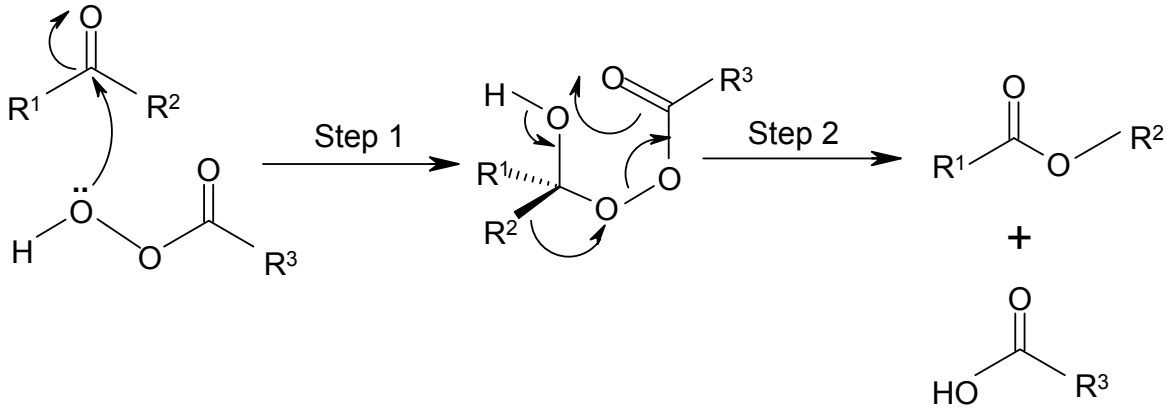


NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)

Scheme 1.2 Monooxygenase catalyzed reactions

1.5.2 Baeyer-Villiger oxidation

The transformation of an acyclic ketone to an ester or a cyclic ketone to a lactone using an oxidant such as peroxyacid was discovered by Baeyer and Villiger in 1899 [28]. The accepted two-step mechanism for the Baeyer-Villiger oxidation is shown in Scheme 1.3.



Scheme 1.3 Mechanism for the Baeyer-Villiger oxidation

Thus addition of peroxyacid to the carbonyl group of the ketone (step 1) creates a tetrahedral 'Criegee' intermediate [29]. Subsequently (step 2), this intermediate rearranges to form the corresponding ester or lactone. When different substituents are attached to the carbonyl carbon atom, the one that can better accommodate a partial positive charge migrates to the incoming oxygen atom (R^2 in Scheme 1.3). Consequently, the usual migratory preferance of alkyl groups in the Baeyer-Villiger oxidation is tertiary> secondary> primary> methyl [30, 31]. This order has been attributed either to the greater electron-releasing power [32] of, or to the steric acceleration of migration by, the larger group [30].

1.5.2.1 Chemical methods and reagents for the Baeyer-Villiger oxidation

Currently the most popular chemical oxidants for the Baeyer-Villiger reaction are *meta*-chloroperoxybenzoic acid, trifluoroperoxyacetic acid, peroxybenzoic acid, peroxyacetic acid and hydrogen peroxide. However, major problems may occur upon scaling up the reaction and extreme caution must be exercised in some of these cases due to the intrinsic instability of many of these reagents. A variety of other, more stable chemical oxidants, such as bis(trimethylsilyl) peroxide [33] and magnesium monoperoxypthalate [34,35], have been introduced in order to circumvent this problem. Many of the oxidizing agents used in the

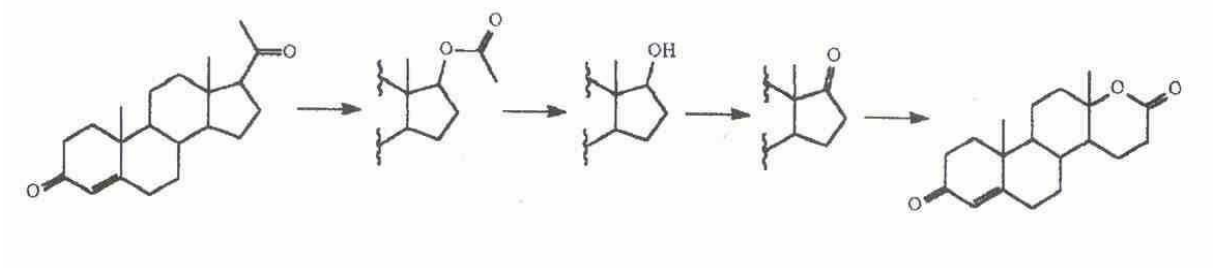
Baeyer-Villiger oxidation also react with a variety of functional groups such as alkene, amines, phosphines, sulfides and selenides, and as such, several reagents have been developed which allow chemoselective oxidation in the presence of other functional groups [36]. Within the last five years, another chemical oxidation system has been established. This new protocol use molecular oxygen as the oxidant, aldehyde as reductants and transition metal complexes, oxides or salts as the catalysts [37]. For example, Mukaiyama [38] has reported the use of nickel(II) complexes as catalysts for the Baeyer-Villiger oxidation.

Additionally iron(III) oxide [39], copper(II) and nickel(II) acetate [40], heteropolyoxometalates [41], hydrotalcites [42], iron(III) complex [43], ruthenium(IV) and manganese(IV) dioxides [44] have been reported to catalyze the Baeyer-Villiger oxidation.

Recently it has been reported that Baeyer-Villiger reaction can be successfully performed by using heterogeneous catalyst such as TS-1 or Sn-beta and H_2O_2 as oxidizing agent [45,46].

1.5.2.2 Monooxygenases enzymes as catalysts for the Baeyer-Villiger oxidation

In the early 1950s, Fried [47] and Peterson [48] noted that, during the incubation of progesterone with *Penicillium* species and *Aspergillus flavus*, testololactone was formed, apparently by sequential Baeyer-Villiger oxidations (Scheme 1.4).



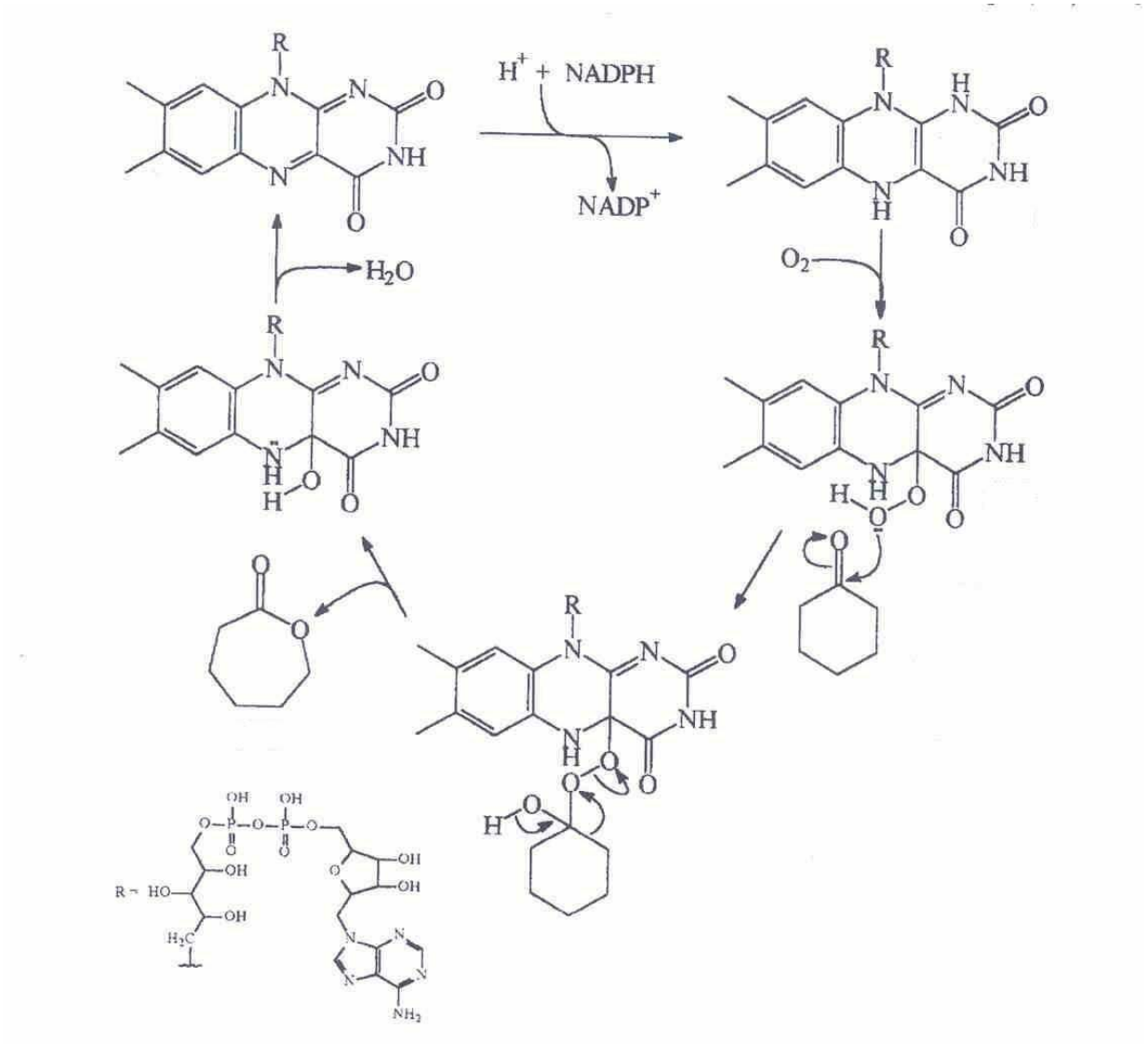
Progesterone Testololactone

Scheme 1.4 Baeyer-Villiger biotransformation of progesterone.

Although the enzyme(s) responsible was (were) not characterized, it was assumed that BVMOs (Baeyer-Villiger monooxygenses) were responsible for the ring-expansion reactions involved in these bioconversions. A large no of microorganisms for BV type oxidation has been reviewed by Roberts *et al*. [49].

Cyclohexanone monooxygenase (CHMO), initially isolated from *Acinetobacter calcoaceticus*, has been purified and characterized. Scheme 1.5 depicts the proposed mechanism of Baeyer-Villiger oxidation by cyclohexanone monooxygenase (CHMO), which is Flavin-dependent monooxygenase. The prosthetic group FAD (which is tightly bounded to the active site of the enzyme) is reduced to $FADH_2$ by NADPH. The $FADH_2$ so formed is oxidized in the presence of molecular oxygen yielding a hydroperoxide species (FAD-4a-OOH) which is a potent oxidizing agent. Addition of 4a-hydroperoxyflavin to the carbonyl group of cyclohexanone creates a tetrahedral intermediate. This tetrahedral intermediate rearranges to give FAD-4a-OH and ε-caprolactone. Finally, a water molecule is eliminated from FAD-4a-OH to regenerate FAD ready for a subsequent catalytic cycle.

In Table 1.3 a list of various biocatalysts (fungi, bacteria, actinomycete) used for Baeyer-Villiger type oxidations is given alongwith corresponding references.



Scheme 1.5 Proposed mechanism of action of CHMO in the enzymic Baeyer-Villiger oxidation (Reproduced from ref. 49)

Table 1.3

Literature search of different whole cell biocatalysts for Baeyer-Villiger type oxidation

Biocatalyst	Type of biocatalyst	Ref.
Acinetobacter calcoaceticus NCIMB 9871	Bacteria	[50-61]
Acinetobacter TD 63	,,	[50-52, 54,55]
Pseudomonas sp. NCIMB 9872	,,	[59,60,62]
Pseudomonas putida NCIMB 10007	,,	[63-67]
Xanthobacter autotrophicus NCIMB 10811	,,	[68]
Pseudomonas putida AS 1	,,	[69]
Acinetobacter junii	,,	[69]
Rhodococcus coprophilus WT1	Actinomycete	[69]
Rhodococcus fascines WT 13	,,	[69]
Curvularia lunata NRRL 2380	Fungus	[70]
Cunninghamella echinulata NRRL 3655	,,	[71]
Drechslera australiensis	,,	[72]
Curvularia sp.	,,	[73]
Cylindrocarpon destructans ATCC 11011	,,	[74]
Beauveria bassiana ATCC 7159	,,	[75]
***Fusarium oxysporum* f. sp. *ciceri* NCIM 1282**	**Fungus (Present work)**	**[76]**

1.6 BIOREDUCTION

1.6.1 Reduction of carbonyl compounds

The synthesis of enantiomerically pure compounds is becoming increasingly important for research and development in chemistry and biochemistry [77], especially in the pharmaceutical industry, as chiral drugs now represent close to one-third of all pharmaceutical sales world wide [78]. In most of the cases, one enantiomer is more effective as a drug than the other. The influence on the environment is also different between the enantiomers; different enantiomers of chiral pollutants in soils are preferentially degraded by microorganisms in various environments [79]. Therefore, synthetic methods exhibiting extremely high enantioselectivities are necessary.

Asymmetric synthesis carried out with chiral metal complexes as catalysts has been successfully used [80,81], however, some difficulties often remain in attaining high optical purity and practical usage in comparison with the ones performed by enzymatic catalysis. In fact, in most cases the use of enzymes as biocatalysts dramatically improves stereochemical quality. Further, for biocatalysis, the reaction and separation steps are significantly simplified [82].

Biocatalytic reduction and oxidation, especially the removal and addition of hydrogen, are catalyzed by oxidoreductase enzymes. Purified reductases, which are useful for synthetic purposes, have the disadvantages that they need expensive cofactors like NADH and NADPH. Although several methods for chemical and biochemical recycling of the coenzymes have been found, this delicate problem still remains the main obstacle to a more general use of this useful category of biocatalysts.

1.6.1.1 Baker's Yeast (BY) mediated reductions

The asymmetric reduction of carbonyl-containing compounds by BY constitutes one of the most widely applicable reactions. Ketones with varying substituents (Me, Et, n-Pr, n-Bu, Bz) were reduced by BY, and the secondary alcohols obtained were mainly of *S*-configuration. Only 3-hydroxyheptanol (from the reduction of 3-heptanone) was predominantly *R*-configured. Sterically hindered ketones (e.g., 4-octanone, *tert*-butyl methyl ketone, isobutyl ketone, or n-amyl phenyl ketone) were not reduced at all. These results [83-84] suggested a hydrogen transfer to the *re* face of the prochiral ketone to yield alcohol. The reduction of aldehyde and ketones by means of fermenting BY is long known, and this subject has been covered in several reviews [85-89].

1.6.1.2 Microorganism mediated reductions

Microbial cells contain multiple dehydrogenases, which are able to accept non-natural substrates, all the necessary cofactors and the metabolic pathways for their regeneration. Thus, cofactor recycling can be omitted since it is automatically done by the cell. As a consequence, cheap carbon sources such as saccharose or glucose can be used as auxiliary substrates for asymmetric reduction reactions. Furthermore, all the enzymes and cofactors are well protected within their natural cellular environment.

The enormous potentiality of the microorganisms as biocatalysts is far from being completely exploited. Besides the general review by Yamada and Shimizu [90], excellent accounts on biocatalyzed asymmetric reductions should be mentioned [91].

These distinct advantages have to be taken into consideration alongside some significant drawbacks.

- The productivity of microbial conversions is usually low since the majority of non-natural substrates are toxic to living organisms and they are more active at the low concentrations range (0.1-0.3 %).
- The large amount of biomass present in the reaction medium causes low overall yields and makes product recovery troublesome, particularly when the product is stored inside the cells and not excreted into the medium.
- Chiral transport phenomena into and out of the cell may influence the specificity of the reaction, particularly when racemic substrates are used.
- Finally, different strains of a microorganism may posses different specificities, thus it is important to use exactly the same culture to obtain comparable results with the literature [92].

1.6.1.3 Reductions catalyzed by plant cell cultures

The biotransformations catalyzed by plant cell cultures have been recently reviewed [93-95]. Early examples of biotransformations were, for instance, oxidative and reductive transformations of biogenetic-like compounds of the terpene series. Thus, these transformations were performed on p-menthane derivatives with cultured cells of *Nicotiana tabacum* [96] or bicyclo[3.3.1]heptane derivatives with the same biocatalyst [97]. Early applications of plant cells to the biotransformation of synthetically important foreign compounds are for instance the reduction of β-ketoesters [98]. In this case, immobilized cells of *Nicotiana tabacum* on calcium alginate beads were used and the method suffered with low yields of the hydroxy esters. In this case of (*S*)-3-hydroxybutanoates the ee was often high and the stereochemistry was identical to the one realized with BY.

1.6.1.4 Dehydrogenase catalyzed reductions

The class of enzymes which can catalyze useful asymmetric reductions is that of oxidoreductases and many purified enzymes are already commercially available. The main disadvantage of these biocatalysts is that they need expensive cofactors, although many methods are currently available for their efficient regeneration. The application of dehydrogenases for the synthesis of chiral compounds has been recently reviewed [99,100]. Most of the application of dehydrogenases is related to the reduction of prochiral carbonyl compounds in order to prepare optically active hydroxy compounds.

1.7 BIOTRANSFORMATION OF METAL IONS

Microorganisms can transform heavy metal and metalloid species by, e.g. oxidation, reduction, methylation and demethylation [101,102]. Many bacteria, algae, fungi, and yeasts can reduce Au^{3+} to elemental Au^{0} [103] and Ag^{+} to elemental Ag^{0}, which deposits on glass surfaces and in and around growing colonies [104,105]. Microbial transformations of arsenic and chromium species are also associated with a decrease in toxicity and may have relevance to wastewater treatment [101]. Treatment of arsenic-loaded sewage with arsenite oxidase-producing bacteria (which oxidize As^{3+} to As^{5+}) can improve certain arsenic removal methods since arsenate (As^{5+}) is more easily precipitated from wastewater by Fe^{3+} than is arsenite (As^{3+}) [101]. Chromate (CrO_4^{2-}) -reducing bacteria, e.g., *Enterobacter cloacae*, are resistant to high levels of chromate (10 mM) and can anaerobically reduce CrO_4^{2-} to Cr(III) that is precipitated [106].

Many organisms, both unicellular and multicellular, are known to produce inorganic materials either intra- or extracellularly [107,108] examples of which include magnetotactic

bacteria (which synthesize magnetite nanoparticles) [109,110], diatoms (which synthesize siliceous materials) [108,109] and S-layer bacteria (which produce gypsum and calcium carbonate layers) [113,114].

1.7.1 Bioreduction of metal ions: Nanoparticle synthesis

Nanoparticles are extremely important materials in different areas ranging from nanotechnology, nonlinear optics, diode lasers, smart sensors, markers in drugs, gene sequencing to catalysis. Nanomaterials can be obtained by various chemical and physical methods. Some examples of physical methods are vapor deposition, lithographic processes and molecular beam epitaxy (MBE). Chemical methods include the popular borohydride and citrate reduction methods for the preparation of colloidal metal (like gold, silver etc.) particles. Reduction of metal ions by radiolysis is also a frequently used method for preparing nanosized metal particles.

However, the methods mentioned above suffer from drawbacks such as being environmentally hazardous (chemical methods) and result in the quick agglomeration of nanoparticles leading to big particles of poor monodispersity. Although specific capping agents are used in some of the above mentioned methods to restrict the size of the colloidal metal particles and to stabilize the particle size distribution, this makes the whole system quite complicated and user-unfriendly. Another disadvantage, particularly of the radiolysis method, is that it is quite complicated and gamma ray sources are not readily available.

Even though microbes have been used with considerable success in biotechnological applications such as remediation of toxic metals [115,116], reports on their use in the synthesis of nanomaterials have been extremely limited. Beveridge and coworkers have demonstrated

that gold particles of nanoscale dimensions may be readily precipitated within bacterial cells by incubation of the cells with Au^{3+} ions [114-119]. A detailed study of the growth of Au nanoparticles using *Bacillus subtilis* 168 revealed the initial precipitation of noncrystalline Au particles which subsequently transformed into crystalline octahedral gold containing sulfur and phosphorus [118]. More recently, Klaus-Joerger and coworkers have demonstrated that the bacteria *Pseudomonas stutzeri* AG259 isolated from a silver mine when placed in a concentrated aqueous solution of $AgNO_3$, resulted in the reduction of the Ag^+ ions and formation of silver nanoparticles of well-defined size and distinct morphology within the periplasmic space of the bacteria [120,121]. Furthermore, they have shown that biocomposites of nanocrystalline silver and the bacteria may be thermally treated to yield a carbonaceous material with interesting optical properties for potential application in functional thin film coatings [122].

Recently, we have observed for the first time that eukaryotic microorganisms such as fungi can reduce various metal salts ($HAuCl_4$, $AgNO_3$, $CdSO_4$) and produce nanomaterials [123-127].

1.8 CHARACTERIZATION OF NANOMATERIALS

A number of techniques have been used to characterize the nanomaterials. Each technique is unique by itself and provides important information for the understanding of different structural features of the nanomaterials. Among all, the most commonly used spectroscopic (UV-Vis, X-ray diffraction) and microscopic (scanning electron microscopy, transmission electron microscope) methods are essential for thorough characterization of these materials.

1.8.1 UV-Vis Spectroscopy

Absorption spectroscopy in the UV-visible region has long been an important tool for the nanomaterial characterization. Color transitions arise due to molecular and structural changes in the substances being examined, leading to corresponding changes in the ability to absorb light in the visible region of the electromagnetic spectrum. Appearance of color arises from the property of the colored material to absorb selectively within the visible region of the electromagnetic spectrum. Absorption of energy leads to a transition of electron from ground state to excited state. Most of the spectra are very broad, smooth curves and not sharp peaks. Colloidal solutions of gold nanoparticles exhibit a deep red color due to the well-known surface plasmon absorption in the range of *ca*. 500-550 nm, whereas in case of silver it absorbs in the range of *ca*. 400-450 nm. This surface plasmon resonance is caused by the coherent oscillation of the free conduction electrons induced by light.

1.8.2 Scanning Electron Microscopy (SEM)

This is another important tool for morphological characterization of nanomaterials. Specially in case of intracellular biosynthesis of nanomaterials the formation of the nanomaterials on the fungal mycelia can be located by this technique.

1.8.3 Transmission Electron Microscopy (TEM)

This is one of the powerful tools for nanomaterial characterization. TEM is unique because it can provide a real space image on the atom distribution in the nanocrystal and on its surface [128]. Nowadays TEM is a versatile tool that provides not only atomic-resolution lattice images, but also chemical information at a spatial resolution of 1 nm or better, allowing

direct identification the chemistry of a single nanocrystal. With a finely focused electron probe, the structural characteristics of a single nanoparticle can be fully characterized. This is quite useful tool for the determination of particle size of the nanomaterials. The diffraction pattern of the single crystal is obtained by using this technique and it helps to derive the orientation of the crystal. It should be mentioned that the voltage of the electron beam is kept limited for the characterization of biosynthesized nanomaterials, as the biofilm can be damaged by high voltage of electron beam.

1.8.4 X-Ray Diffraction (XRD)

X-ray methods of characterization represent a powerful approach to the study of nanophase materials. The advantage of these techniques is to provide meaningful ensemble-averaged information about both medium range, and local, atomic structure in nanosystems.

The very small grain size of clusters in nanophase materials gives their diffraction pattern the appearance of an amorphous material. The difficulty in determining their structure by X-ray diffraction, however, is imposed at a fundamental level by two features of these systems: the small size of structural domains that characterize the diffraction pattern; and the occurrence of highly symmetric, but, noncrystalline structures. In general, regardless of structure, there is a steady evolution in the aspect of diffraction profiles: as particles become larger, abrupt changes do not occur, features grow continuously from the diffraction profile and more detail is resolved. These observations form the basis for a direct technique of diffraction pattern analysis that can be used to obtain structural information from experimental diffraction data.

The size of the nanoparticle can also be obtained by this technique using the Debye-Scherrer equation ($D = n\lambda / \beta\cos\theta$) by determining the width of the (111) Bragg reflection in the XRD pattern.

1.9 SCOPE / OBJECTIVES OF THE THESIS

In the present investigation, around 24 fungal strains were screened for the biotransformation of ketone taking cyclohexanone as a model substrate. Fungus *Fusarium oxysporum* f. sp. *ciceri* NCIM 1282 has been found to be a new biocatalyst among them for the biotransformation of carbonyl compounds, both cyclic aliphatic and aromatic ketones. The effect of different parameters such as time, biomass concentration was studied in detail for cyclohexanone reaction. The adaptability of the fungus toward different ring sized cyclic ketone was studied. It was observed that Baeyer-Villiger action of the above biocatalyst was specifically active for C_4-C_6 cyclic ketones. The potentiality of the biocatalyst toward different substrate (cyclopentanone and cyclohexanone) concentration was studied, results shown that it could act upto a certain level of the substrate, beyond that it (substrate) became toxic to the biocatalyst. The effect of inorganic solid as additive in the above biotransformations was also studied. It worked in case of cyclobutanone and cyclohexanone. The above fungus converts the cyclic ketone to its corresponding alcohols followed by the lactone formation.

Exploration of different fungi for the enantioselective bioreduction of ketones also forms an important part of the present dissertation. In this context, 21 fungal strains were screened for the bioreduction of ketone taking acetophenone as a model substrate and the results are tabulated in the present dissertation. The fungus *Trichothecium* was found to be an effective biocatalyst for such type of bioreduction, for the first time. Reaction time, amount of

biomass and growth of the fungus was standardized for acetophenone reduction. The addition of surfactant was investigated whether it could improve the conversion of acetophenone, in vain. The potentiality of the fungus, *Trichothecium* was studied towards higher concentration of acetophenone and thus checked the toxicity of the substrate for the above fungus. The fungus has been exploited for the reduction of some other prochiral ketones also.

Observing the reducing property of the fungus it was applied for metal ion reduction. Interestingly it worked and gold nanoparticles were obtained from aqueous solution of chloroauric acid when exposed to the same fungus, *Fusarium oxysporum* f. sp. *ciceri* NCIM 1282. Hence, the scope of the present work was extended to study the bioreduction of metal ions by using fungi producing metal (Au, Ag) nanoparticles, both intracellularly [123,124] as well as extracellularly [125,126]. The bioreduction of aqueous $AuCl_4^-$ and $AgNO_3$ by another fungus *Verticillium* has been studied during present work, where the reduction of the noble metal ions occurs on the surface of the mycelia as well as on the cytoplasmic membrane leading to the formation of gold and silver nanoparticles of fairly well defined dimensions and good monodispersity.

The major objective of the thesis was to explore some reduction / oxidation properties of fungi for the biotransformations of organic carbonyl group and metal ions.

1.10 OUTLINE OF THE THESIS

The thesis has been divided into five chapters.

Chapter one represents a general introduction to biocatalysis and earlier literature on the different oxidation/reduction properties of the microorganisms towards carbonyl compounds. It also describes the reduction of inorganic compounds by different microorganisms. The purpose and aim of fungal mediated oxidation/reduction reactions of carbonyl compounds and inorganic compounds are described at the end of this chapter.

Chapter two describes the screening of different fungi for the Baeyer-Villiger type biotransformation of carbonyl compounds where cyclohexanone was taken as model substrate. The fungus *Fusarium oxysporum* was found to be a new biocatalyst that can perform this type of biotransformation. This chapter also describes the detailed study of Baeyer-Villiger type biotransformations of different simple monocyclic ketones with the above fungus. The effect of different inorganic solid such as alumina, silica and zeolites in the Baeyer-Villiger type biotransformation by the fungus *F. oxysporum* has also been studied. It has been observed that these types of materials enhance the reaction rate.

Chapter three describes the screening of fungi for the bioreduction of prochiral carbonyl compounds taking acetophenone as model substrate. The fungus *Trichothecium* was obtained as an effective biocatalyst for this type of bioreduction. Acetophenone can be converted to (*R*)-1-phenylethanol (ee 93 %) by this biocatalyst. The effect of different parameters such as time, biocatalyst concentration, surfactant and substrate concentration has been studied for the acetophenone bioreduction. Some other prochiral ketones have also been investigated with this biocatalyst. High enantiomeric excesses (90-98 %) were obtained in the above cases.

Chapter four accounts the interesting observation that fungi can reduce the metal ions to its corresponding metal particles, which are in the size of nanometer range either intracellularly or extracellularly. This fungal-mediated green chemistry approach towards the synthesis of gold nanoparticles has many advantages such as ease with which the process can be scaled up, economic viability, possibility of easily covering large surface areas by suitable growth of the mycelia etc. to form new bioinorganic composite materials.

Chapter five summarizes and concludes the results obtained and the basic finding of the present work.

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Lactones are important flavor compounds in many foods and beverages because of their potency and varied sensory properties. Biodegradable polyesters with controlled molecular weights and polydispersity ratios can be obtained by the ring opening polymerization of lactones. Aliphatic polyesters are of great interest for their applications in the medical field as biodegradable surgical sutures or as a delivery medium for controlled release of drugs due to their biocompatible and permeable properties [1]. The oxidation of cyclohexanone to ε-caprolactone is of particular interest since the product is extensively used in the synthesis of polycaprolactone (PCL), which is a well-known biodegradable polyester that can be prepared by the ring opening insertion polymerization of caprolactone. The oxidation of ketones to esters and lactones by the Baeyer-Villiger reaction is a reliable and highly useful transformation in synthetic chemistry. The most common reagents used for this reaction are peroxycarboxylic acids (e.g., peracetic acid, m-chloroperbenzoic acid). These are commercially available on a large scale, but their industrial use is either costly or requires significant safety considerations. Moreover, these peracids yield the corresponding carboxylic acids, raising separation and recycling concerns.

The Baeyer-Villiger type biotransformations of different cycloalkanones derivatives carried out by using microorganisms has been reviewed in Chapter 1. Some of the recommended strains such as *Acinetobacter calcoaceticus* are pathogenic and therefore have to be handled with care. Although, some strains of the fungus *Fusarium oxysporum* are reported to be active biocatalyst for epoxidation of styrene [2], steroid hydroxylation [3] and sulfoxidation of alkyl aryl sulfides [4], we have found that fungal strain *Fusarium oxysporum* f. sp. *ciceri* NCIM 1282 can be used as a biocatalyst in the Baeyer-Villiger type biotransformation of cyclohexanone [5,6] and other cyclic ketones using the whole biomass (mycelia) as biocatalyst instead of isolated enzyme. This was done to avoid the cofactor addition, the complicated pathway of cofactor recycling and cumbersome and costly

isolation of enzyme from the biomass. The fungus *F. oxysporum* exhibits redox activity (reduction followed by oxidation). Such dual (redox) activity has also been observed in the biotransformation of ketones by fractured cells of *Acinetobacter* [7] and whole-cells of *Beauveria bassiana* [8].

In this chapter, Baeyer-Villiger type biotransformations of different cyclic ketones as well as aromatic ketones such as acetophenone has been studied using whole cell of the fungus *Fusarium oxysporum* as a biocatalyst. The use of whole cells can provide some advantages. For example, enzyme may be more stable when present in whole cells than when isolated [9], perhaps because the cell wall affords a degree of protection from the organic phase. Most importantly, however, whole cell biocatalysis is especially advantageous when enzyme cofactors are required, because the cost of typical cofactors often exceeds that of the enzyme or the value of the product. With whole cell systems, we can avoid the addition of purified cofactors since they are already present and generated within the cells.

But one of the drawbacks of whole cell biotransformation, however, is that the conversion of the substrate is rather slow. Among the parameters that affect the course of a biotransformation like pH [10], the nature of the nutrients [11], the cell immobilization [12], the heat treatment [13] are well known. Immobilization of various enzymes on zeolite supports has been documented [14,15]. The use of these solids for application in other areas of biotechnology is also known [16,17]. Moreover, it was also reported that the rate of disappearance of acenaphthene could be increased by suspended solids (organic particulate matter) in water [18]. Recently, Kim *et al*. [19] reported that catalytic efficiency of enzymes in organic media could be enhanced with the addition of the solid support such as silica gel.

However, the effect of the addition of inorganic solids in whole cell biocatalysis was not studied. Here attempts were made to enhance the conversion of substrate by adding

inorganic additives in the whole-cell method. Zeolites, alumina, silica gel and clay were used as additives. Quite surprisingly, it was found that the conversion of the substrates is significantly enhanced upon the addition of a zeolite in the reaction mixture compared to other additives.

2.1 EXPERIMENTAL

The fungal strain *Fusarium oxysporum* was obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. Cyclobutanone, cyclopentanone, cycloheptanone (Lancaster), cyclohexanone (Merck), acetophenone (SD Fine Chem, India) were used as received without further purification. Culture ingredients like glucose, malt extract, yeast extract and peptone were purchased from Hi Media, India.

2.1.1 Preparation of microbial culture

The fungus was maintained on potato-dextrose agar (PDA) slants at 25 °C. The MGYP (Malt-Glucose-Yeast-Peptone) medium was prepared by mixing malt extract (0.3 %), glucose (1.0 %), yeast extract (0.3 %), and peptone (0.5 %) in distilled water. The conical flask containing 100 mL medium was sterilized at 120 °C and 1 atmosphere pressure. The fungus from PDA slant was incubated in the medium and allowed to grow at 25-28 °C under shaking condition (200 rpm) for 96 h.

2.1.2 Biotransformation of ketone

After 96 h of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 10 °C for 20 min and settled mycelia were washed twice with sterile distilled water. The harvested mycelial mass (30 g wet wt.) was then resuspended in 100 mL sterile distilled water in 500 mL conical flasks at pH = 5.5-6.0 and the biotransformation was started by adding the substrate (100 mg) which was predissolved in

ethanol (500 mg) under sterile conditions. The whole mixture was put into a shaker at 28 °C (200 rpm).

The biotransformations were routinely monitored by periodic sampling of aliquots (2 mL), which were extracted with dichloromethane and analyzed by GC. After completion of the biotransformation the mycelia were removed by centrifugation (5000 rpm) and the supernatant was extracted with dichloromethane. Extracted solvent was dried and concentrated under vacuum. Crude reaction mixture was analyzed by GC (gas chromatography Agilent 6890 series) using Supelco BETA-DEX 110 capillary column (30M X 0.32 X 0.25). The products were also confirmed by GC-MS (Shimadzu, GCMS-QP 2000A).

2.1.3 Determination of dry weight of biomass

For measuring the dry weight of the wet biomass, the wet biomass was filtered, dried at room temp for 24 h and then kept at 60 °C for 24 h. The dry weights of 30 g, 20 g and 10 g wet wt. biomass were found to be 1.0 g, 0.7 g and 0.35 g respectively, indicating that fungal biomass contains *ca*. 96.5 % water.

2.2 RESULTS AND DISCUSSION

2.2.1 Screening of fungus

Twenty four fungal strains were screened for the Baeyer-Villiger type biotransformation of ketone where cyclohexanone was taken as a model substrate. The results are shown in Table 2.1. It was observed that the fungus *Fusarium oxysporum* f. sp. *ciceri* NCIM 1282 could transform cyclohexanone to cyclohexanol followed by the formation of caprolactone, the Baeyer-Villiger product of cyclohexanone.

Table 2.1

Screening of fungi for the biotransformation of cyclohexanone

Entry	Fungal Strain	Reaction type Redn. /Oxidn.	Time, h	Conversion, %
1.	*Aspergillus flavus* NCIM 536	Reduction	48	71.5
2.	*Aspergillus flavus* NCIM 537	"	"	38.3
3.	*Aspergillus flavus* NCIM 538	"	"	36.5
4.	*Aspergillus flavus* NCIM 542	"	"	63.0
5.	*Aspergillus oryzae* NCIM 637	"	"	30.0
6.	*Aspergillus oryzae* NCIM 643	"	"	85.0
7.	*Aspergillus oryzae* NCIM 649	"	"	41.5
8.	*Aspergillus oryzae* NCIM 1032	"	"	2.5
9.	*Aspergillus terreus* NCIM 659	"	"	78.0
10.	*Aspergillus niger* NCIM 545	"	"	25.7
11.	*Chloridium* sp.	"	"	33.8
12.	*Colletotrichum* sp.	"	"	89.8
13.	*Cunninghamella echinulata* NCIM 691	"	"	36.4
14.	*Curvularia lunata* NCIM 716	"	"	2.0
15.	*Curvularia fallax* NCIM 714	"	"	2.0
16.	*Curvularia cymbopogonis* NCIM 695	"	"	4.4
17.	*Trichoderma reesei* NCIM 1052	"	"	2.7
18.	*Penicillium notatum*	"	"	6.5
19.	*Penicillium fellutanum* NCIM 1227	"	"	10.5
20.	*Verticillium* AAT-TS-3	"	"	16.0
21.	*Fusarium moniliforme* NCIM 1100	"	"	28.6
22.	*Fusarium oxysporum* NCIM 1043	"	"	63.3
23.	*Fusarium oxysporum* NCIM 1072	"	"	53.8
24.	***Fusarium oxysporum* f. sp. *ciceri* NCIM 1282**	**Reduction-oxidation**	**"**	**100**

Reaction conditions: Substrate concentration 100 mg/100 mL reaction medium, biocatalyst 1.0 g dry wt., room temperature, shaker speed 200 rpm.

It has been observed from the screening (Table 2.1) that most of the fungi have reducing property. They can reduce cyclohexanone to cyclohexanol, which is a favorable step energetically. Among the screened strains some (entry 1, 6, 9, 12, 24) can reduce cyclohexanone significantly, whereas few strains (entry 8, 14-18) have negligible reducing properties. Only one strain *Fusarium oxysporum* f. sp. *ciceri* NCIM 1282 is remarkably active (complete conversion) for the biotransformation of cyclohexanone to cyclohexanol followed by the formation of ε-caprolactone, the Baeyer-Villiger product of cyclohexanone.

2.2.2 Biotransformation of cyclohexanone

2.2.2.1 Effect of time

The effect of reaction time on the conversion of cyclohexanone and product distribution using whole cells of *Fusarium oxysporum* as biocatalyst is depicted in Fig. 2.1. It was found that *ca*. upto 50 % of the conversion of cyclohexanone, the only product was cyclohexanol (with 100 % selectivity, when compared with an authentic sample). Later on with further increase in ketone conversion, ε-caprolactone, a Baeyer-Villiger oxidation product of cyclohexanone, started forming at the expense of cyclohexanol and cyclohexanone. At complete conversion, the caprolactone was obtained with +99 % selectivity as assessed by GC analysis.

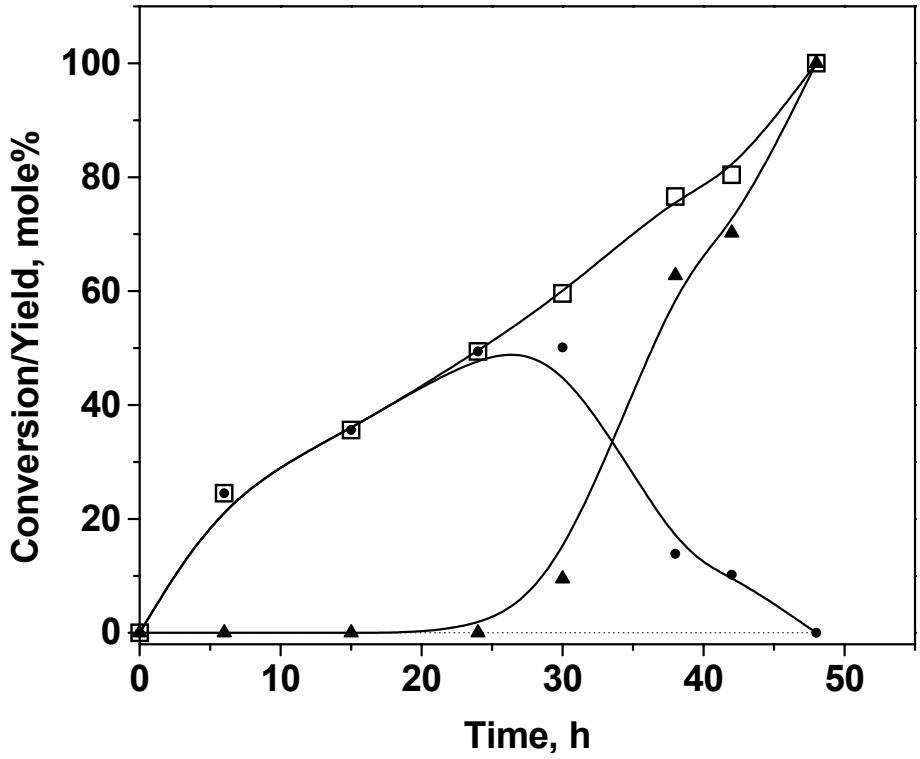


Fig. 2.1 Progress curve in cyclohexanone biotransformation. [(□) Conversion of cyclohexanone, (●) Yield of cyclohexanol, (▲) Yield of caprolactone. Reaction conditions: substrate concentration 100 mg/100 mL reaction mixture, biocatalyst 1 g dry wt., room temperature, shaker speed = 200 rpm].

It seems that initially the system tries to attain an equilibrium between cyclohexanone and cyclohexanol before the onset of lactone formation. The pathway of lactone formation alongwith significant amount of cyclohexanol formation indicates that at least two types of enzymes were present in the microorganism. For maintaining the equilibrium between ketone and alcohol an oxidoreductase enzyme is needed and for lactone formation a lactonization enzyme must be present there.

In order to corroborate this observation, we have carried out a control experiment where cyclohexanol was used as a substrate. The results are shown in Fig. 2.2.

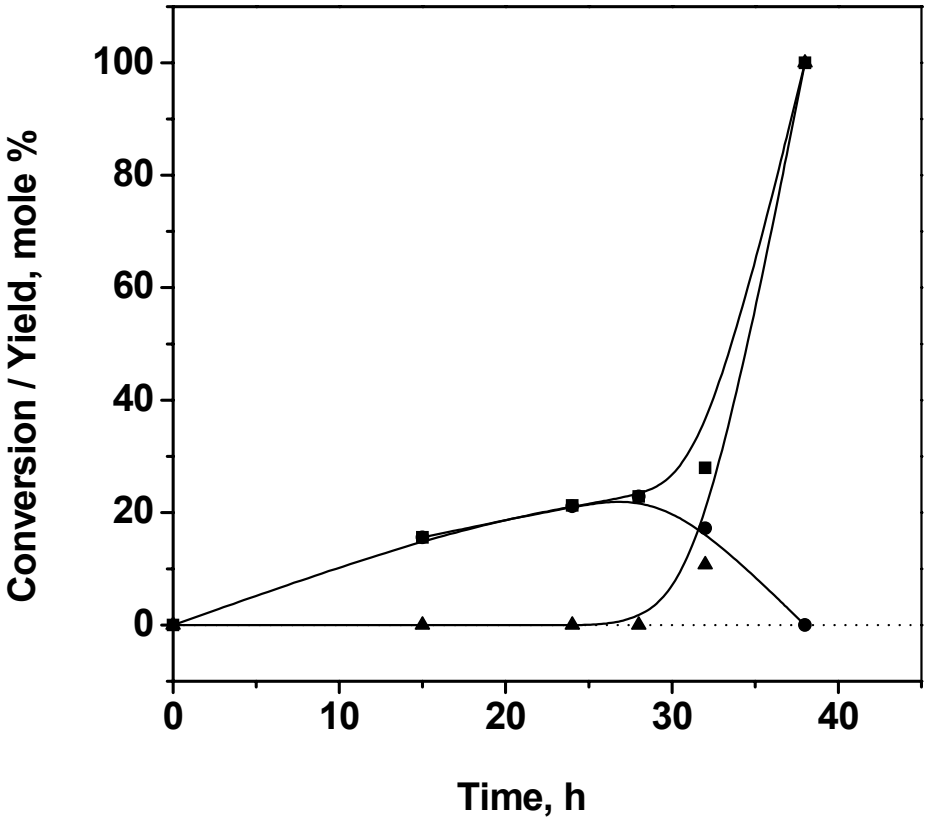


Fig. 2.2 Progress curve in cyclohexanol biotransformation. [(■) conversion of cyclohexanol, (●) yield of cyclohexanone, (▲) yield of caprolactone. Reaction conditions: substrate concentration 100 mg/100 mL reaction mixture, biocatalyst 1 g dry wt., room temperature, shaker speed 200 rpm].

It is clear from these results that upto *ca*. 25 % cyclohexanol conversion no caprolactone was formed and the sole product was cyclohexanone. However, with increasing reaction time and conversion, caprolactone started forming at the expense of the ketone. It must be noted that while upto *ca*. 28 h, no lactone formation was observed (conv. 25 %), both conversion as well as the formation of lactone was complete (*ca*. 100 %) in just next 10 h. This indicates that the redox reaction is rather slow compared to lactonization.

Since, the relative concentration of cyclohexanone-cyclohexanol could be the initiator for the lactone formation, another controlled biotransformation with 1:1 mixture of cyclohexanone and cyclohexanol as substrate was also performed. Quite interestingly, we observed that initially the concentration of cyclohexanol was increased as usual, that means cyclohexanone was getting reduced first (Fig. 2.3). From the above results, no definite equilibrium value between cyclohexanone and cyclohexanol could be ascertained, which could be considered as initiator for lactone formation, eliminating this assumption. However it is clear that while reduction of cyclohexanone to cyclohexanol is relatively slow, the lactone formation is rather very fast.

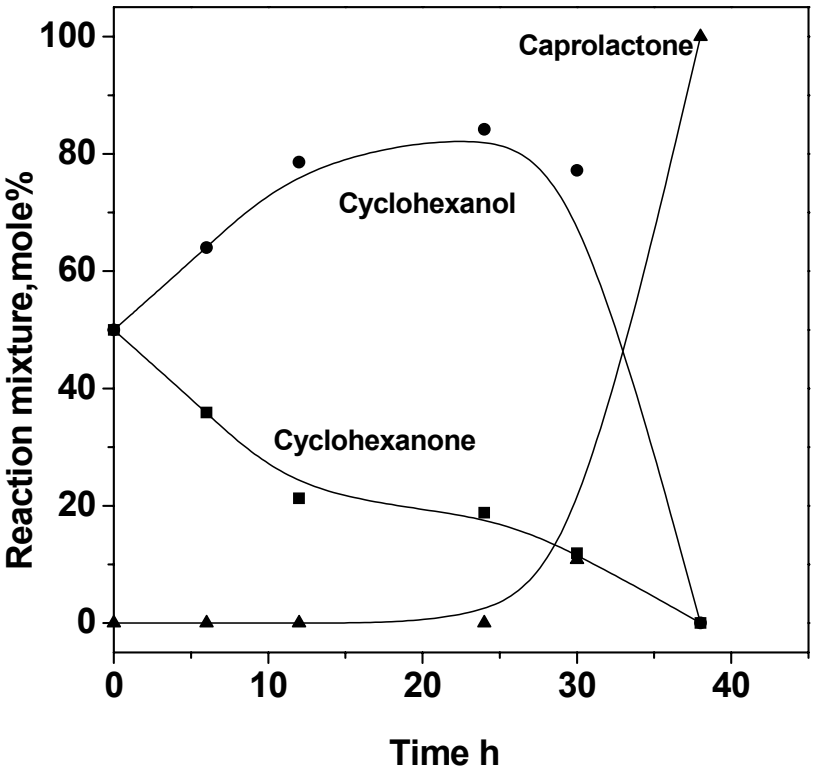


Fig. 2.3 Composition of reaction mixture. [Reaction conditions: Biocatalyst 1.0 g dry wt, cyclohexanone 50 mg, cyclohexanol 50 mg, shaker speed 200 rpm, room temperature].

Moreover, the lactone was not further hydrolyzed by the fungus after prolonged reaction time. To corroborate this, another control reaction was performed where a lactone (γ- valerolactone) was taken as starting material with the fungus and observed to be stable without being converted any product.

2.2.2.2 Effect of biomass

The effect of biomass concentration on the conversion of cyclohexanone was studied starting from 0.35 g dry wt. to 1.0 g dry wt. of the biomass. It was observed that the conversion of cyclohexanone depends on the amount of biomass of the grown culture. If the transformation is carried out in the presence of 0.55 g dry wt. of biomass +99 % lactone can be obtained in 72 h, whereas in the presence of 1.0 g dry biomass complete conversion of cyclohexanone into caprolactone was obtained in 48 h.

The effect of biocatalyst concentration on cyclohexanone transformation and selectivity of products is shown in Table 2.2.

Table 2.2

Effect of biomass on the biotransformation of cyclohexanone

Biocatalyst (Dry wt. in g)	Time, h	Conversion, mole %	Selectivity of products, %	
			cyclohexanol	caprolactone
0.35	24	19.8	100	0.00
	48	31.5	100	0.00
	72	39.0	100	0.00
	96	86.8	67.0	33.0
0.55	24	27.4	100	0.00
	48	45.6	100	0.00
	60	76.5	18.2	81.8
	72	100	0.00	100
0.7	24	35.5	100	0.00
	36	48.6	100	0.00
	48	69.4	68.6	31.3
	60	100	0.00	100
1.0	15	35.5	100	0.00
	30	59.6	84.1	15.9
	42	80.4	12.7	87.3
	48	100	0.00	100

Reaction conditions: Substrate concentration 100 mg/100 mL reaction medium, room temperature, shaking speed 200 rpm.

2.2.2.3 Effect of substrate concentration

The potentiality of the biocatalyst was investigated by varying the cyclohexanone concentration from 100 mg to 800 mg / 100 mL of the reaction medium. Up to *ca*. 300 mg/ 100 mL of cell suspension the conversion was almost same in 48 h. With increasing substrate concentration (300-800 mg/100 mL of reaction medium) the conversion became steadily slower. The effect of substrate concentration on cyclohexanone transformation with

the biocatalyst is shown in Fig. 2.4. This decrease in activity of the biocatalyst at higher concentration is probably due to the toxic effect of higher levels of cyclohexanone.

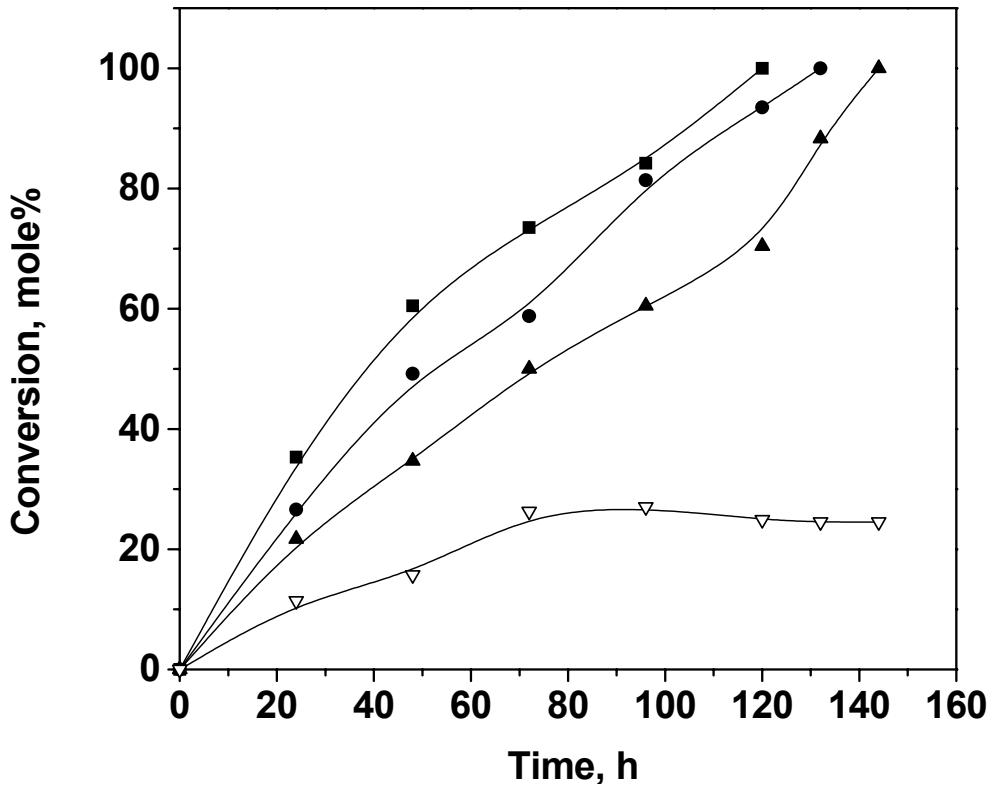


Fig. 2.4 Effect of substrate concentration in cyclohexanone transformation. [(■) 4 mmol, (●) 6 mmol, (▲) 8 mmol, (▽) 10 mmol. Reaction conditions: biocatalyst 1.0 g dry wt., room temperature, shaker speed 200 rpm].

In order to study the effect of the ring size of the different cyclic aliphatic ketones such as cyclopentanone, cyclobutanone as well as cycloheptanone, they were also compared as substrates for Baeyer-Villiger biotransformation in the following section.

2.2.3 Biotransformation of cyclopentanone

2.2.3.1 Effect of time

The biotransformation of cyclopentanone is quite interesting due to its lower reactivity. Though upto 24 h of the reaction only 2.3 % cyclopentanol was first obtained. After that valerolactone, the Baeyer-Villiger product of cyclopentanone, started forming very rapidly. The reaction pattern is depicted in Fig. 2.5.

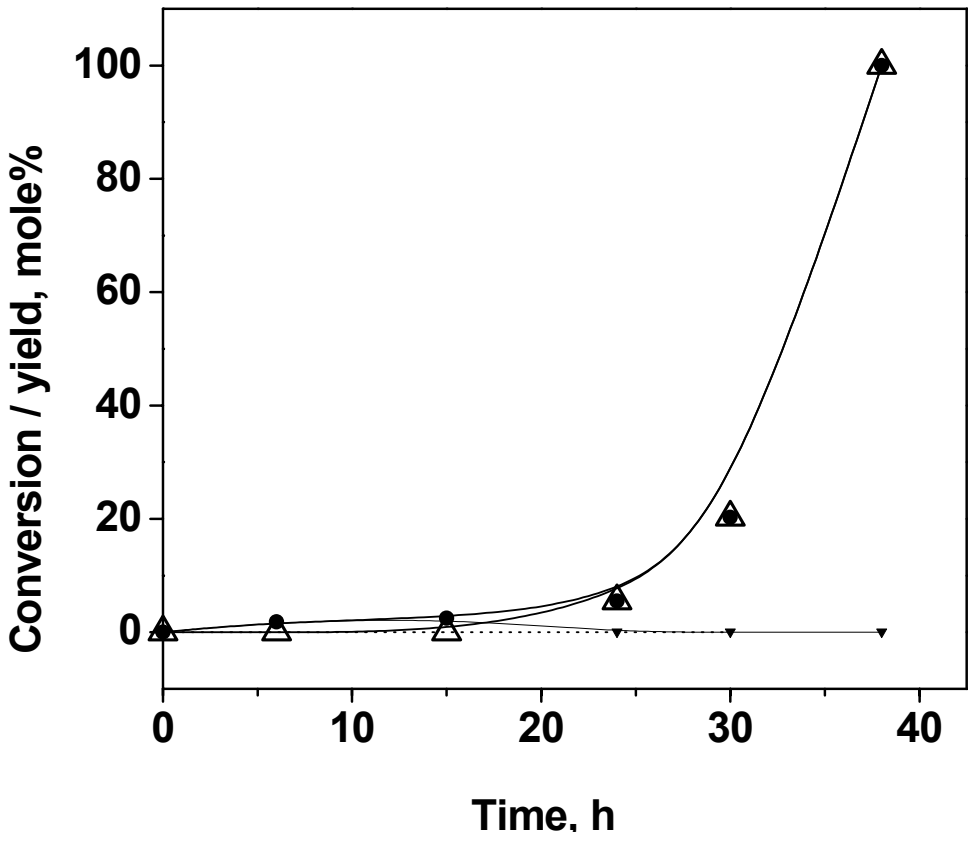


Fig. 2.5 Biotransformation of cyclopentanone. [(●) conversion of cyclopentanone, (▼) yield of cyclopentanol, (△) yield of valerolactone. Reaction conditions: biocatalyst 1 g (dry wt.), substrate 100 mg, room temperature, shaker speed 200 rpm].

2.2.3.2 Effect of biocatalyst concentration

One of the problems usually encountered in such biocatalytic processes is the requirement of large amount of biomass [20]. The effect of biocatalyst was studied from 0.35 g to 1.35 g. It was observed that cyclopentanone conversion did not differ significantly as the biocatalyst was varied from 0.7 g to 1.35 g. But the conversion was low at lower biocatalyst concentration (0.35 g). The effect of biocatalyst concentration on cyclopentanone is shown in Fig. 2.6.

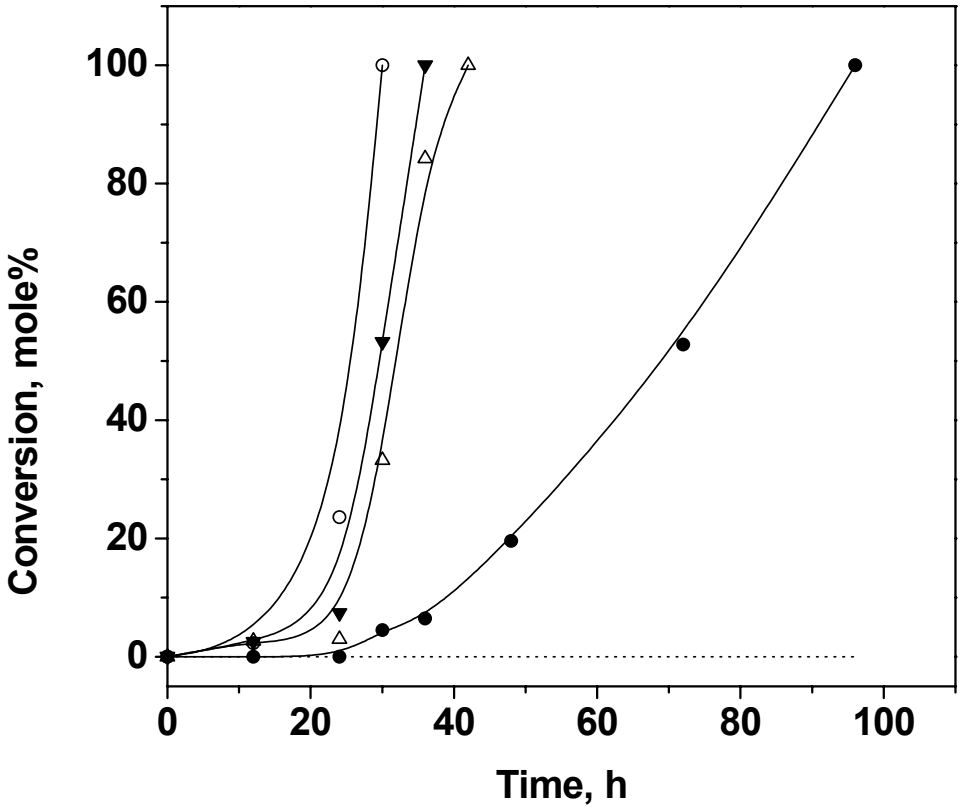


Fig. 2.6 Effect of biocatalyst concentration on the biotransformation of cyclopentanone. [(●) 0.35 g, (△) 0.7 g, (▼) 1.0 g, (○) 1.35 g. Reaction conditions: substrate 100 mg/100 mL reaction medium, room temperature, shaker speed 200 rpm].

2.2.3.3 Effect of substrate concentration

The toxicity of cyclopentanone concentration was studied towards the standard amount of the biocatalyst. It has been observed that the biocatalyst can act up to 10 mmol of the substrate in 100 mL of reaction medium (Fig. 2.7).

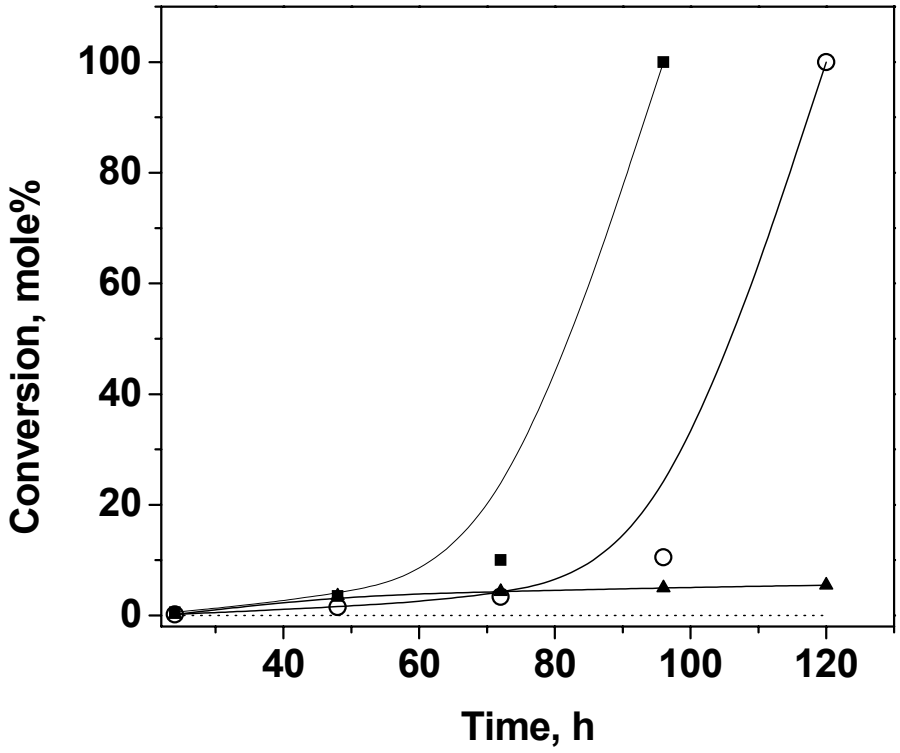


Fig. 2.7 Effect of substrate concentration on cyclopentanone biotransformation. [(▲) 12 mmol, (○) 9.5 mmol, (■) 6 mmol. Reaction conditions: biocatalyst 1 g (dry wt.), room temperature, shaker speed 200 rpm].

The activity of oxidoreductase enzyme, which is responsible for the ketone-alcohol transformations, was checked in case of cyclopentanol also. Interestingly, the disappearance of cyclopentanol was occurred very quickly and cyclopentanone was formed at the expense of cyclopentanol and most of the alcohol (*ca*. 95 %) was observed to be converted to its corresponding ketone within 15 h followed by quick decrease with corresponding increase

in valerolactone formation. The reaction trend in above case resembles the established Baeyer-Villiger type oxidation. Here the alcohol was oxidized to the carbonyl form that was subsequently transformed, probably by the monooxygenase type of enzyme, to the lactone. Cyclopentanol biotransformation by the above fungus is depicted in Fig. 2.8.

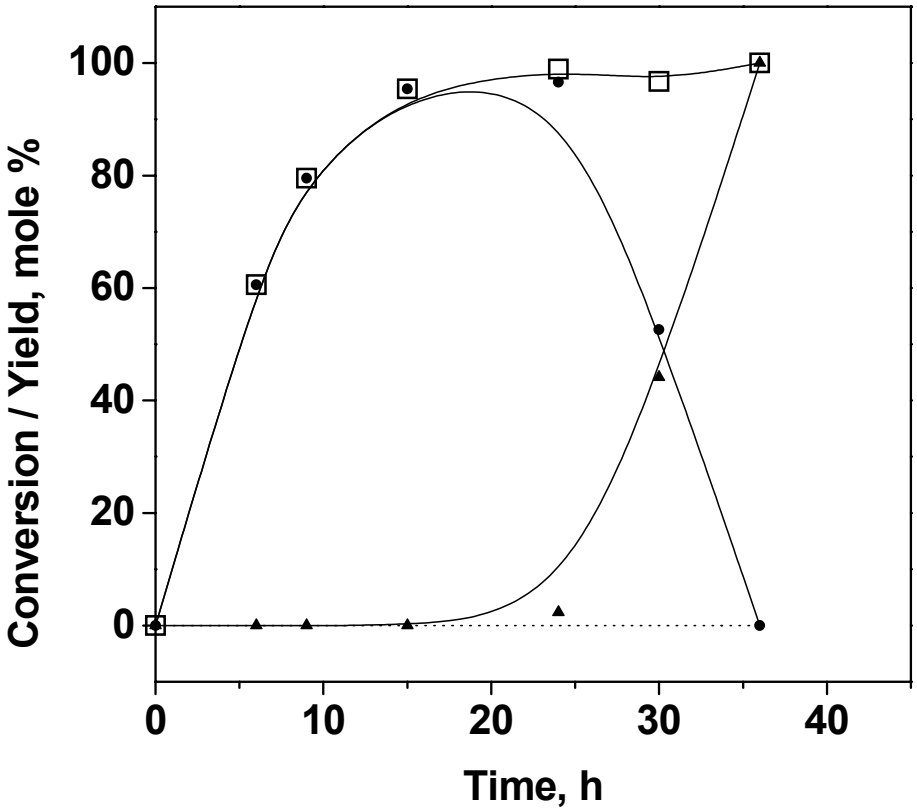


Fig. 2.8 Biotransformation of cyclopentanol. [(□) conversion of cyclopentanol, (●) yield of cyclopentanone, (▲) yield of valerolactone. Reaction conditions: substrate 100 mg/100 mL reaction medium, biocatalyst 1 g (dry wt.), room temperature, shaker speed 200 rpm].

2.2.4 Biotransformation of cyclobutanone

2.2.4.1 Effect of time

The ring size of ketone was further reduced for the biotransformation and it was observed that in case of smaller ring size (cyclobutanone) the conversion becomes slow. It required 72 h for complete conversion (in presence of standard amount of biocatalyst) of cyclobutanone where upto 24 h the product was only cyclobutanol. The effect of time in cyclobutanone transformation is shown in Fig. 2.9. It is clear that in this case while the formation of cyclobutanol (reduction) was rather fast and the formation of butyrolactone is slow, contrary to the reaction pattern in the case of C_5 and C_6 ketones.

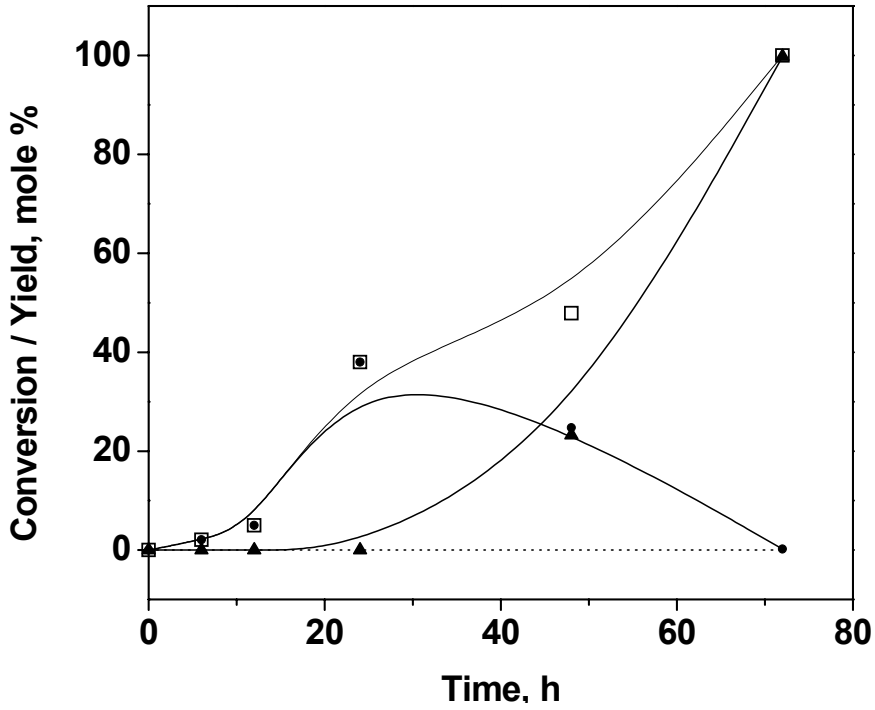


Fig. 2.9 Biotransformation of cyclobutanone. [(□) conversion of cyclobutanone, (●) yield of cyclobutanol, (▲) yield of butyrolactone. Reaction conditions: substrate 100 mg, biocatalyst 1 g (dry wt.), room temperature, shaker speed 200 rpm].

2.2.5 Biotransformation of cycloheptanone

In the case of higher ring size ketone than cyclohexanone e.g., cycloheptanone (7-membered ring), it was converted slowly and 100 % conversion could be achieved after 60 h (Fig. 2.10). Interestingly, in the case of cycloheptanone, the only product was cycloheptanol till the completion of the reaction and there was no formation of corresponding lactone. Moreover, the conversion was so slow that upto *ca*. 30 h the amount of the substrate remained unchanged. It indicates that the Baeyer-Villiger monooxygenase enzymatic activity is specific upto cyclohexanone only. Perhaps, the size of this cyclic ketone is not adaptable to the enzyme site.

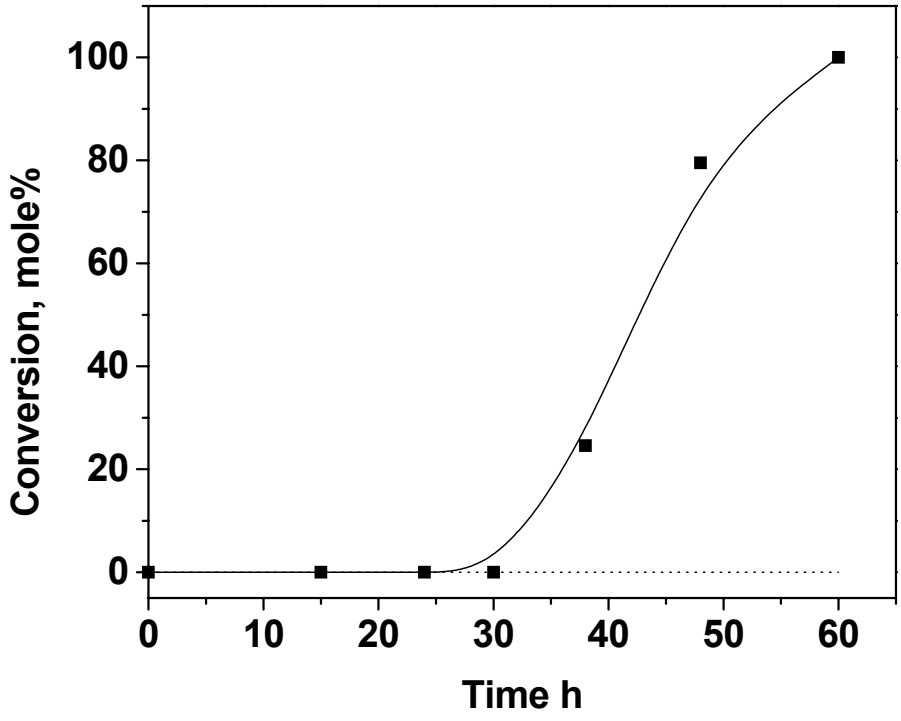
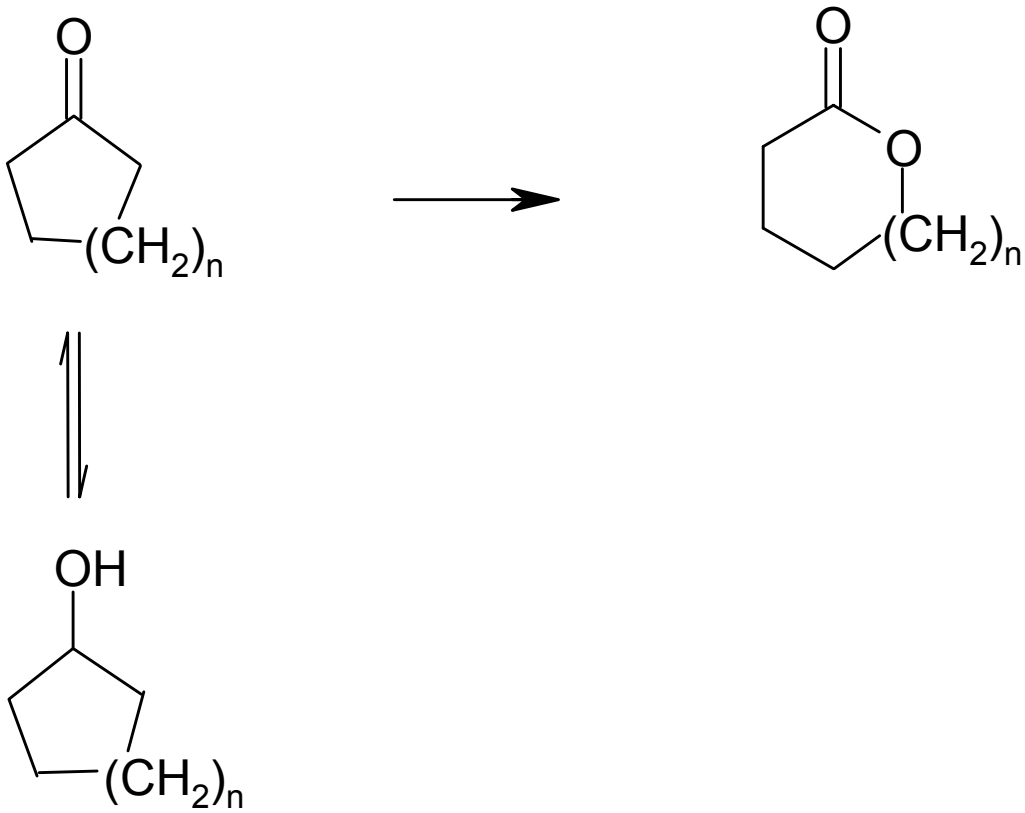


Fig. 2.10 Biotransformation of cycloheptanone. [Reaction conditions: substrate 100 mg/100 mL reaction medium, biocatalyst 1.0 g dry wt., room temperature, shaker speed 200 rpm].

It is very clear from the above observation that the reaction patterns are almost same in case of cyclobutanone and cyclohexanone. As far as the conversion is concerned the adaptability order of this fungus towards different cyclic ketones could be like this: cyclopentanone ≃ cyclohexanone > cycloheptanone > cyclobutanone. This order indicates that cyclopentanone and cyclohexanone are more suitable substrates for this fungus for Baeyer-Villiger type biotransformation. So, the reaction sequence of various cyclic ketones with the fungus, *Fusarium oxysporum* schematically can be represented as below (Scheme 2.1).



Scheme 2.1 Biotransformation of cyclic ketones by *F. oxysporum* [n = 0, 1, 2].

The inter-conversion between cycloalkanones and cycloalkanols depends on the number of carbon atoms in the carbocyclic ring of the substrates; the equilibrium in the interconversion of the 6-membered compound (cyclohexanone) and 4-membered compound (cyclobutanone) lying towards cycloalkanol, whereas in the case of 5-membered ring (cyclopentanone) the same lies toward the side of cycloalkanone. This difference in the reduction of cyclohexanone and cyclobutanone with that of cyclopentanone can be explained on the basis of the higher strain energy of the formers over that of the latter [21].

2.2.6 Biotransformation of acetophenone

To study whether aromatic ketones like acetophenone can also be subjected to Baeyer-Villiger biotransformation using the same fungus, similar experiments with acetophenone were also carried out.

Unlike the cyclic ketones, acetophenone shows different reaction pattern with the fungus *Fusarium oxysporum*. Though some reduction of acetophenone to the corresponding alcohol (1-phenylethanol) was observed, no detectable formation of phenyl acetate, the Baeyer-Villiger product of acetophenone could be measured with the fungus (Fig. 2.11). It can be assumed that the intermediate ester is immediately converted into phenol by ester hydrolases (unable to hydrolyze lactone) present in the microorganism. Indeed, upon incubation of phenyl acetate (100 mg) with the fungus rapid hydrolysis was observed under the conditions employed (*ca*. 95 % conv. in 2.5 h).

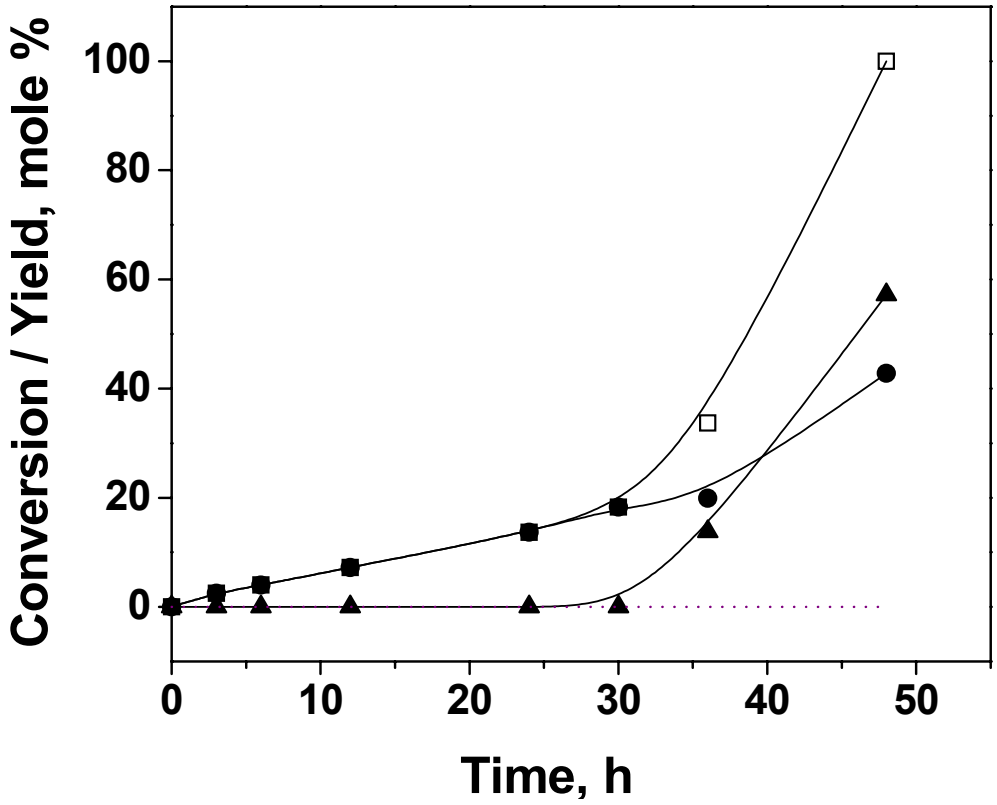


Fig. 2.11 Biotransformation of acetophenone. [(□) conversion of acetophenone, (▲)yield of phenol and acetic acid, (●) yield of 1-phenylethanol. Reaction conditions: biocatalyst 0.7 g, substrate 100 mg, room temperature, shaker 200 rpm].

Later it was observed that with change in media ingredients conversion of the substrate was improved. As it was changed from malt / yeast extract to malt / yeast extract powder, the complete conversion of cyclohexanone with 0.7 g dry wt. of biomass reduced to 45 h instead of 60 h. So, experiments were performed thereafter with the improved media composition.

2.3 EFFECT OF INORGANIC SOLID IN BIOTRANSFORMATION

2.3.1 Experimental

2.3.1.1 Synthesis of ZSM-5

In a typical preparation, 6.0 g SiO_2 (fumed silica) was first slurried in an alkali solution containing 0.5 g NaOH in 20 mL water. Then a template solution containing 5.32 g tetrapropylammonium bromide (TPABr) in 15 mL water was added to it and stirring was continued for 2 h, followed by the addition of another solution of 0.43 g $NaAlO_2$ dissolved in 10 mL water under vigorous stirring. Finally the promoter solution, 1.36 g NaH_2PO_4 dissolved in 9 mL water was added to the synthesis mixture and stirring was continued for 1 h at room temperature. The starting gel for this ZSM-5 sample was transferred to a stainless steel autoclave and kept in a static oven at 433 K for crystallization for 8 h. The solid thus obtained was washed thoroughly with deionized water, dried at 393 K and calcined at 813 K under the flow of air for 16 h. The molar gel composition of the synthesis mixture was:

$$40SiO_2: Al_2O_3: 8NaOH: 5TPABr: 1200H_2O: 4NaH_2PO_4$$

2.3.1.2 Characterization of Zeolites

X-ray diffraction patterns are the fingerprint of individual zeolite structures. We can study the uniqueness of structure, presence of single phase or mixture of phases, incorporation of other elements into structural framework structures and the level of crystallinity.

Powder XRD patterns of calcined ZSM-5 samples were recorded on a Rigaku DMAX III VC instrument using Ni-filtered Cu K_α radiation in the 2θ range of 5-50° at a scan rate of 4° per min.

2.3.1.3 Maintenance and growth of the fungus

The fungus was maintained on potato-dextrose agar (PDA) slants at 25 °C. The MGYP (Malt-Glucose-Yeast-Peptone) medium was prepared by mixing malt extract powder (0.3 %), glucose (1.0 %), yeast extract powder (0.3 %), and peptone (0.5 %) in distilled water. The conical flask containing 100 mL medium was sterilized at 120 °C and 1 atmosphere pressure. The fungus from PDA slant was incubated in the medium and allowed to grow at 25-28 °C under shaking condition (200 rpm) for 96 h.

2.3.1.4 Biotransformation

Harvested and washed mycelial mass (0.7 g dry wt. unless stated otherwise) was then resuspended in 100 mL sterile distilled water with the inorganic materials in 500 mL Erlenmeyer flasks at pH = 5.5-6.0 and the suspension was used immediately for biotransformation. The substrate (100 mg) that was predissolved in ethanol (0.5 g) in sterile tube was added to the cell suspension. The whole mixture was put into a shaker at 28 °C (200 rpm).

The biotransformations were routinely monitored by the same procedure as stated earlier.

2.3.2 Results and discussion

Fig. 2.12 depicts the influence of zeolite (ZSM-5 and zeolite Na-A) compared to the controlled experiment for the biotransformation of cyclohexanone. It was observed that the long induction time of the reaction could be reduced significantly by the addition of zeolites.

The effect of different inorganic additives such as zeolite (ZSM-5, Zeolite Na-A), alumina, silica, montmorillolite clay has been investigated on catalytic efficiency of the fungus *Fusarium oxysporum* f. sp. *ciceri* NCIM 1282 in the biotransformation of

cyclohexanone (where cyclohexanone was taken as model substrate). Higher conversion was obtained with Zeolite Na-A apart from ZSM-5. While for silica and montmorillolite clay the effect is not pronounced and time taken for 100 % conversion was comparable than that taken by the blank.

To find out the optimum amount of the zeolite the amount of zeolite ZSM-5 was varied between 50-200 mg in the total reaction mixture (containing 100 g water, 0.7 g dry wt. biomass, 100 mg cyclohexanone and 500 mg ethanol). The comparative effect of different zeolite (ZSM-5) concentration is shown in the Fig. 2.13. 150 mg zeolite was observed to be the optimum amount for that reaction. It has been found that the reaction with 150 mg of ZSM-5 gets almost completely converted within 30 h whereas the control (without ZSM-5) takes about 45 h for completion of the reaction. Initially the conversion of cyclohexanone in both the cases was almost same but later on with further increase in ketone conversion lactone formation became faster in presence of zeolite (150 mg). With further increase in zeolite concentration the conversion of substrate became slower.

This type of additive effect was also studied for other cyclic ketones such as cyclobutanone, cyclopentanone and cycloheptanone. For the later two substrates it was found that the addition of zeolite did not show any effect. As it may be recalled that the control (without additive) reaction pattern is different in case of cyclopentanone and cycloheptanone when these are incubated with the above fungus whereas it worked for cyclobutanone transformation. The effect of ZSM-5 (150 mg) on the biotransformation of cyclobutanone is depicted in Fig. 2.14.

The structure of the zeolite remains the same after completion of the reaction and it was confirmed by powder XRD (Fig. 2.15).

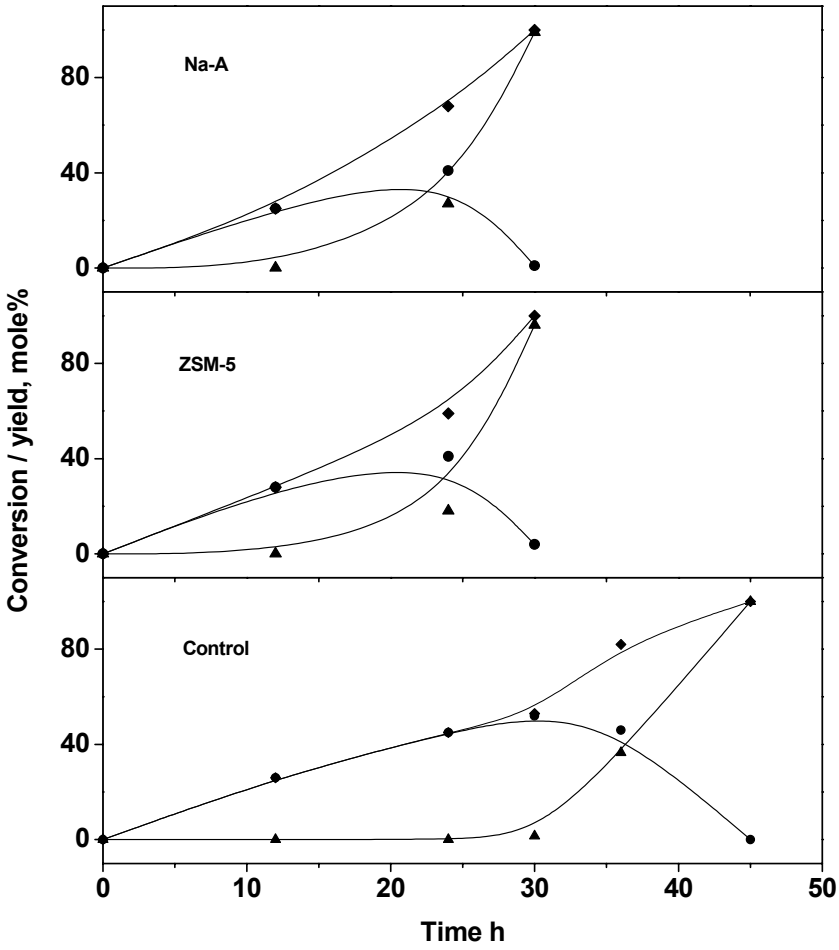


Fig. 2.12 Effect of different zeolite on the biotransformation of cyclohexanone. [(■) conversion of cyclohexanone, (●) yield of cyclohexanol, (▲) yield of caprolactone. Reaction conditions: substrate 100 mg, biocatalyst 0.7 g (dry wt.), zeolite 150 mg, room temperature, shaker 200 rpm].

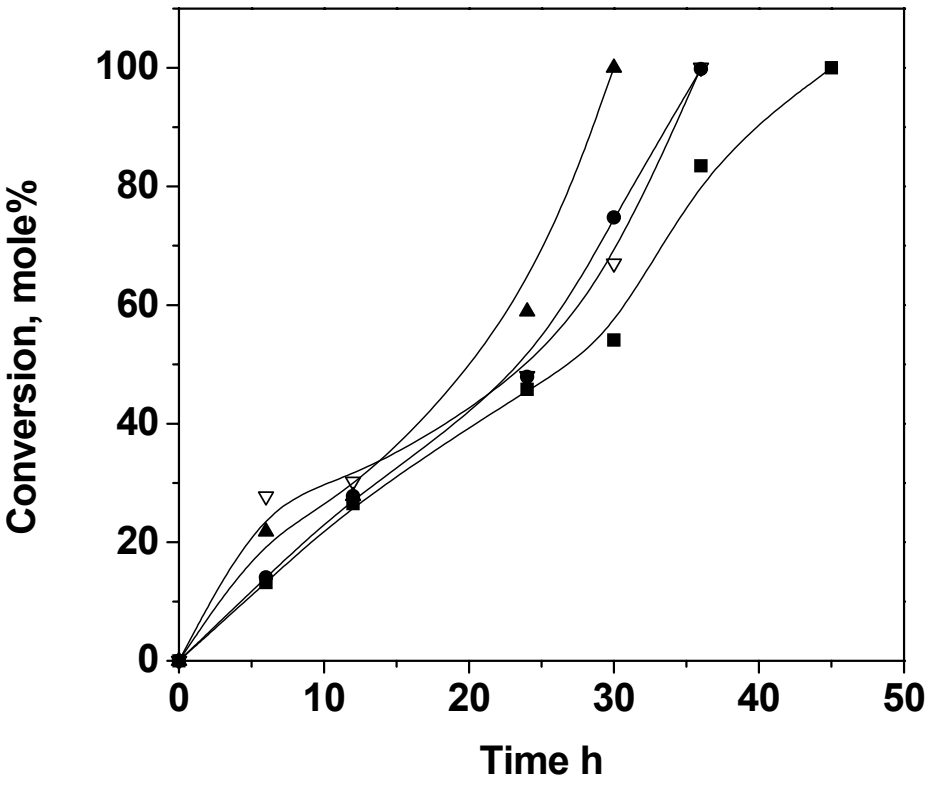


Fig. 2.13 Optimization of zeolite concentration in biotransformation of cyclohexanone. [(■) without zeolite, (●) 50 mg, (▲) 150 mg, (▽) 200 mg zeolite ZSM-5. Reaction conditions: Substrate 100 mg, biocatalyst 0.7 gm dry wt., room temperature, shaker speed 200 rpm].

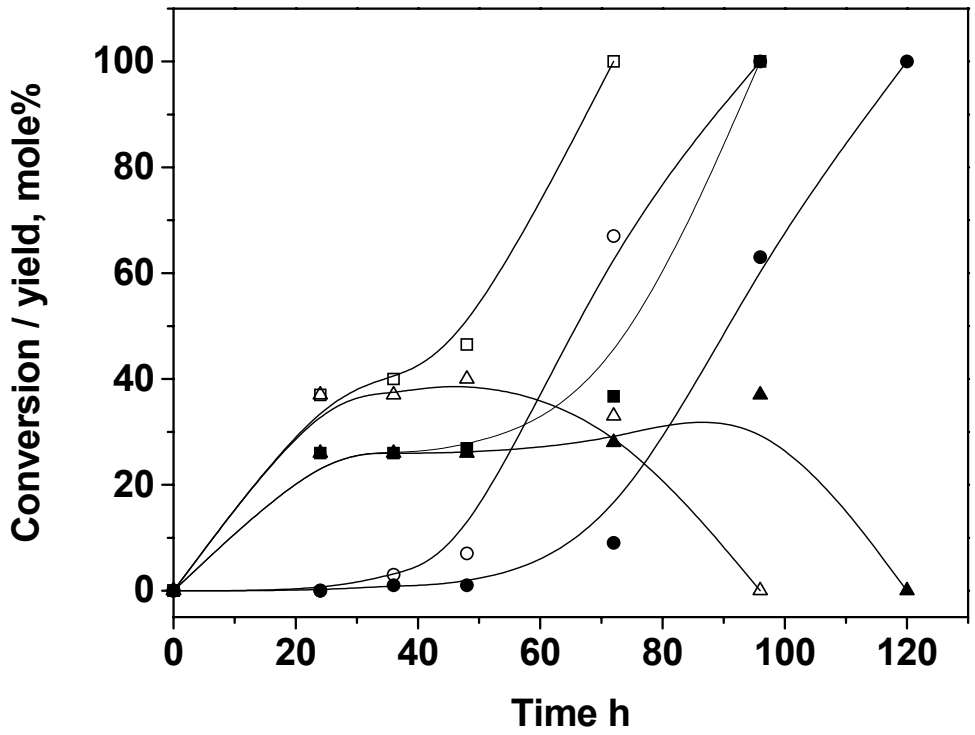


Fig. 2.14 Effect of zeolite (ZSM-5) on biotransformation of cyclobutanone. [(■) conversion of cyclobutanone (control), (□) conversion of cyclobutanone (zeolite), (▲) yield of cyclobutanol (control), (△) yield of cyclobutanol (zeolite), (●) yield of butyrolactone (control), (o) yield of butyrolactone (zeolite). Reaction conditions: Substrate 100 mg, biocatalyst 0.7 g dry wt., zeolite 150 mg, room temperature, shaker speed 200 rpm]

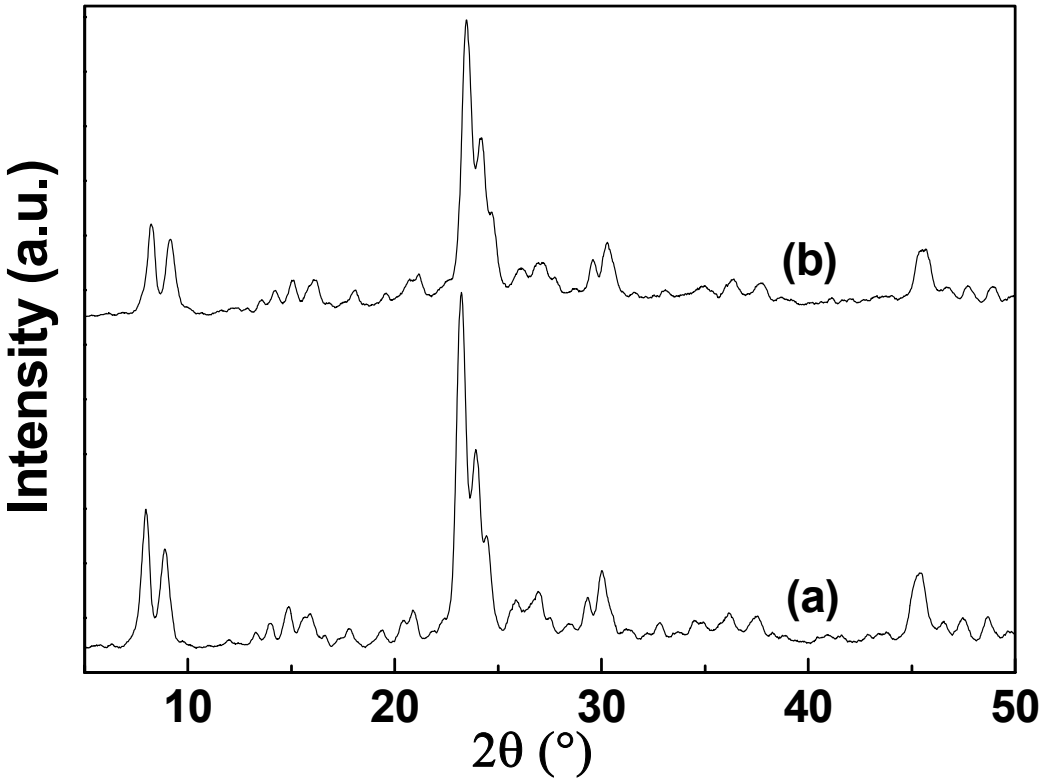


Fig. 2.15 XRD patterns of ZSM-5 (a) before and (b) after the biotransformation.

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During the past few decades, enormous attention has been paid towards enantioselective synthesis of optically pure compounds or chiral synthons that are increasing in demand for the development of modern drugs and agrochemicals. Among the chiral compounds, enantiomerically pure alcohols are particularly useful as building blocks for the synthesis of natural products, pharmaceuticals and agricultural chemicals.

Asymmetric synthesis carried out with chiral metal complexes as catalysts has been successfully used [1,2]. Tables 3.1-A and 3.1-B summarize the work done on asymmetric reduction of acetophenone and other aromatic ketones respectively, by using various chemical reagents. However, some difficulties remain in attaining high optical purity and practical usage in comparison with the ones performed by enzymatic catalysis. Generally, enzymatic reactions have been carried out by employing either whole cells or isolated enzyme. Enzymatic or microbial transformation of synthetic compounds is a convenient method for preparing chiral compounds. Microbial reductions have been widely used for synthesizing chiral alcohols [3,4]. The use of microbial whole cells is particularly advantageous for carrying out the desired reduction since they do not require addition of cofactors for their regeneration.

Baker's yeast is by far the most widely used microorganism for reduction of prochiral ketones yielding the corresponding optically active alcohols with fair-to-excellent enantioselectivity. Although Baker's yeast has several reducing enzymes, it generally gives the (*S*)-alcohol on reduction of ketones, which is governed by Prelog's rule. Recently, there are some reports on the bioreduction of ketones by fungi. For example, the whole cells of the fungus *Rhizopus arrhizus* have been reported [5,6] for reducing acetophenone and its derivatives to corresponding (*S*)-alcohols. Asymmetric reduction of acetophenone and its derivatives was also investigated by whole cells of the fungus *Geotrichum candidum* IFO 4597

by varying the cultivation medium to produce (*S*)-alcohols, but ee was rather poor [7]. In contrast, the acetone powder of the same fungus [8-10] reduces aromatic and aliphatic ketones with excellent ee and with same absolute configuration (*S*). Jian-Xin *et al.* [11] have reported another strain of *Geotrichum* (*Geotrichum* sp. G38) for the reduction of oxo-esters and diketones to produce corresponding (*R*)-alcohols. Both enantiomers of secondary alcohols from aromatic ketones could be obtained by using the fungus *Geotrichum candidum* IFO 5767 by modifying the reaction conditions [12].

Simple aromatic ketones are usually very poor substrates for Baker's yeast mediated reductions, often furnishing very low yields of desired chiral alcohols [13,14]. To overcome such drawbacks of Baker's yeast, Molinari *et al.* [15] tried to reduce prochiral ketones to its corresponding alcohols by using some other yeasts. They obtained both enantiomers (*R/S*) of alcohols with different yeasts. In case of acetophenone, one of the yeasts produced (*R*)-alcohol with high ee (95 %). But it should be mentioned that the same yeast produced different enantiomers with different substrates. Bacterial cells [16] have also been used successfully for some aromatic ketones to its corresponding (*S*)-alcohols. But drawback of these protocols involves external addition of the costly coenzyme.

Previous bioreduction protocols have shown that microorganisms with Prelog specificity are prevalent while those with anti-Prelog specificity are less common to some extent. In this context, we have screened various fungal strains, which reduces carbonyl compounds in an anti-Prelog manner. It was found that *Trichothecium* sp. is a useful strain, which promotes the reduction of acetophenone and its analogous compounds and exhibits good enantioselectivity (90-98 %). It should be emphasized that the above biocatalyst is easy to

handle, the reaction proceeds under mild conditions and the product isolation is simple. Another advantage of this system is that it is environmentally friendly.

There are four stereochemical pathways in the transfer of the hydride from the coenzyme, NAD(P)H, to the substrate as shown in Fig. 3.1 [17]. With E1 and E2 enzymes, the hydride attacks the *si* face of the carbonyl group, whereas with E3 and E4 enzymes, the hydride attacks the *re* face, which results in the formation of *R* and *S* alcohols, respectively. On the other hand, E1 and E3 enzymes transfer the pro-*R* hydride of the coenzymes, and E2 and E4 enzymes use the pro-*S* hydride.

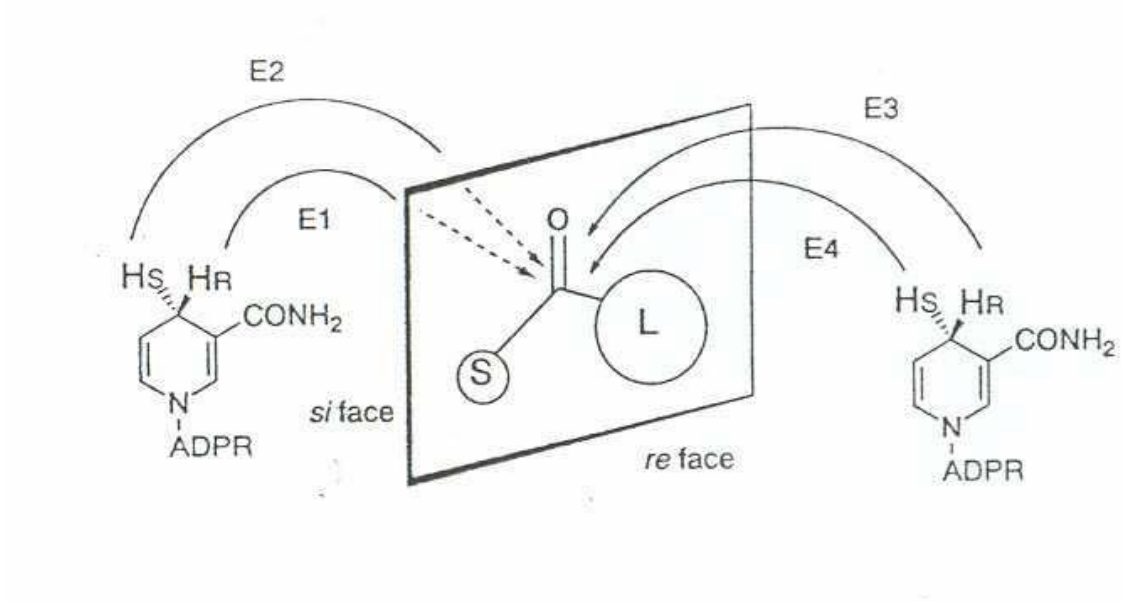


Fig. 3.1 Stereochemistry of the hydride transfer from NAD(P)H to the carbonyl carbon on the substrate (S is a small group and L is a large group). (Reproduced from ref. 17)

Table 3.1-A

Chemical reagents used as catalyst for enantioselective reduction of acetophenone

Reagent	ee (%)	confg	Ref.
Catecholborane, Ti-bicyclo[2.2.2]octanediols	96	*R*	[18]
$His(OR)_3$, (*S*)-$LiOCH_2CH(NHLi)R$, [R= (*S*)-Phenylalaninol]	77	*R*	[19]
BH_3 and nickel-oxazaborolidine	92 94	*R* *S*	[20]
Polymer bound oxazaborolidine	94	*R*	[21]
Glycol-Lithium Aluminium Hydride	24	*R*	[22]
$NaBH_4$ on dendrimer	99	*S*	[23]
Ir complexes of (1*R*,2*R*)-(-)-N-tosyl-1,2-diphenyl-1,2-ethanediamine	92	*S*	[24]
β-methyl-(1*R*)-amino-(2*S*)-indanol	84	*S*	[25]
Trimethoxysilane and N-benzylquininium fluoride	51	*R*	[26]
Chiral thiourea with $(RuCl_2C_6H_6)_2$	87	*S*	[27]
Chiral aminodiol	82	*R*	[28]
$NaBH_4$ and $(CH_3)_2CHCOOH$	64	*R*	[29]
K-glucoride	78	*R*	[30]
(+)-1,2,2-Trimethyl-1,3-bis(hydroxymethyl)-cyclopentane-$LiAlH_4$	18.5	*R*	[31]
BINAP-Ru(II) complex-Chiral diamine-KOH	87	*R*	[32]
Chiral Ru-complex	97	*S*	[33]
(*R*)-(+)-1-phenylethylamine-borane	13.5	*R*	[34]
Amino ester-borane	17	*S*	[34]
Chiralamine-borane complex	51.1	*R*	[35]
Catecholborane, Ti-bicyclo[2.2.2]octanediols	97	*R*	[36]
Chiral hydride reagent	92	*S*	[37]
Chiral hydride reagent	22	*S*	[38]
Chiral hydride reagent	92	*S*	[39]
(*S*)-(-)-2-amino-3-(*p*-hydroxyphenyl)-1,1-diphenyl-1-propanol	66	*R*	[40]

Table 3.1-B

Chemical reagents used as catalyst for enantioselective reduction of other prochiral ketones

Substrate	Reagent	ee (%)	confg	Ref.
Propiophenone	Catecholborane, Ti-bicyclo[2.2.2]-octanediols	95	*R*	[18]
,,	$His(OR)_3$, (*S*)-$LiOCH_2CH(NHLi)R$, [R = (*S*)-1,1-diphenylvalinol]	72	*R*	[19]
,,	β-methyl-(1*R*)-amino-(2*S*)-indanol	69	*S*	[25]
,,	Trimethoxysilane and N-benzylquininium fluoride	65	*R*	[26]
,,	Chiral thiourea with $(RuCl_2C_6H_6)_2$	91	*S*	[27]
,,	Chiral aminodiol	77	*R*	[28]
,,	$NaBH_4$ and $(CH_3)_2CHCOOH$	63	*R*	[29]
,,	K-glucoride	92	*R*	[30]
,,	Chiral Ru-complex	97	*S*	[33]
,,	Chiralamine-borane complex	41.1	*R*	[35]
,,	Catecholborane, Ti-bicyclo[2.2.2]-octanediols	76	*R*	[36]
,,	Chiral hydride reagent	85	*S*	[37]
,,	(*S*)-(-)-2-amino-3-(*p*-hydroxyphenyl)-1,1-diphenyl-1-propanol	72	*R*	[40]
p-Chloroacetophenone	Trimethoxysilane and N-benzylquininium fluoride	35	*R*	[26]
,,	BINAP-Ru(II) complex-Chiral diamine-KOH	94	*R*	[32]
,,	Chiral Ru-complex	93	*S*	[33]
p-Methylacetophenone	BINAP-Ru(II) complex-Chiral diamine-KOH	91	*S*	[32]
Cyclohexylmethylketone	K-glucoride	23	*R*	[30]

3.1 EXPERIMENTAL

All fungal strains were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India and *Trichothecium* sp. was isolated from soil. Acetophenone (SD Fine Chem, India), propiophenone, *p*-chloroacetophenone, *p*-methylacetophenone, cyclohexylmethylketone, cyclopropylphenylketone (Lancaster), trifluoromethylphenylketone (Aldrich) were purchased and used as such without further purification. Culture ingredients like glucose, malt extract powder, yeast extract powder and peptone were purchased from Hi Media, India.

3.1.1 Preparation of microbial culture

The fungus was maintained on potato-dextrose-agar (PDA) slants at 25 °C. The medium MGYP (malt-glucose-yeast-peptone) was prepared by mixing malt extract powder (0.3 %), glucose (1.0 %), yeast extract powder (0.3 %), and peptone (0.5 %) in distilled water. The medium (100 mL) in 500 mL Erlenmeyer flask was sterilized at 120 °C and 1 atmosphere pressure. The fungus from PDA slant was incubated in the medium and allowed to grow at 25-28 °C under shaking condition (200 rpm) for 72 h.

3.1.2 Biotransformation of ketones

After 72 h of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 10 °C for 20 min and settled mycelia were washed twice with sterile distilled water. The harvested mycelial mass (40 g wet wt.) was then resuspended in 100 mL sterile distilled water in 500 mL conical flasks at pH = 5.5-6.0 and the biotransformation was started by adding the substrate (100 mg) which was predissolved in ethanol (500 mg) under sterile conditions. The whole mixture was put into a shaker at 25-28 °C (200 rpm).

The biotransformations were routinely monitored by periodic sampling of aliquots (2 mL), which were extracted with dichloromethane and analyzed by GC. After completion of the biotransformation the mycelia were removed by centrifugation (5000 rpm) and the supernatant was extracted with dichloromethane. Extracted solvent was dried and concentrated under vacuum. Crude reaction mixture was analyzed by GC (Agilent 6890 series) using HP chiral (20 % permethylated β-cyclodextrin) (30M X 0.32 X 0.25) capillary column. The products were also confirmed by GC-MS (Shimadzu, GCMS-QP 2000A) and ^{1}H NMR.

The products (alcohols) were purified by preparative TLC using pet ether / ethyl acetate solvent system. Optical rotations of purified alcohols were recorded on a JASCO DIP-1020 digital polarimeter and absolute configuration was determined by comparing with literature [41,42].

3.1.3 Determination of dry weight of biomass

For measuring the dry weight of the wet biomass of fungus *Trichothecium*, the wet biomass was filtered, dried at room temperature for 24 h and then kept at 60 °C for 24 h. The dry weights of 50 g, 40 g, 30 g and 20 g wet weight biomass were found to be 0.9 g, 0.7 g, 0.5 g and 0.35 g respectively, indicating that fungal biomass contains *ca*. 98.2 % water.

3.2 RESULTS AND DISCUSSION

3.2.1 Bioreduction of acetophenone

3.2.1.1 Screening of fungus

21 fungal strains have been screened for enantioselective bioreduction of prochiral ketone taking acetophenone as model substrate. The results are tabulated in Table 3.2 on the basis of maximum ee values produced by different fungal strains. It was observed that the fungus *Trichothecium* was a useful strain for such type of biotransformation. Acetophenone could be reduced to 1-phenylethanol in good enantioselectivity (ee 93 %) by that fungus. It produced (*R*)-alcohol, which suggests that the fungus *Trichothecium* secretes some specific enzymes that catalyze a hydride transfer to the *Si* face of the carbonyl group (Fig. 3.1). Enantiomers of 1-phenylethanol were separated by chiral column (Fig. 3.2).

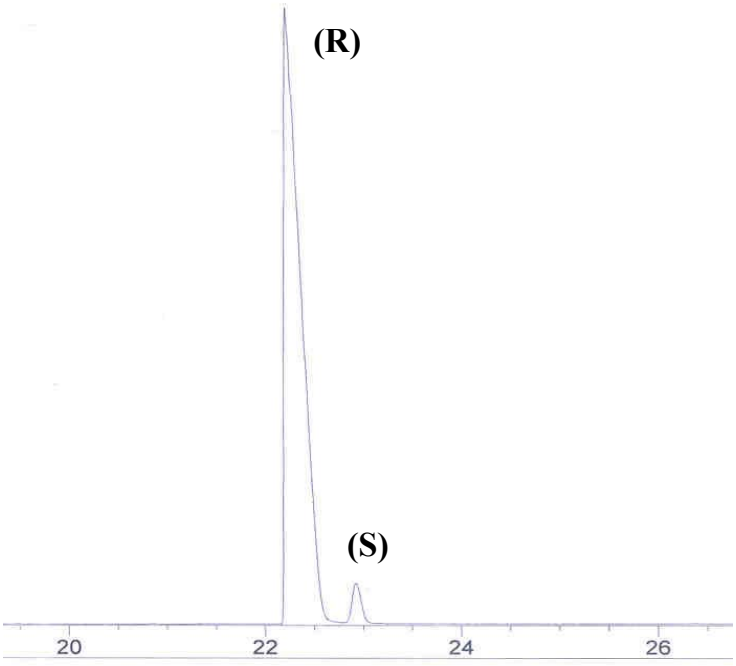


Fig. 3.2 Enantiomeric separation of 1-phenylethanol by HP chiral column (20 % permethylated β-cyclodextrin).

Table 3.2

Screening of fungi for the bioreduction of acetophenone

Entry No.	Fungal strain	Conversion, %	Time, h	ee, % .
1	*Fusarium oxysporum* NCIM 1072	5.4	0.5	86 (*S*)
2	*Fusarium moniliforme* NCIM 1099	11.0	12	80 (*S*)
3	*F. oxysporum* NCIM 1043	6.2	0.5	75 (*S*)
4	*F. oxysporum* f. sp. *ciceri* NCIM 1282	100	42	74 (*S*)
5	*Curvularia lunata* NCIM 716	30.0	24	55 (*S*)
6	*Curvularia fulcatum* NCIM 714	6.8	3	54 (*S*)
7	*F. oxysporum* NCIM 1008	22.0	12	51 (*S*)
8	*Colletotrichum*	3.5	3.0	40 (*S*)
9	*Trichoderma* NCIM 1052	7.3	24	25 (*S*)
10	***Trichothecium* sp.**	**85.0**	**72**	**93 (*R*)**
11	*Aspergillus oryzae* NCIM 649	100	48	81 (*R*)
12	*Aspergillus oryzae* NCIM 637	78.5	36	79 (*R*)
13	*Aspergillus oryzae* NCIM 643	61.8	30	75 (*R*)
14	*Aspergillus flavus* NCIM 546	32.7	36	72 (*R*)
15	*Aspergillus flavus* NCIM 542	100	48	67 (*R*)
16	*Aspergillus flavus* NCIM 536	40.0	30	61 (*R*)
17	*Aspergillus flavus* NCIM 545	11	48	52 (*R*)
18	*Fusarium* sp. NCIM 1075	25.5	24.0	51 (*R*)
19	*Aspergillus flavus* NCIM 537	21.5	24	45 (*R*)
20	*Verticillium*	78.0	48.0	41 (*R*)
21	*Fusarium moniliforme* 1100	18.7	36.0	32 (*R*)

Reaction conditions: Substrate 100 mg/100 mL reaction medium, biocatalyst 0.7 g dry wt., room temperature, shaker 200 rpm.

Among the strains screened (Table 3.2), *Trichothecium* sp. (entry **10**) was found to yield very good ee (93 %) followed by *Fusarium oxysporum* NCIM 1072, *Fusarium moniliforme* and *Aspergillus oryzae* NCIM 649 (entry **1**, **2**, **11** respectively) with good ee (≥ 80 %) for the enantioselective reduction of acetophenone. As far as the *Fusarium* strains are concerned, one strain (entry **4**) could reduce acetophenone to (*S*)-1-phenylethanol in quantitative yield as well as with good enantioselectivity (74 %). Except that (entry **4**) all of the *Fusarium* strains reduced acetophenone in low yield. *Verticillium* (entry **20**) also gave moderate ee (41 %) at 78 % conversion. All the strains of *Aspergillus* reduced acetophenone to 1-phenylethanol in the range of moderate to good ee (45 % to 81 %). It is also noticeable that in all cases of *Aspergillus* the predominant enantiomer of 1-phenylethanol was (*R*).

3.2.1.2 Effect of biomass

The amount of the fungal mycelia was optimized for the bioreduction of acetophenone. 0.35 – 0.9 g dry wt (20–50 g wet wt.) of biomass was used for this standardization as depicted in Fig. 3.3. It was observed that conversion varied with the amount of biomass. At 0.35 g and 0.5 g dry weight of biomass, the maximum conversion of *ca*. 20 % and 33 %, respectively, was obtained during first 24 h and there was insignificant increase in conversion when reaction was further continued for next 72 h (curves a and b, Fig. 3.3). But interestingly, there was a rapid change in the conversion as the biomass was increased from 0.5 g to 0.7 g dry wt., whereas moderate conversion (53 %) was obtained in first 24 h and it reached upto 82 % in next 72 h (total 96 h) (curve c, Fig. 3.3). However, insignificant increase in conversion was observed with further increase in biomass amount from 0.7 g to 0.9 g wt. (curve d, Fig. 3.3).

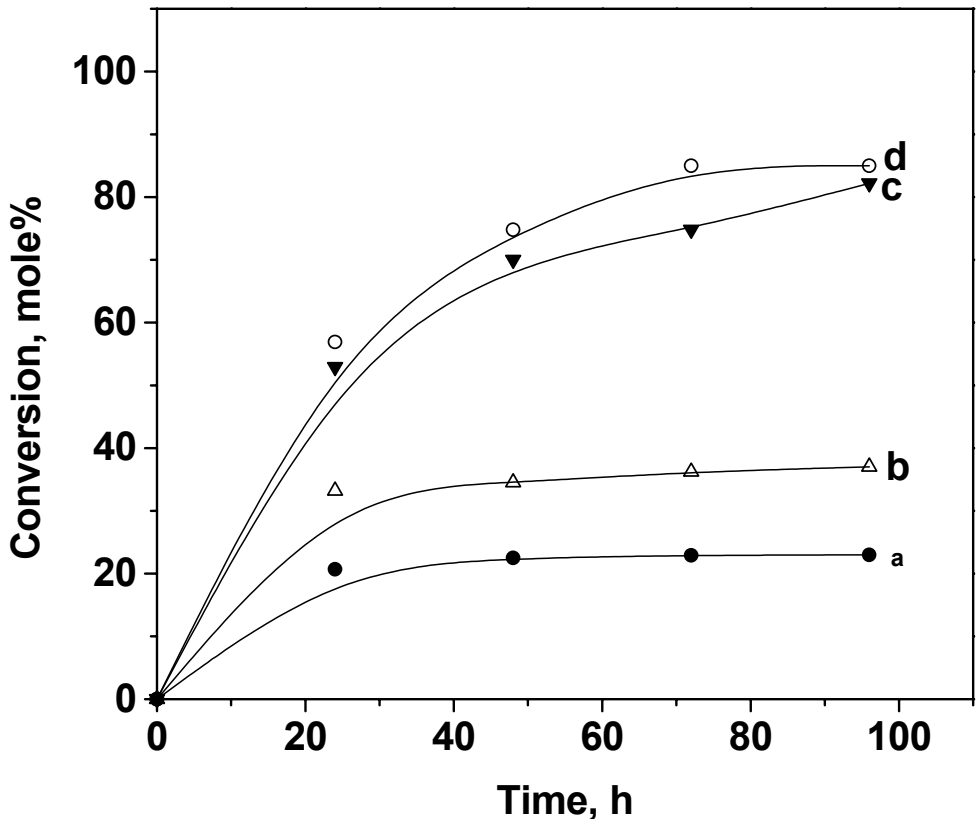


Fig. 3.3 Effect of biomass in the bioreduction of acetophenone by *Trichothecium* sp. [(a) 0.35 g dry wt., (b) 0.5 g dry wt., (c) 0.7 g dry wt., (d) 0.9 g dry wt. Reaction conditions: substrate 100 mg, room temperature, shaker 200 rpm].

3.2.1.3 Effect of time

Effect of reaction time was studied on the bioreduction of acetophenone with different fungi using 0.7 g dry wt. of biomass (the amount of biomass remains same unless stated otherwise). The time course of reduction of acetophenone with the fungus *Trichothecium* is depicted in Fig. 3.4. In this case ee remains same (93 %) during the reaction period. In some cases it was observed that the enantioselectivity of 1-phenylethanol was greatly affected by the

reaction time. In the case of fungus *Fuasrium oxysporum* NCIM 1043 (Fig. 3.5), initially ee was 75 % in just 0.5 h (though the conv. was less, 6.2 %), but elongation of the reaction time leads to the decrease in ee; whereas in case of *Colletotrichum* (Fig. 3.6) the product slowly got racemized. The enantioselectivity is gradually increased upto *ca.* 50 % and remained constant thereafter in case of *Fusarium* sp. NCIM 1075 (Fig. 3.7). The reason for such observations might be the presence of at least two types of major enzymes in the fungus. If two enzymes, each with high but opposite stereochemical preferences, compete for the same substrate, the optical purity of the product is determined by the relative rates of the individual reactions [43].

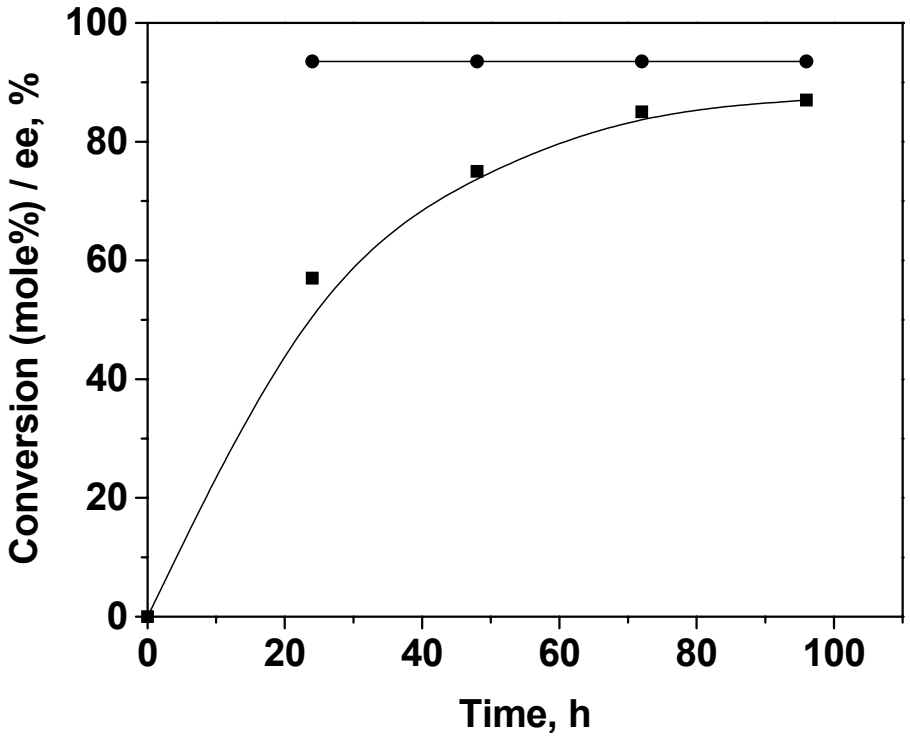


Fig. 3.4 Time course in bioreduction of acetophenone by *Trichothecium.* [(■) Conversion of acetophenone, (●) ee of 1-phenylethanol. Reaction conditions: substrate 100 mg, biocatalyst 0.7 g (dry wt.), room temperature, shaker 200 rpm].

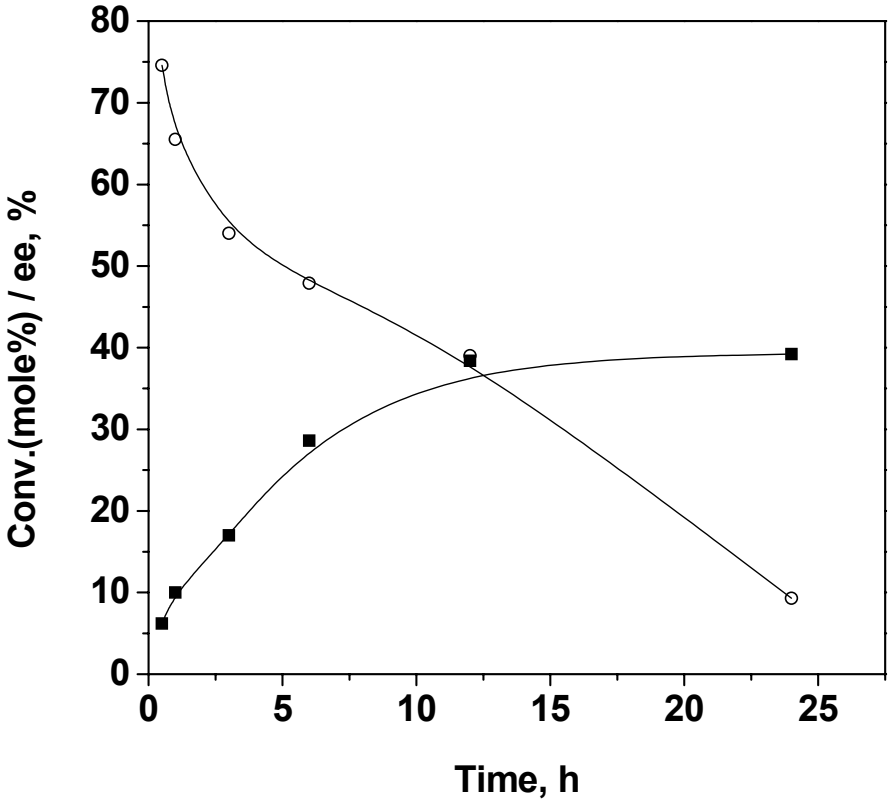


Fig. 3.5 Effect of reaction time on enantioselectivity in the bioreduction of acetophenone by *Fusarium oxysporum* NCIM 1043. [Reaction conditions: substrate 100 mg, biocatalyst 0.7 g, room temperature, shaker 200 rpm. (■) conversion of acetophenone, (○) ee of 1-phenylethanol].

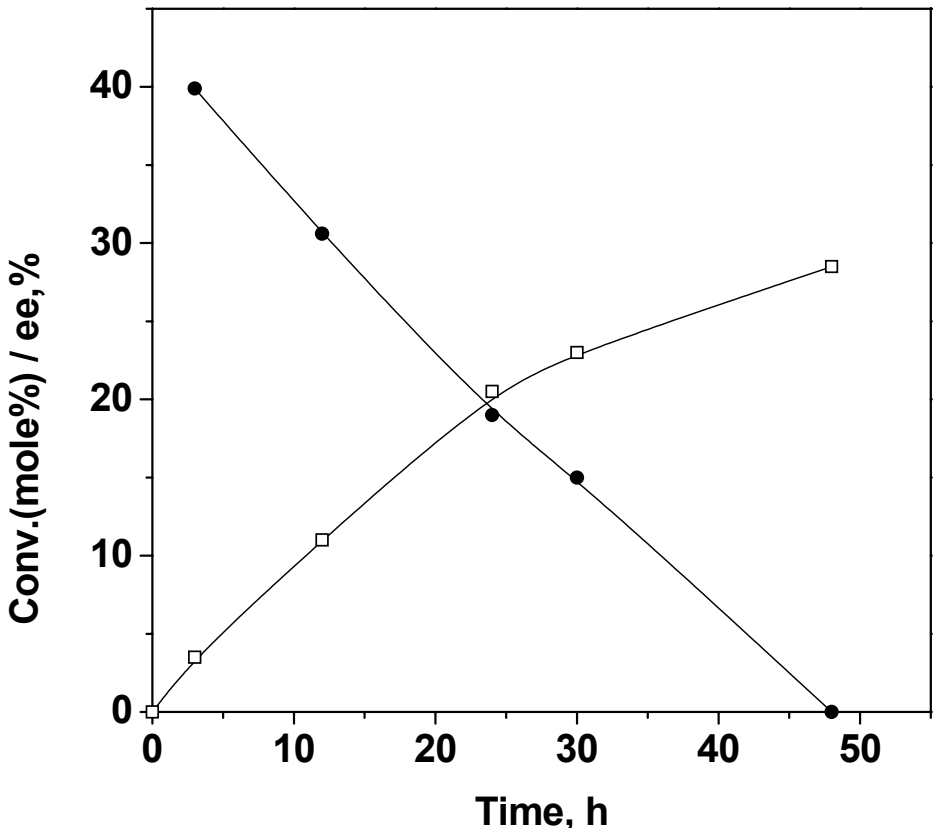


Fig. 3.6 Effect of reaction time on enantioselectivity in the bioreduction of acetophenone by *Colletotrichum.* [Reaction conditions: substrate 100 mg, biocatalyst 0.7 g, room temperature, shaker 200 rpm. (□) conversion of acetophenone, (●) ee of 1-phenylethanol].

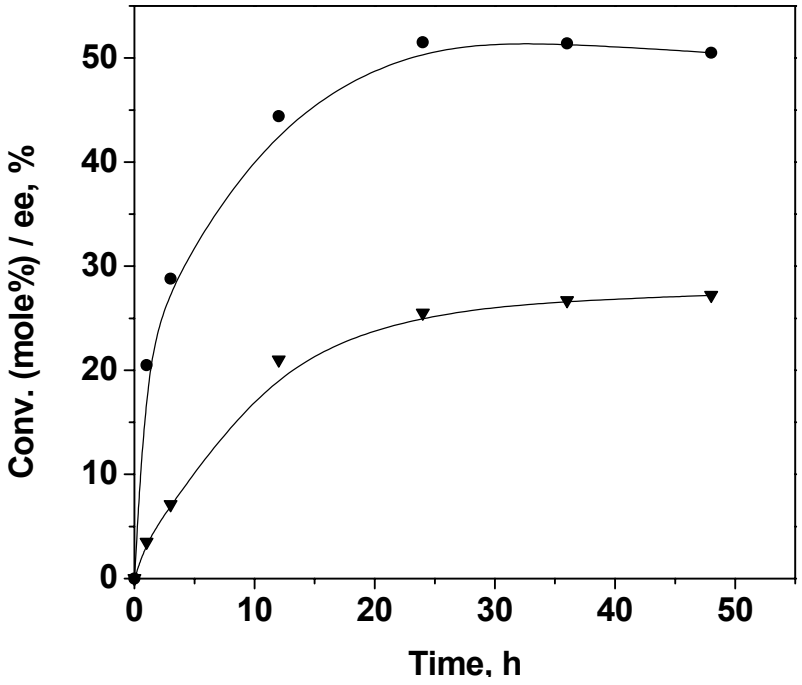


Fig. 3.7 Effect of reaction time on enantioselectivity in the bioreduction of acetophenone by *Fusarium* sp. NCIM 1075. [Reaction conditions: substrate 100 mg, biocatalyst 0.35 g, room temperature, shaker 200 rpm. (▼) conversion of acetophenone, (●) ee of 1-phenylethanol].

Usually, in whole cell biotransformation the enantioselectivity does not get affected with the variation of biomass amount. But it was observed that in the case of *Fusarium* sp. NCIM 1075, 0.5 g of dry biomass initially produced (*S*)-1-phenylethanol (ee ≈ 14 %), which got slowly converted to (*R*)-1-phenylethanol (conv. 45 %, ee 18 %). In contrast, 0.25 g of dry biomass produced (*R*)-1-phenylethanol with better ee, 51.5 % (Fig. 3.7). So, a slight improvement in enantioselectivity with decrease in biomass amount was observed in this case.

3.2.1.4 Effect of substrate concentration

The potentiality of the fungus towards high substrate concentration was investigated with increase in acetophenone concentration. As the concentration of acetophenone was increased from 100 mg to 200 mg the conversion changed drastically. Maximum conversion obtained for 200 mg was 24 % instead of 85 % in case of 100 mg substrate. Interestingly, enantioselectivity remains the same (92 ± 1 %) irrespective of substrate concentration.

Table 3.3

Effect of substrate concentration in the bioreduction of acetophenone by *Trichothecium*

Substrate conc. (g / 100 mL reaction medium)	Time (h)	Conversion (mole %)
0.2	24	17
	48	17
	120	24
0.3	24	15
	48	15
	120	18
0.4	24	14
	48	14
	120	17
0.5	24	10
	48	10
	120	12

Reaction conditions: Biocatalyst 0.7 g (dry wt.), room temperature, shaker speed 200 rpm.

3.2.1.5 Effect of solvent

As the organic compounds are insoluble or sparingly soluble in water, usually ethanol is used as solvent for the solubilizing the compound in the whole cell biotransformation. To investigate whether the hydride ion for reduction is coming from the solvent as occurs in chemical reduction, the biotransformation was performed with other aprotic solvents like DMF (dimethyl formamide). Interestingly, it was observed that the conversion of acetophenone was comparable in both the cases, which suggests that the hydride ion might be coming from the cofactor, which is generated *in situ* by the whole cell.

3.2.1.6 Effect of surfactant

It was reported by Goswami *et al*. [44] that surfactant could increase both the conversion of acetophenone derivatives and the enantioselectivity of the products. In this context, the effect of different surfactants such as SDS, CTAB, IGEPAL, Tritan, Tween 20, Tween 80, was studied in the bioreduction of acetophenone by *Trichothecium*. However, no such effect in conversion was observed.

3.2.2 Bioreduction of other prochiral ketones

3.2.2.1 *Bioreduction by Trichothecium*

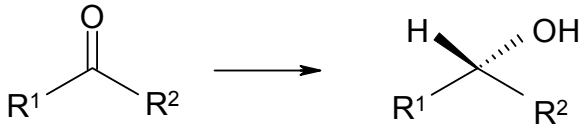
The fungus *Trichothecium* sp. was also applied to reduce some other prochiral ketones for investigating the substrate range. The results are summarized in Table 3.4. It can be seen from table that the acetophenone (entry **1**) could be reduced to (*R*)-1-phenylethanol with 93 % ee, whereas no reaction was observed in the case of ketones, where $R^2 = CF_3$ (entry **2**) and cyclopropyl (entry **4**) and R^1 = Ph.

In case of propiophenone (entry **3**), 1-phenyl-1,2-propanediol was formed alongwith 1-phenyl-1-propanol, the reduced product of propiophenone. Further, the role of substituents in

the aromatic moiety (R^1) on the course of the biotransformation was also studied (entry **6-7**). In this case, para-substituted phenylethanones were consistently converted to the corresponding (*R*)-alcohols. Introduction of an electron withdrawing substituent is expected to assist the reaction. Indeed, the *p*-chloro-substituted acetophenone was reduced with high enantioselectivity possibly due to the -I effect of the substituent. Acetophenone gets reduced 56 % in 24 h by *Trichothecium*, whereas upon methyl substitution (+I effect) at para position (entry **6**) the reaction becomes slow (conv. 45.5 % in 24 h). In contrast, para-chloro substitution (entry **7**) accelerates the reaction resulting 72 % conversion during same reaction time of 24 h. To further substantiate this observation, two electron donating substrates namely para-hydroxy acetophenone and para-methoxy acetophenone (entry **8**, **9**) were used as reactants. Expectedly, there was almost negligible conversion. It appears that electronic effect of the substituent in the aromatic ring has a definite role in the enantioselectivity of the reaction.

Table 3.4

Bioreduction of different prochiral ketones by the fungus *Trichothecium*



I - IX **Ia – IXa**

Entry No	Substrate	R^1	R^2	Product	Yield[a] (mol %)	Time, h	ee (%)[b]
1	**I**	Ph	CH_3	**Ia**	85	72	93 *(R)*
2	**II**	Ph	CF_3		No reaction		
3	**III**	Ph	CH_2CH_3	**IIIa**	13[c]	24	92 *(R)*
4	**IV**	Ph	Cyclopropyl		No reaction		
5	**V**	Cyclohexyl	CH_3	**Va**	85	96	97 *(R)*
6	**VI**	*p*-MeC_6H_4	CH_3	**VIa**	45.5	24	90 *(R)*
7	**VII**	*p*-ClC_6H_4	CH_3	**VIIa**	72	24	98 *(R)*
8	**VIII**	*p*-OHC_6H_4	CH_3		No reaction		
9	**IX**	*p*-$MeOC_6H_4$	CH_3		No reaction		

Reaction conditions: Biocatalyst 0.7 g (dry wt.), substrate 100 mg, room temperature, shaker speed 200 rpm.

[a,b]Yield and ee were determined by GC analyses by using HP chiral (20 % permethylated β-cyclodextrin) capillary column. Absolute configuration was assigned by measuring optical rotation.

[c]Rest is 1-phenyl-1,2-propanediol (yield 71 %)

Optical rotations ($[\alpha]_d$) of the products: **Ia** (+ 45.73, $CHCl_3$), **IIIa** (+34.22, $CHCl_3$) **Va** (-3.92, $CHCl_3$), **VIa** (+ 35.92, MeOH), **VIIa** (+65.49, $CHCl_3$).

3.2.2.2 Bioreduction by Aspergillus oryzae

In continuation to the earlier experiment, reduction of prochiral ketones was also studied by another fungus *Aspergillus oryzae* NCIM 649, which is next to *Trichothecium* to produce (*R*)-1-phenylethanol in good enantioselectivity. The results are tabulated below in Table 3.5. As it may be recalled that acetophenone could be reduced by the same fungus to (*R*)-1-phenyl ethanol with 81 % ee, there is an improvement in enantiomeric excess with the introduction of substituent at para position of the aromatic moiety. Moreover, it can reduce acetophenone and other two derivatives with better yields as compared to *Trichothecium*.

Table 3.5

Bioreduction of aromatic ketones by *Aspergillus oryzae*

Entry	Substrate	Product	Yield, mole %	Time, h	ee, %
1	**I**	**Ia**	100	48	81 (*R*)
2	**VI**	**VIa**	68	24	93 (*R*)
3	**VII**	**VIIa**	88	24	93 (*R*)

Reaction conditions: Biocatalyst 0.7 g (dry wt.), substrate 100 mg, room temperature, shaker speed 200 rpm.

Yield and ee were determined by GC analyses by using HP chiral (20 % permethylated β-cyclodextrin) capillary column. Absolute configuration was assigned by comparing with earlier experiments.

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The area of nanotechnology, which spans through the synthesis of nanoscale matter, understanding/utilizing their exotic physicochemical and optoelectronic properties and organization of nanoscale structures into predefined superstructures, promises to play an increasingly important role in many key technologies of the new millennium [1,2]. As far as the synthesis of nanoparticles is concerned, there is an ever-growing need to develop clean, nontoxic and environmentally friendly ("green chemistry") synthesis procedures. Consequently, researchers in the field of nanoparticle synthesis and assembly have been seriously looking at biological systems for inspiration. The above factor, combined with purely academic curiosity, has lead to the development of biomimetic approaches for the growth of advanced materials. Many organisms, both unicellular and multicellular, are known to produce inorganic materials either intra- or extracellularly [3,4], examples of which include magnetotactic bacteria (which synthesize magnetite nanoparticles) [5,6], diatoms (which synthesize siliceous materials) [7,8] and S-layer bacteria (which produce gypsum and calcium carbonate layers) [9,10].

Even though microbes have been used with considerable success in biotechnological applications such as remediation of toxic metals [11,12], reports on their use in the synthesis of nanomaterials have been extremely limited. Beveridge and coworkers have demonstrated that gold particles of nanoscale dimensions may be readily precipitated within bacterial cells by incubation of the cells with Au^{3+} ions [13-15]. A detailed study of the growth of Au nanoparticles using *Bacillus subtilis* 168 revealed the initial precipitation of noncrystalline Au particles which subsequently transformed into crystalline octahedral gold containing sulfur and phosphorus [14]. More recently, Klaus-Joerger and coworkers have demonstrated that the bacteria *Pseudomonas stutzeri* AG259 isolated from a silver mine when placed in a

concentrated aqueous solution of $AgNO_3$, resulted in the reduction of the Ag^+ ions and formation of silver nanoparticles of well-defined size and distinct morphology within the periplasmic space of the bacteria [16,17]. Furthermore, they have shown that biocomposites of nanocrystalline silver and the bacteria may be thermally treated to yield a carbonaceous material with interesting optical properties for potential application in functional thin film coatings [18].

Observing the reducing property of the fungus *Fusarium oxysporum* discussed in Chapter 2, it was applied for metal ion reduction. Interestingly it worked and gold nanoparticles were obtained from aqueous solution of chloroauric acid when exposed to the same fungus [19]. The idea was extended to other fungi. In this chapter, it is demonstrated that the fungus, *Verticillium* (AAT-TS-4), when exposed to aqueous $HAuCl_4$ or aqueous $AgNO_3$ solution results in the reduction of the metal ions and formation of gold or silver nanoparticles of *ca*. 20-25 nm diameter [20,21]. The nanoparticles are formed on the surface as well as within the fungal cells (on the cytoplasmic membrane) with negligible reduction of the metal ions observed in solution.

4.1 EXPERIMENTAL

4.1.1 Biotransformation of metal ions

The fungi, *Fusarium oxysporum* was obtained from National Collection of Industrial Microorganisms (NCIM), NCL, Pune and *Verticillium*, was isolated from the *Taxus* plant and maintained on potato-dextrose agar slants at 25 °C. The fungus was grown in 500 mL Erlenmeyer flasks each containing 100 mL MGYP media, composed of malt extract (0.3 %), glucose (1.0 %), yeast extract (0.3 %), and peptone (0.5 %) at 25-28 °C under shaking

condition (200 rpm) for 96 h. After 96 h of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 10 °C for 20 min and the settled mycelia was washed thrice with sterile distilled water. 10 g of the harvested mycelial mass was then re-suspended in 100 mL sterile distilled water in 500 mL conical flasks at pH = 5.5 - 6.0. To this suspension, $AgNO_3$ or $AuCl_4^-$ was added in such a way so that the strength of the solution becomes 10^{-4} M. The whole mixture was thereafter put into a shaker at 28 °C (200 rpm) and the reaction carried out for a period of 72 h.

4.1.2 UV-Vis Spectroscopy

The biotransformation was routinely monitored by visual inspection of the biomass as well as measurement of the UV-vis spectra from the fungal cells and the aqueous medium in the reaction mixture. After this reaction period, the *Verticillium* biomass was washed thrice with copious amounts of sterile distilled water prior to preparation of the biofilms for analysis. UV-vis spectroscopic measurements of the films were made on a Shimadzu dual-beam spectrophotometer (model UV-1601PC) operating in the reflection mode at a resolution of 2 nm. Since the films of the bio-nanocomposite were rough, the results are not quantitative and have been used to merely detect the presence of gold/silver nanoparticles in the biomaterial. UV-vis spectra of the aqueous $AgNO_3$ / $AuCl_4^-$ solution as a function of time of reaction as well as after 72 h of reaction with the mycelial cells were also carried out in the transmission mode on the same instrument.

4.1.3 XRD

The powder X-ray diffractograms of nanomaterials were recorded on a Rigaku D MAX VC diffractometer using a Ni-filtered monochromatic Cu K_α radiation (λ = 1.5406 Å). The

nanomaterials were scanned at 2θ ranges 5-70° with a scanning rate of 4°/min. The samples were prepared as thin layers on glass/aluminum slides, prior to scanning.

4.1.4 SEM and EDX

Scanning electron microscopy (SEM) and energy dispersive X-ray analyses (EDX) of the fungal cells of *Verticillium* after formation of gold/silver nanoparticles were carried out on a Leica Stereoscan-440 SEM equipped with a Phoenix EDX attachment. EDX spectra were recorded in the spot-profile mode by focusing the electron beam onto a region on the surface of the mycelial cells rich in gold/silver nanoparticles.

4.1.5 TEM

TEM measurements were carried out on a JEOL Model 1200EX instrument operated at an accelerating voltage of 60 kV. A low operating voltage was used to minimize damage to the thin sections by electron beam heating.

Thin sections of the *Verticillium* cells after reaction with $AgNO_3$ or $AuCl_4^-$ ions were prepared for transmission electron microscopy (TEM) analysis as follows. Approximately 1 mm^3 bits of the Au/Ag nano-*Verticillium* biomass were taken and fixed in 2.5 % gluteraldehyde in distilled water for 2 h at room temperature. After fixation, the cells were sedimented (1,500 rpm, 10 min) and washed thrice with distilled water. Without postfixation, the pellet was subjected to dehydration with 30, 50, 70 and 90 % ethanol for 15 min at each concentration followed by two changes in absolute ethanol. Since ethanol does not possess good miscibility with epoxy resins, propylene oxide was used as a linking agent. The dehydrated pellet was kept in propylene oxide for 15 min following which the infiltration of the resin was done by placing the pellet in a 1:1 mixture of propylene oxide and Epon 812 overnight at room temperature. Embedding was carried out using a mixture of the resin (Epon 812) and hardeners (DDSA –

dodecynyl succinic anhydride and MNA – methyl nadic anhydride) in the ratio 1:1.5. To this, two drops of tridimethylaminomethyl phenol (DMP30) were added to accelerate the polymerization process. Polymerization was carried out using this mixture at 60 °C for 3 days. Ultrathin sections were cut using an ultramicrotome (Leica Ultracut UCT) and were taken on copper TEM grids (40 μm X 40 μm mesh size). The sections were slightly stained with uranyl acetate and lead citrate prior to TEM analysis.

4.2 RESULTS AND DISCUSSION

4.2.1 Extracellular Synthesis of gold nanoparticles

Fig. 4.1 shows two conical flasks with the *Fusarium oxysporum* biomass before (A) and after reaction with chloroaurate ions for 72 h (B). It is observed that the biomass has a pale yellow color before reaction with the gold ions (A), which changes to a dark purple color on completion of the reaction (B). The appearance of a purple color is a clear indication of the formation of gold nanoparticles in the reaction mixture. The characteristic pink-purple color of colloidal gold solutions is due to excitation of surface plasmon vibrations in the nanoparticles and provides a convenient spectroscopic signature of their formation. Upon filtration, it was observed that the biomass was still pale yellow and the aqueous solution contained the gold nanoparticles. Fig. 4.2 shows the UV-vis spectra recorded from the reaction solution as a function of time of reaction. The strong surface plasmon resonance centered at *ca*. 545 nm is clearly seen which increases in intensity with time, stabilizing after 48 h of reaction. Quite interestingly, the solution was extremely stable with no evidence of flocculation of the particles even a month after the reaction. The plasmon resonance is sharp and indicates little aggregation of the particles in solution.

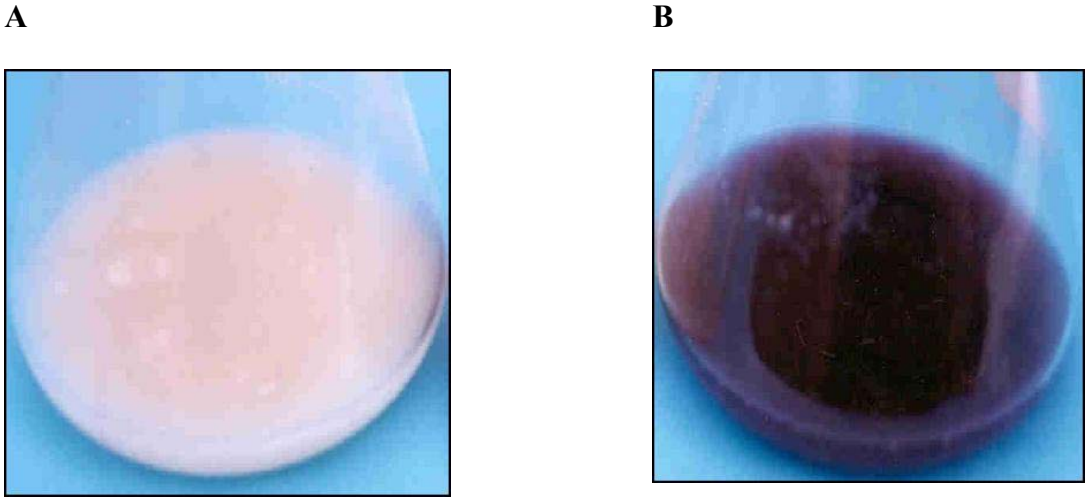

Fig. 4.1 Conical flasks with *Fusarium oxysporum* biomass before (A) and after exposure to $AuCl_4^-$ ions for 72 h (B).

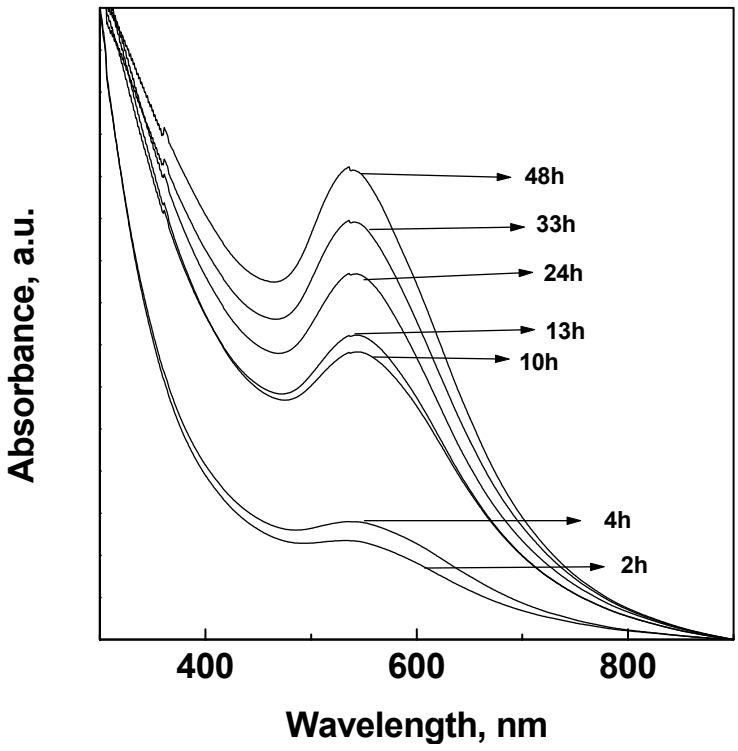

Fig. 4.2 UV-Vis spectra of the filtrate after immersion of fungal biomass in $HAuCl_4$ solution.

4.2.2 Intracellular synthesis of gold nanoparticles

Fig. 4.3 (A) shows a conical flask of the *Verticillium* fungal cells after removal from the culture medium and before immersion in $HAuCl_4$ solution. The pale yellow color of the fungal cells can clearly be seen in the figure. A picture of the conical flask containing the fungal cells after exposure to 10^{-4} M aqueous solution of $HAuCl_4$ for 72 h is shown in Fig. 4.3 (B). A vivid purple color is clearly seen in the fungal cells and indicates formation of Au nanoparticles in the cells. It is to be noted that Fig. 4.3 (B) shows the fungal cells along with the $HAuCl_4$ solution after incubation for 72 h. The aqueous medium is observed to be almost colorless, strongly indicating that extracellular reduction of $AuCl_4^-$ ions had not occurred (Fig. 4.4). It is well known that gold nanoparticles absorb radiation in the visible region of the electromagnetic spectrum (*ca*. 520 nm) due to excitation of surface plasmon vibrations and this is responsible for the striking color of gold nanoparticles in various media [22]. Fig. 4.5 shows the UV-vis spectra recorded from a film of the fungal cells before (curve 1) and after immersion in 10^{-4} M $HAuCl_4$ solution for 72 h (curve 2). While there is no evidence of absorption in the spectral window (400 – 800 nm) in the case of the as-harvested fungal cells, the fungal cells exposed to $AuCl_4^-$ ions show a distinct absorption at *ca*. 540 nm. This resonance is close to that observed for thin films of gold nanoparticles grown by different techniques [22,23-26]. The UV-vis spectrum recorded from the aqueous $HAuCl_4$ solution after immersion of the fungal cells for 72 h in the electrolyte solution is also shown in Fig. 4.5 (dashed line). As in the case of the as-harvested fungal cells (curve 1), there is no discernable absorption in this spectral region. This result thus supports the visual evidence provided by the photograph shown in Fig. 4.3 (B) that negligible extracellular reduction of the $AuCl_4^-$ ions had occurred in solution. Further evidence

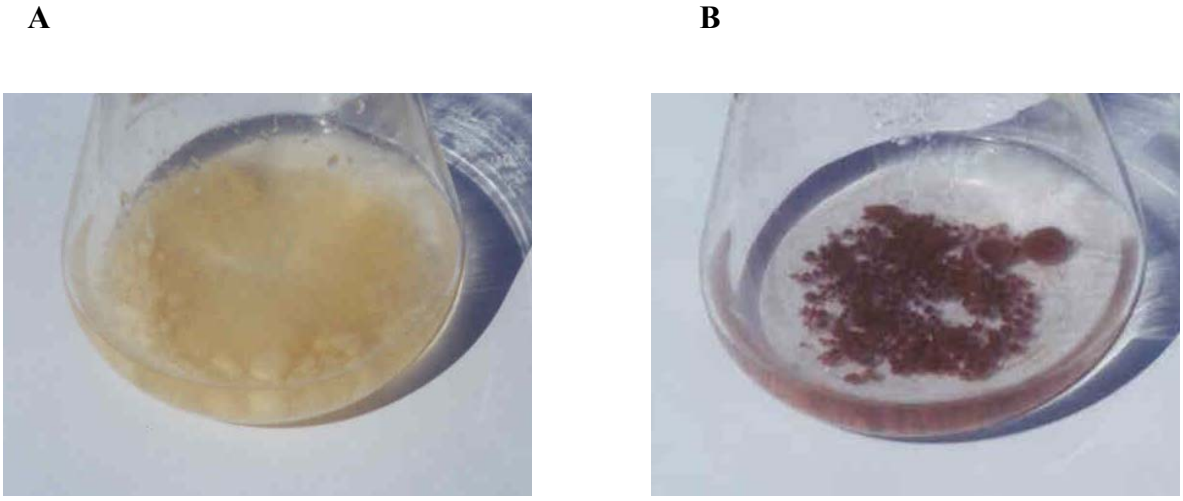

Fig. 4.3 A) Conical flask showing the *Verticillium* fungal cells after removal from the culture medium, B) Conical flask showing the *Verticillium* fungal cells after exposure to 10^{-4} M aqueous solution of $HAuCl_4$ for 72 h.

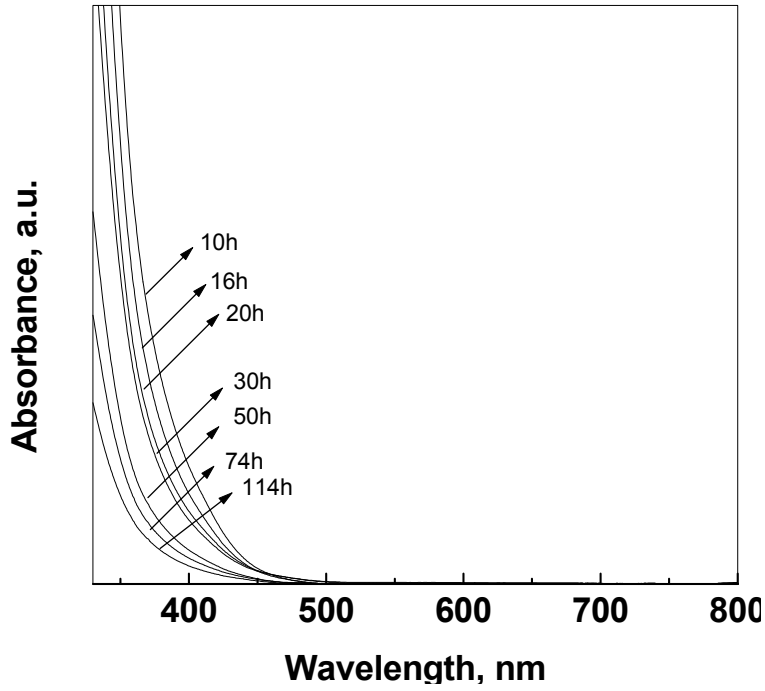

Fig. 4.4 UV-Vis of the filtrate of the biomass after immersion in $HAuCl_4$ solution.

for the intracellular formation of gold nanoparticles is provided by XRD analysis of the biofilm deposited on a Si(111) substrate (Fig. 4.6). The presence of intense peaks corresponding to the (111), (200) and (220) Bragg reflections of gold are identified in the diffraction pattern. The peak positions agree with those reported in the literature for gold nanocrystals [27]. An estimate of the mean size of the gold nanoparticles formed in the *Verticillium* cells was made using the Debye-Scherrer equation by determining the width of the (111) Bragg reflection [28]. The size of the gold nanoparticles was determined in this manner to be *ca*. 25 nm.

Fig. 4.7 shows an SEM picture of the fungal cells after exposure to $AuCl_4^-$ ions for 72 h. The presence of mycelia covered with well-dispersed gold nanoparticles can clearly be observed in the figure. The gold nanoparticles appear to be on the surface of the mycelia. A spot profile EDX analysis of one of the gold nanoparticles on the surface of the mycelia was carried out and is shown in Fig. 4.8. The presence of strong signals from the gold atoms in the nanoparticles together with weaker signals from C, O and Cl atoms is seen. The C and O signals are likely to be due to X-ray emission from proteins/enzymes either directly bound to the gold nanoparticle or present in the vicinity of the particle, while the presence of a weak Cl signal indicates the presence of a small fraction of unreacted $AuCl_4^-$ ions in the region being investigated.

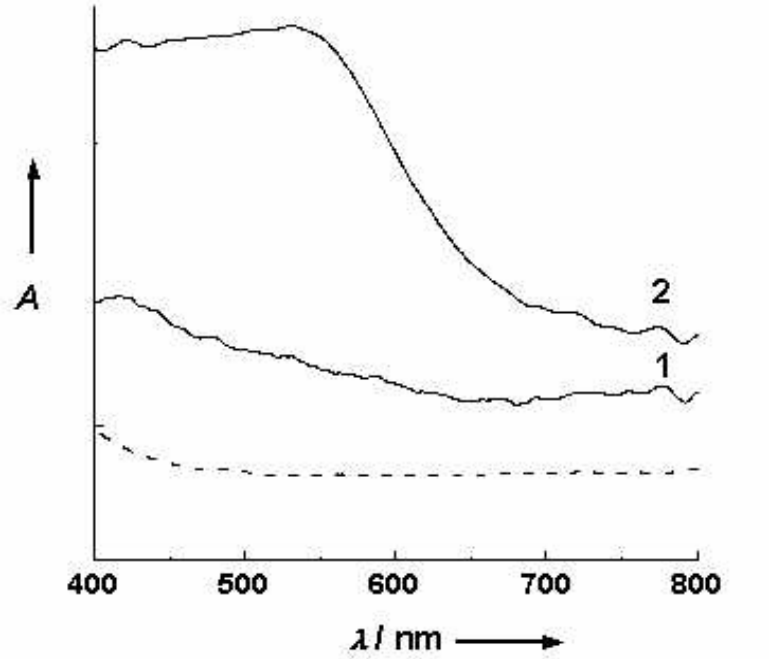

Fig. 4.5 UV-Vis spectra recorded from biofilms of *Verticillium* fungal cells before (curve 1) and after exposure to $HAuCl_4$ solution for 72 h (curve 2). Dashed line indicates the spectrum of $HAuCl_4$ solution obtained after immersion of the fungal cells for 72 h and removing fungal cells.

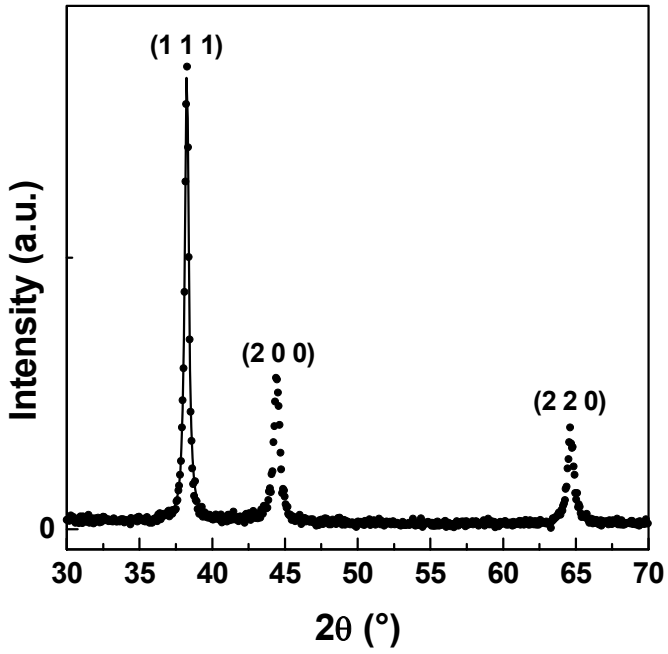

Fig. 4.6 XRD pattern recorded from an Au nano-*Verticillium* biofilm on a Si(111) wafer.

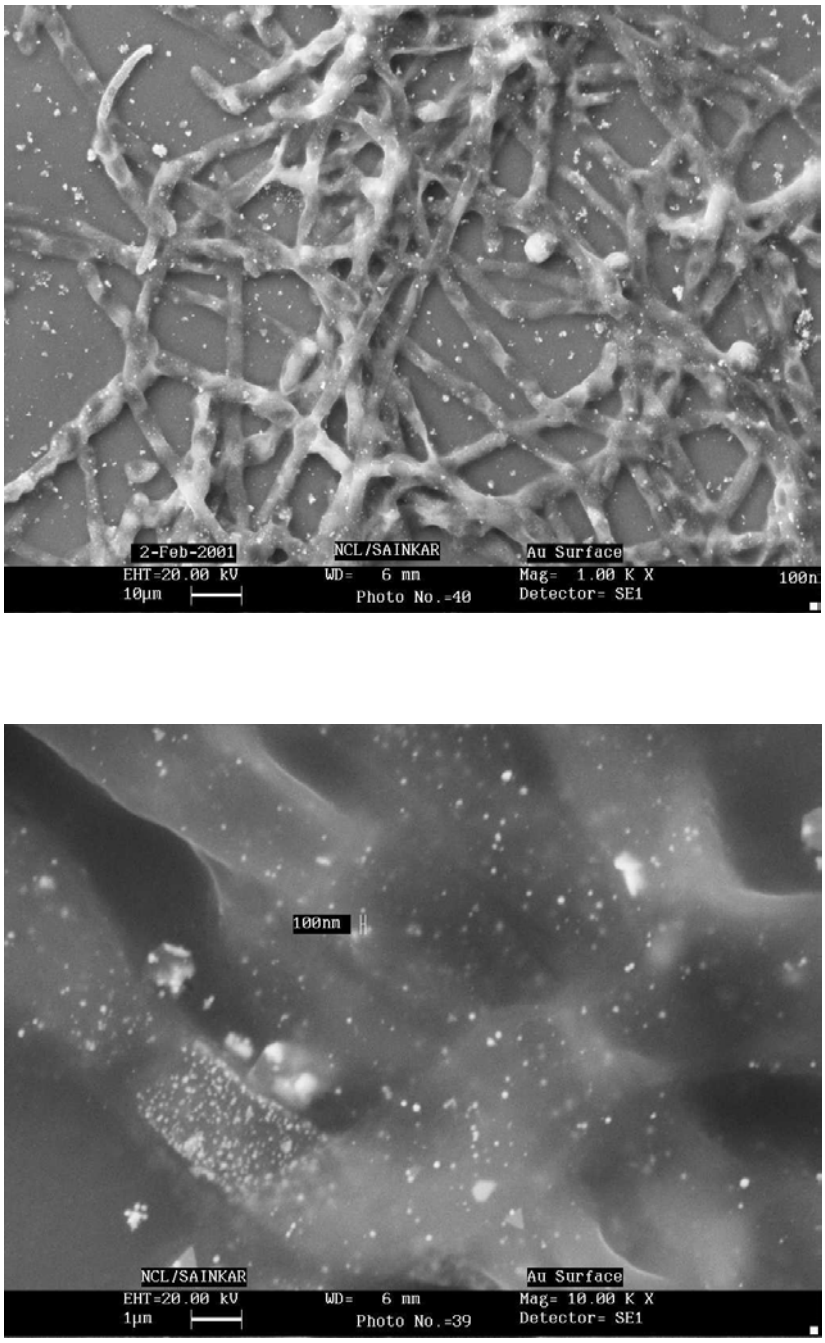

Fig. 4.7 SEM images with different magnification of a biofilm of the *Verticillium* fungal cells deposited on a Si(111) wafer after immersion of the cells in $HAuCl_4$ solution for 72 h.

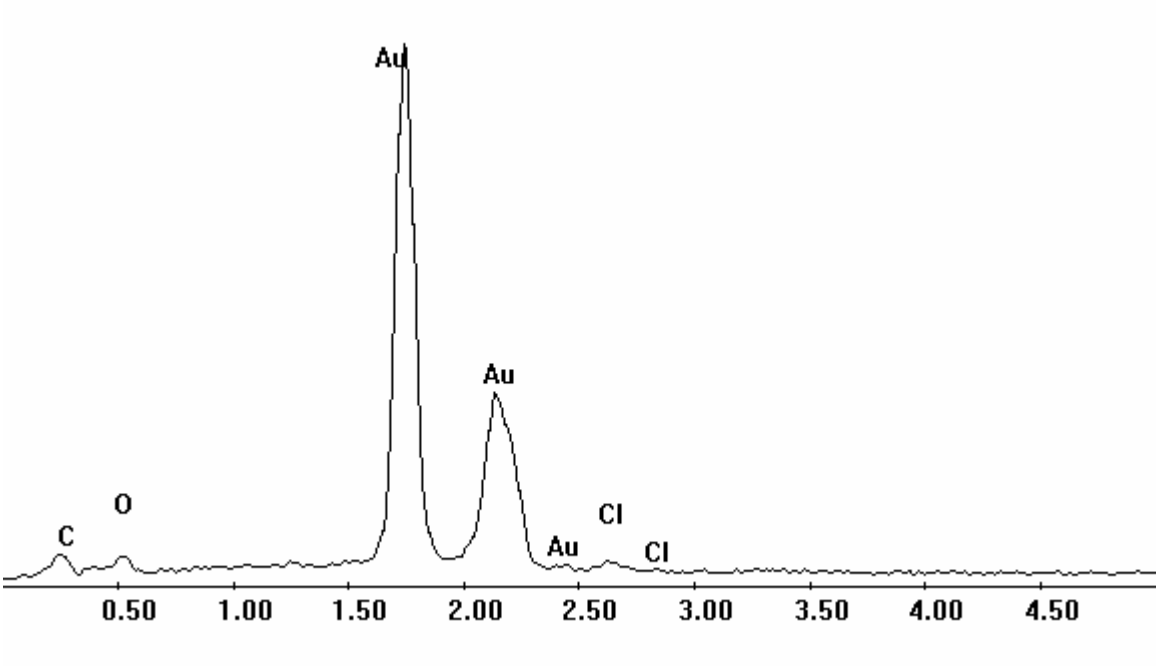

Fig. 4.8 Spot profile EDX spectrum recorded from one of the gold nanoparticles shown in Fig. 4.7.

In order to understand, albeit tentatively, the mechanism of formation of the gold nanoparticles in the biofilm, information on the location of the gold nanoparticles relative to the fungal cells is important. This is conveniently done by TEM analysis of thin sections of the Au nano-*Verticillium* cells, images of which are shown at various magnifications in Fig. 4.9. At lower magnification, a number of *Verticillium* cells can be seen in Fig. 4.9 (A). Careful inspection of the picture shows extremely small particles of gold organized beautifully on the walls of the different cells. Larger particles of gold are seen within the cells and are in considerably smaller number. Fig. 4.9 (B) shows a TEM image of a single *Verticillium* cell

where it is observed that the gold nanoparticles are formed both on the cell wall (outer boundary) as well as on the cytoplasmic membrane (inner boundary). The density of gold nanoparticles is clearly higher on the cytoplasmic membrane than on the cell wall. As observed in Fig. 4.9 (A), a very small percentage of larger gold particles are observed within the cytoplasm. A statistical analysis of the size of gold nanoparticles formed both on the cell wall and on the cytoplasmic membrane from many TEM images of single *Verticillium* cells resulted in an average gold nanoparticle size of 20 ± 8 nm. It is to be mentioned that in this analysis, the bigger gold particles (often larger than 100 nm, Figs. 4.9 C and D) have been excluded.

In order to better understand the morphology of the gold nanoparticles formed on the cytoplasmic membrane, higher magnification TEM images were recorded from one of the cells and are shown in Figs. 4.9 C and D. The highly organized assembly of gold nanoparticles on the membrane surface is clearly observed in Fig. 4.9 (C). What is striking is the density of the gold nanoparticles on the membrane surface – this aspect marks a significant point of departure from metal particle densities observed in bacterial cells where often not more than 10-15 nanoparticles are observed within one cell [17,18]. As can be seen in the higher magnification TEM thin section image (Fig. 4.9 D), the gold nanoparticles are mostly spherical in nature with occasional occurrences of triangular and hexagonal particles. The cytoplasmic membrane is clearly observed in Fig. 4.9 D and a large, quasi-hexagonal gold particle is observed within the cytoplasm.

While the exact mechanism leading to the formation of gold nanoparticles by reaction with the fungal cells is not understood at the moment, based on the results presented earlier it can be speculated the following. Recently, Esumi *et al*. have shown that sugar persubstituted poly(amidoamine) dendrimers spontaneously reduced $AuCl_4^-$ ions leading to the formation of

gold nanoparticles bound to the dendrimers [29]. In order to rule out the reduction of $AuCl_4^-$ ions by sugars in the cell wall, reaction of the metal ions with different genera of fungi such as *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Curvularia lunata*, etc. were carried out in a manner identical to that for *Verticillium*. No reduction of the $AuCl_4^-$ ions was observed in these cases on the surface of the cell thus ruling out a non-specific mechanism for the growth of the nanoparticles. Since the nanoparticles are formed on the surface of the *Veriticillium* cells and not in solution, it is believed that the first step involves trapping of the $AuCl_4^-$ ions on the surface of the fungal cells. This could occur *via* electrostatic interaction with positively charged groups (such as for example, lysine residues) in enzymes present in the cell wall of the mycelia. Thereafter, the gold ions are reduced by enzymes within the cell wall leading to the aggregation of the metal atoms and formation of gold nanoparticles. TEM analysis of thin sections also shows the presence of gold nanoparticles on the cytoplasmic membrane (Fig. 4.9 B, D) indicating that some of the $AuCl_4^-$ ions/small gold particles diffuse across the cell wall barrier and are localized on the cytoplasmic membrane. It is possible that enzymes present in the cytoplasmic membrane also participate in the reduction of the gold ions.

The full potential of using microorganisms for the synthesis of nanoscale matter may be realized if the ability of the cells to multiply (and thus cover large surface areas with nanomaterials) is not compromised by the presence of metal ions. Consequently, a small number of the Au nano-*Verticillium* cells were removed from the biofilm and placed on an agar plate in the culture medium. Within a week, the seed fungal cells had multiplied to cover the surface of the agar plate of diameter 8 cm (picture shown in Fig. 4.10) thus clearly demonstrating that the $AuCl_4^-$ ions and Au nanoparticles do not lead to the death of the cells.

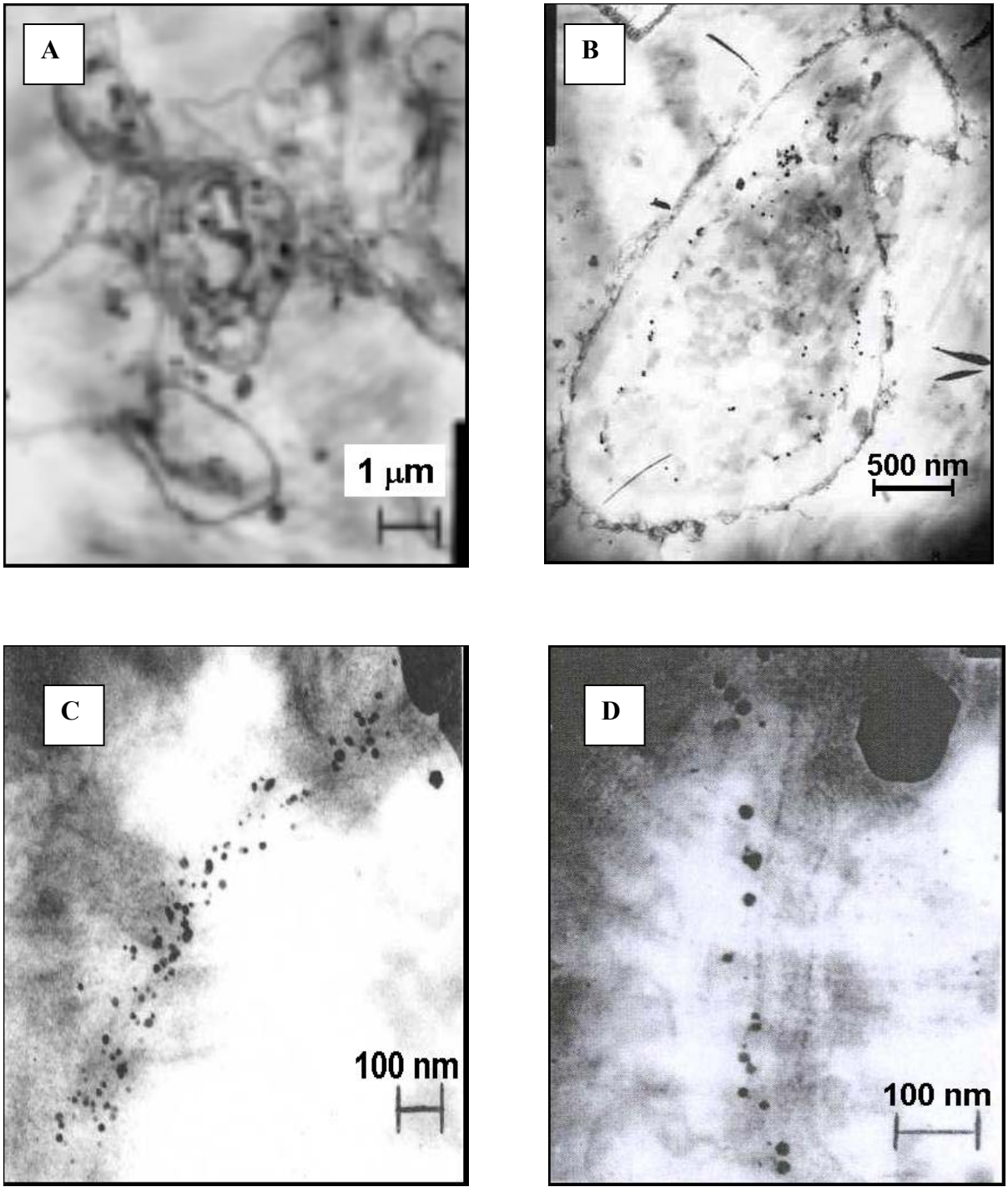

Fig. 4.9 TEM images at different magnifications of thin sections of stained *Verticillium* cells after reaction with $HAuCl_4$ for 72 h.

Fig. 4.10 Growth of *Verticillium* after reaction with $AuCl_4^-$ ions for 72 h.

4.2.3 Intracellular Synthesis of silver nanoparticles

Fig. 4.11 (A) shows a conical flask of the fungal cells after removal from the culture medium and before immersion in $AgNO_3$ solution. The pale yellow color of the fungal cells can clearly be seen in the figure. A picture of the conical flask containing the fungal cells after exposure to 10^{-4} M aqueous solution of $AgNO_3$ for 72 h is shown in Fig. 4.11 (B). A dark brown color is clearly seen in the fungal cells and indicates the synthesis of Ag nanoparticles by the cells. It is to be noted that Fig. 4.11 (B) shows the fungal cells along with the $AgNO_3$ solution. It is clear that the aqueous medium is colorless, thereby strongly indicating that extra-cellular reduction of Ag^+ ions had not occurred. It is well known that silver nanoparticles absorb radiation in the visible region of the electromagnetic spectrum (*ca*. 380-450 nm) due to excitation of surface plasmon vibrations and this phenomenon is responsible for the striking yellow-brown color of silver nanoparticles in various media [30-33]. Fig. 4.12 shows the UV-vis spectra recorded from a film of the fungal cells before (curve 1) and after immersion in 10^{-4} M $AgNO_3$ solution for 72 h (curve 5). While there is no evidence of absorption in the spectral window (400–800 nm) in the case of the as-harvested fungal cells, the fungal cells exposed to Ag^+ ions show a distinct and fairly broad absorption band centered at *ca*. 450 nm. The presence of the broad resonance indicates an aggregated structure of the silver particles in the film. As mentioned earlier, scattering from the rough biomass surface would also contribute to the broadening of the resonance. It is important to note that the UV-vis spectrum recorded from the fungal cells after immersion in $AgNO_3$ solution (Fig. 4.12, curve 5) is characteristic of aggregated silver nanoparticles and not silver sulfide nanoparticles [32].

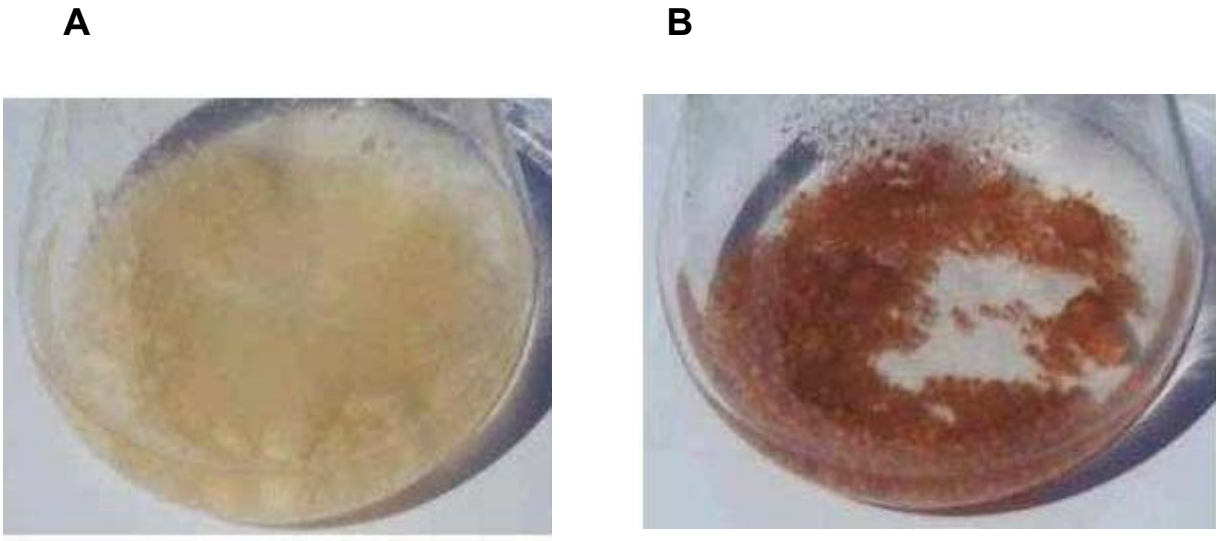

Fig. 4.11 A) Conical flask showing the *Verticillium* fungal cells after removal from the culture medium, B) Conical flask showing the *Verticillium* fungal cells after exposure to10^{-4} M aqueous solution of $AgNO_3$ for 72 h.

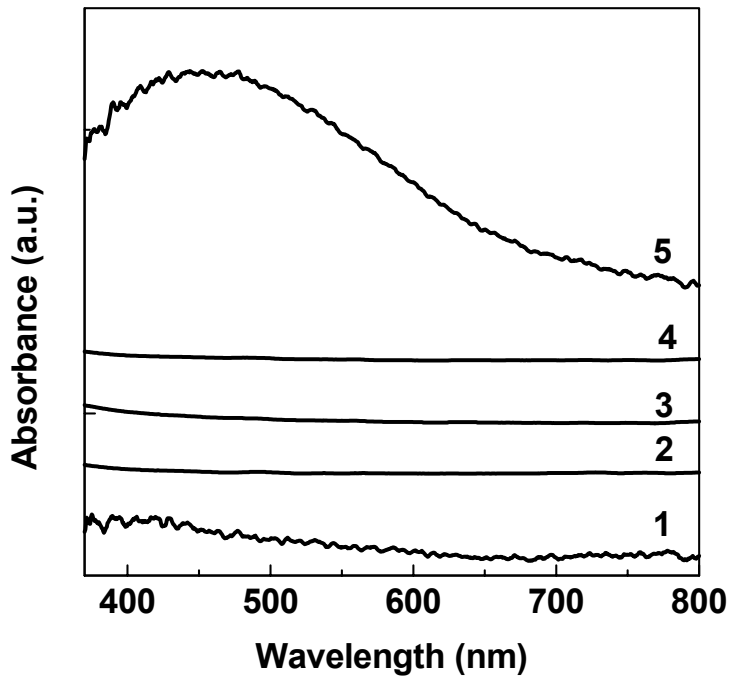

Fig. 4.12 UV-Vis spectra recorded from films of the fungal cells before (curve 1) and after immersion in 10^{-4} M $AgNO_3$ solution for 72 h (curve 5). Curves 2-4 corresponds to spectra recorded from the $AgNO_3$ phase after 6, 24 and 48 h of exposure to the biomass, respectively.

This thus rules out the role of peptides such as glutathione which have been implicated in the formation of metal sulfide nanoparticles such as Ag_2S [32] and CdS [33] by reacting yeast cells with the appropriate metal ions. The mechanism for the formation of silver nanoparticles by the fungus *Verticilium* is thus radically different and is an important result of this investigation.

A possible mechanism for the presence of silver nanoparticles in the fungal biomass could be the extracellular reduction of the Ag^+ ions in solution followed by precipitation onto the cells. UV-vis spectra recorded from the aqueous $AgNO_3$ solution after 6 h, 24 h and 48 h of reaction with the biomass are shown as curves 2, 3 and 4 respectively in Fig. 4.12. The curves have been displaced vertically for clarity. It is clear that there is negligible presence of silver particles in solution thus clearly pointing to intracellular/surface reduction of the Ag^+ ions as the most probable mechanism for the synthesis of the silver nanoparticles by the fungus.

Fig. 4.13 shows an SEM picture of the fungal cells after exposure to 10^{-4} M aqueous $AgNO_3$ solution for 72 h. The highly filamentary nature of the mycelia is clearly seen in the figure. The presence of uniformly distributed silver nanoparticles on the surface of the fungal cells is observed indicating that the nanoparticles formed by the reduction of Ag^+ ions are bound to the surface of the cells. The silver nanoparticles seen outside the mycelia may be due to weakly bound silver nanoparticles dislodged from the biomass during preparation of the films for SEM investigation which, it may be recollected, involves washing the biomass thrice with distilled water. It is also possible that the cell walls of a small fraction of the mycelia rupture during the wash treatment due to osmotic pressure.

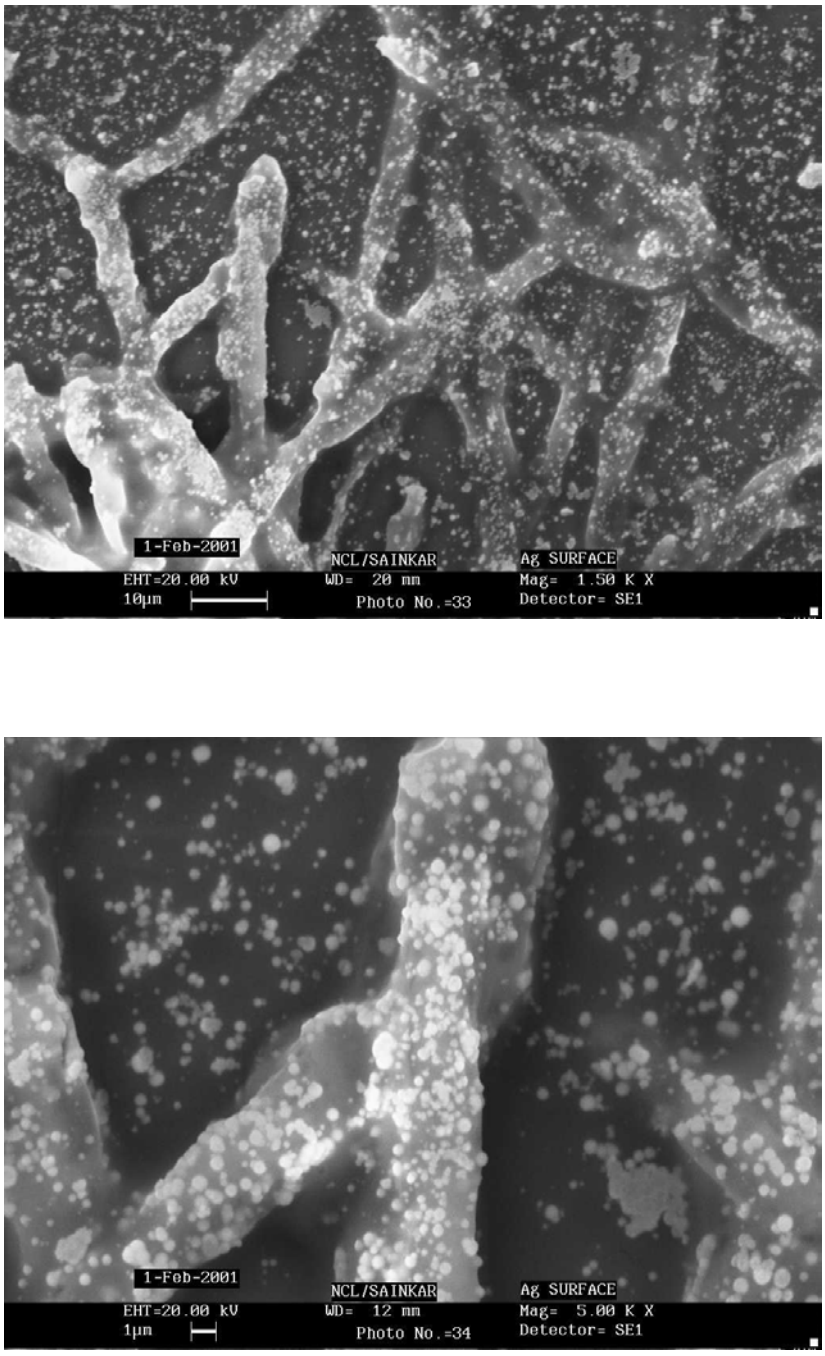

Fig. 4.13 SEM images with different magnification of a biofilm of the *Verticillium* fungal cells deposited on a Si(111) wafer after immersion of the cells in $AgNO_3$ solution for 72 h.

Fig. 4.14 shows the EDX spectrum recorded in the spot profile mode from one of the densely populated silver nanoparticle regions on the surface of the fungal cells. Strong signals from the silver atoms in the nanoparticles are observed while weaker signals from C, O, S, P, Mg and Na atoms were also recorded. The C, O, S, P, Mg and Na signals are likely to be due to X-ray emission from proteins/enzymes present in the cell wall of the biomass.

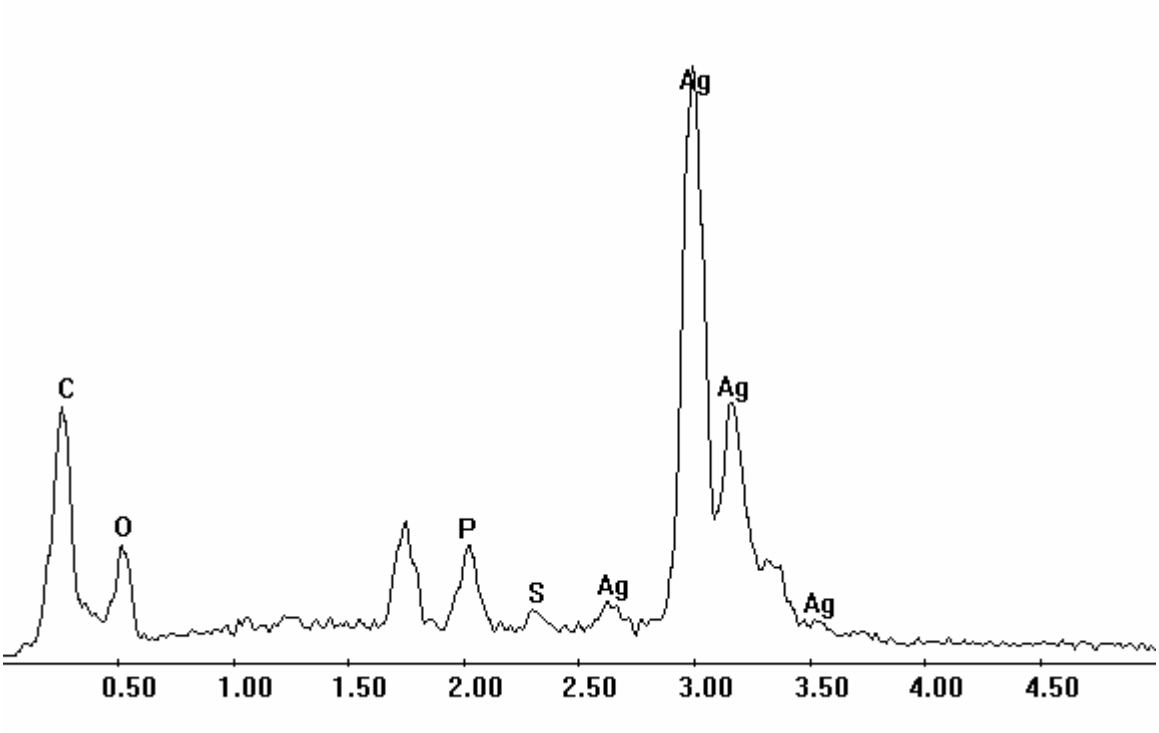

Fig. 4.14 Spot profile EDX spectrum recorded from one of the silver nanoparticles shown in Fig. 4.13.

Information on the location of the silver nanoparticles relative to the fungal cells would be important in elucidating the mechanism of their formation and may be obtained by TEM analysis of thin sections of the Ag nano-*Verticillium* cells. Fig. 4.15 shows representative TEM images at various magnifications of the *Verticillium* cells. At lower magnification, a number of mycelia can be observed, which on closer examination reveal assemblies of *Verticillium* cells within the mycelia (Fig. 4.15 A). This image shows small particles of silver organized on the mycelial walls as well as some larger particles within the cells. Fig. 4.15 (B) shows a slightly higher magnification image of the junction between two mycelia wherein the individual cells are more clearly resolved. A number of silver particles can be seen on the mycelia wall surface. The mycelia in the upper right corner also shows an individual *Verticillium* cell with silver particles clearly bound to the surface of the cytoplasmic membrane. Fig. 4.15 (C) shows a single *Verticilium* cell with silver nanoparticles both on the cell wall (external boundary) and on the cytoplasmic membrane (inner boundary). Occasionally, large silver particles could be observed within the cytoplasm of the *Verticillium* cells (Fig. 4.15 C). Analysis of the size of the silver nanoparticles from many different TEM images of the Ag nano-*Verticillium* cells yielded a particle diameter of 25 ± 12 nm.

Fig. 4.15 D shows a higher magnification TEM image of the cell wall of one of the *Verticillium* cells after Ag nanoparticle formation. A number of silver particles are observed that are essentially spherical in morphology with fairly good monodispersity.

While the exact mechanism leading to the formation of silver nanoparticles by reaction with the fungal cells is not understood at the moment, based on the results presented above we speculate the following. Since the nanoparticles are formed on the surface of the mycelia and not in solution, we believe that as in the case of gold (section 4.2.2) the first step involves

trapping of the Ag^+ ions on the surface of the fungal cells. This may occur via electrostatic interaction between the Ag^+ and negatively charged carboxylate groups in enzymes present in the cell wall of the mycelia. Thereafter, the silver ions are reduced by enzymes present in the cell wall leading to the formation of silver nuclei which subsequently grow by further reduction of Ag^+ ions and accumulation on these nuclei. The TEM results indicate the presence of some silver nanoparticles on the cytoplasmic membrane as well as within the cytoplasm (Fig. 4.15). It is possible that some Ag^+ ions diffuse through the cell wall and reduced by enzymes present on the cytoplasmic membrane and within the cytoplasm. It may also be possible that some of the smaller silver nanoparticles diffuse across the cell wall to be trapped within the cytoplasm.

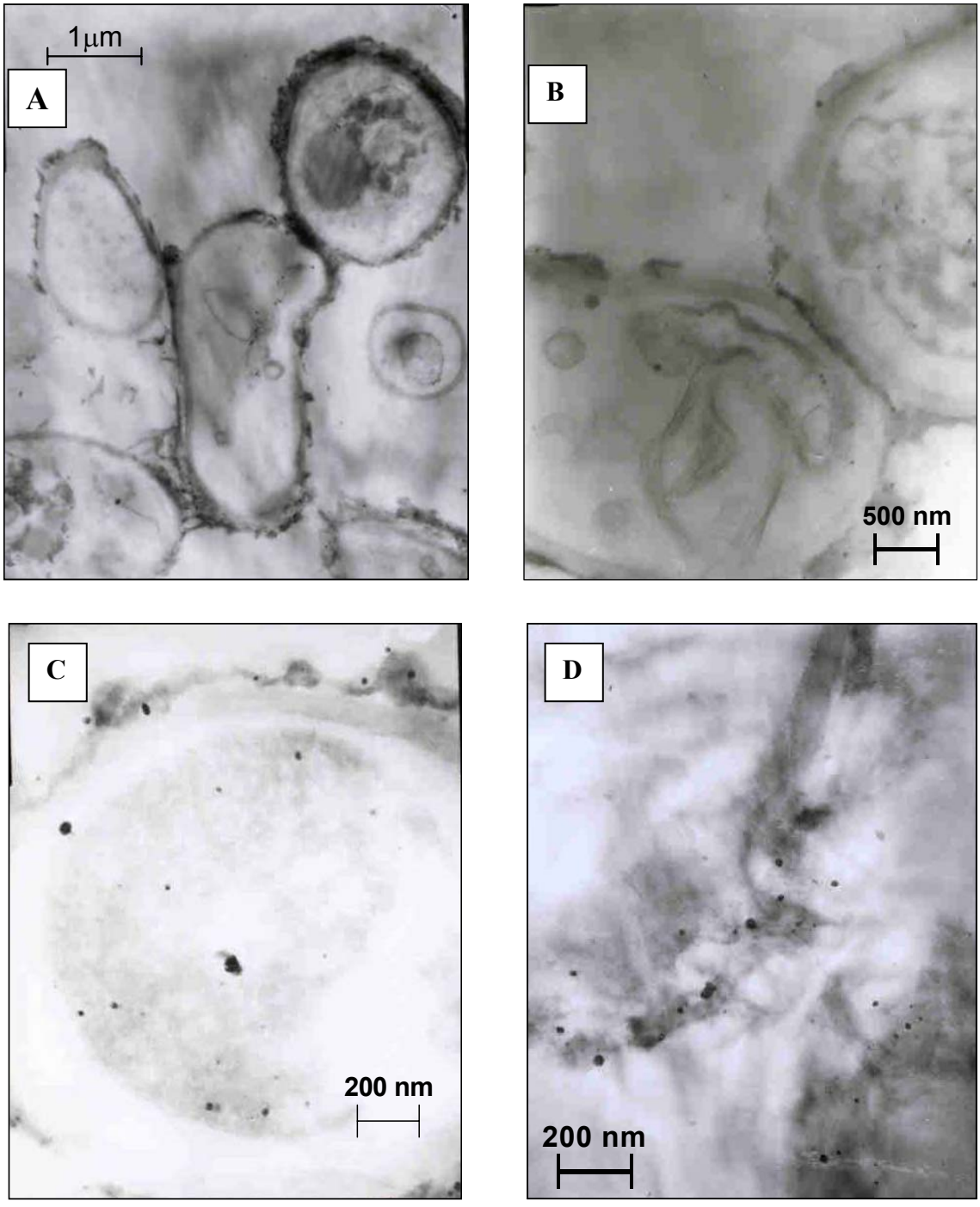

Fig. 4.15 TEM images at different magnifications of thin sections of stained *Verticillium* cells after reaction with $AgNO_3$ for 72 h.

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5.1 SUMMARY

The major objective of the thesis was to explore some reduction / oxidation properties of fungi for the biotransformation of organic carbonyl group and metal ions.

Chapter one represents a general introduction to biocatalysis and earlier literature on the different oxidation/reduction properties of the microorganisms towards carbonyl compounds. It also describes the reduction of inorganic compounds by different microorganisms. The purpose and aim of fungal mediated oxidation/reduction reactions of carbonyl compounds and inorganic compounds are described at the end of this chapter.

Chapter two describes the screening of different fungi for the Baeyer-Villiger type biotransformation of carbonyl compounds where cyclohexanone was taken as model substrate. The fungus *Fusarium oxysporum* was found to be a new biocatalyst that can perform this type of biotransformation. This chapter also describes the detailed study of Baeyer-Villiger type biotransformations of different simple monocyclic ketones with the above fungus. The effect of different inorganic solid such as alumina, silica and zeolites in the Baeyer-Villiger type biotransformation by the fungus *F. oxysporum* has also been studied. It has been observed that these types of materials enhance the reaction rate.

Chapter three describes the screening of fungi for the bioreduction of prochiral carbonyl compounds taking acetophenone as model substrate. The fungus *Trichothecium* was obtained as an effective biocatalyst for this type of bioreduction. Acetophenone can be converted to (*R*)-1-phenylethanol (ee 93 %) by this biocatalyst. The effect of different parameters such as time, biocatalyst concentration, surfactant and substrate concentration has been studied for the acetophenone bioreduction. Some other prochiral ketones have also been

investigated with this biocatalyst. High enantiomeric excesses (90-98 %) were obtained in the above cases.

Chapter four accounts the interesting observation that fungi can reduce the metal ions to its corresponding metal particles, which are in the size of nanometer range, either intracellularly or extracellularly. This fungal-mediated green chemistry approach towards the synthesis of gold nanoparticles has many advantages such as ease with which the process can be scaled up, economic viability, possibility of easily covering large surface areas by suitable growth of the mycelia etc. to form new bioinorganic composite materials.

5.2 CONCLUSIONS

A. Baeyer-Villiger type biotransformation

- Fungus *Fusarium oxysporum f*. sp. *ciceri* NCIM 1282 was found to be an active and selective biocatalyst for the Baeyer-Villiger type of biotransformation of cyclic aliphatic and aromatic ketone, among the fungi screened.
- Some strains of *Acenetobacter*, most commonly used in literature, are pathogenic and therefore precautions have to be taken, whereas *Fusarium oxysporum*, used in this work is not pathogenic and are safe to use.
- The formation of cyclohexanol followed by caprolactone in case of cyclohexanone biotransformation indicates the possibility of the presence of at least two types of enzymes, one oxidoreductase enzyme responsible for ketone-alcohol interconversion and another is lactonization enzyme for lactone formation.
- The above fungus catalyzes $C_4 - C_6$ cyclic ketones to corresponding lactones. However, in the case of larger ring size ketones (e.g., cycloheptanone), no lactone formation was observed. Instead, cycloheptanone was converted to cycloheptanol in quantitative yield.

The absence of Baeyer-Villiger type reaction in the case of cycloheptanone reflects that the enzyme may not capable to accommodate larger ring size of ketones for lactonization.

- According to the conversion of different cyclic ketone with the fungus the adaptability order is like this: cyclopentanone ≃ cyclohexanone > cycloheptanone > cyclobutanone. This order indicates that cyclopentanone and cyclohexanone are more suitable substrates for this fungus for Baeyer-Villiger type biotransformation.
- The formation of phenol in the biotransformation of acetophenone indicates the presence of an ester hydrolase enzyme in the fungus apart from those two responsible for oxidation/reduction step and lactonization step respectively.

B. Bioreduction of prochiral ketone

- The fungus *Trichothecium* sp. was found to be a suitable biocatalyst for the enantioselective bioreduction of prochiral ketone such as acetophenone, produces (*R*)-1-phenylethanol in good enantioselectivity (93 %). Here it is pertinent to mention that this fungus was used for the first time as a biocatalyst in the reduction of ketones.
- The fungus works for other prochiral acetophenone analogues also depending on the branching of the side chain and substitution on the aromatic ring. The results obtained in the case of trifluoromethylphenylketone and cyclopropylphenylketone indicate that the reductase enzyme, present in the fungus *Trichothecium,* is specific for some ketones.

C. Bioreduction of metal ions

- Eukaryotic microorganisms such as fungi were used for the biosynthesis of nanomaterials for the first time. Depending on the fungi the nanomaterials are formed extracellularly or intracellularly.
- The shift from bacteria to fungi as a means of developing natural "nanofactories" has the added advantage as the processing and handling of the biomass would be much simpler.
- The formation of extracellular nanoprticles in the case of *Fusarium oxysporum* indicates the presence of enzymes in aqueous medium and these acts as capping agents for stabilization of nanoparticles. Whereas the formation of intracellular nanoparticles in the case of *Verticillium* reflects the presence of cell wall enzymes.

5.3 FUTURE OUTLOOK

As lactones are important flavor compounds in many foods and beverages, this 'green chemistry' method can be used for the preparation of lactones from cyclic ketones. The fungus *Fusarium oxysporum* can be further investigated for the regio / stereoselective synthesis of lactones from substituted cyclic ketones. As chiral alcohols are useful building blocks in organic synthesis, these can be prepared by using the fungus *Trichothecium*. Substrate range can be increased in the bioreduction of prochiral ketones by *Trichothecium*. The same fungus can be checked for diketone compounds whether these can be regioselectively reduced. Though the whole cell method is cheaper with easy downstream processing, the method is rather slow and yields are also poor. So, in order to enhance the efficiency of the reaction more work is

needed in this area. The enzyme responsible for the above transformations can be isolated, genetically engineered and further can be immobilized on solid support so that it can be reused.

In this thesis while, synthesis of nanoparticles has been demonstrated, the detailed mechanistic were beyond the scope of the present work. This work can be extended by studying those parameters. In case of extracellular synthesis of nanoprticles the particles can be immobilized on solid matrix such as silica or other zeolites and catalytic activity can be investigated. The nanoparticles bound to the surface of the fungal cells may be used for catalysis and precursors for synthesis of coatings for electronic applications. These fungi can be further extended to other metals reductions to synthesize useful nanomatarials such as Pt and Pd.

LIST OF PUBLICATIONS

1. Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*.
   A. Ahmad, P. Mukherjee, S. Senapati, **D. Mandal**, M. I. Khan, R. Kumar and M. Sastry, *Coll. and Surf. B: Biointerface* **2003**, *28*, 313.

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PATENTS

1. Process for preparation of lactone from a cyclic ketone.
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2. Process for preparation of nanosized colloidal metal particle.
   P. Mukherjee, **D. Mandal**, A. Ahmad, M. Sastry and R. Kumar
   US pat. **6, 537, 344** (2003)
3. An improved process for the preparation of immobilized nanoparticles.
   P. Mukherjee, A. Ahmad, **D. Mandal**, S. Senapati, M. I. Khan, M. Sastry and R. Kumar
   US application no. 10/0322206, date of filing 21/12/2001.
4. Process for the preparation of metal sulfide nanoparticles.
   P. Mukherjee, **D. Mandal**, A. Ahmad, M. Sastry and R. Kumar
   Indian application no. 412/DEL/2001.

CONFERENCE TALK

1. Biocatalytic transformation of cyclohexanone by *Fusarium* sp.
   Oral presentation at International Conference (IPCAT-2) on Catalysis at NCL, Pune, India, Jan 2001.
2. Fungal mediated synthesis of gold nanoparticles: a novel biological approach to nanoparticle synthesis
   Presented at Fourth National Symposium in Chemistry (NSC-4) under the auspices of Chemical Research Society of India, at NCL, Pune, February 1-3, 2002.