Bioreactors are the core of any biotechnological based production processes for vaccines, proteins, enzymatic or microbial biotransformation, bioremediation and biodegradation (Andleeb et al., 2010). Immobilization can be defined as the fixation of the biocatalysts (microorganisms, enzymes and organelles) to insoluble solid supports. The most important advantage of immobilization is that it makes continuous bioreactor operations possible, which is useful in the production of fine chemicals and bio-treatment of industrial wastes (Arica et al., 2011). Compared to many unicellular microbes, filamentous cultures present special challenges in the optimization and scale-up because of the varying morphological forms (Wang et al., 2007). Fungal reactors have been developed for dye decolorization (Schliephake et al., 1993; Yang and Yu 1996). The challenge in designing such reactors for wastewater treatment is maintaining an adequate enzyme concentration for high dye decolorization efficiencies, as lignin-degrading enzymes are not continuously secreted during the secondary phase and are digested by extracellular proteases (Dosoretz et al., 1990; Staszczak et al., 2009). In addition, fungi in reactors entangle with the impeller, increasing medium viscosity and consequently impede oxygen transfer because of their excessive growth and adhesion to surfaces (Zhang et al., 1999; Moreira et al., 2003).

Immobilized cell systems make repeated batch culture possible and simplify both the continuous production and the subsequent downstream processes.
Wastewater treatment has attracted increasing interest using immobilized cell bioreactors. Several types of bioreactors have been developed for treatment of textile dye effluents, most efficient decolorization was achieved when fungal mycelium was immobilized in the reactor. The widely used systems were stirred tank reactor, air lift, bubble column, fixed bed bioreactor, rotating disk reactor, silicon membrane bioreactor, column reactor, laboratory reactors, semi-continuous reactors, three-phased fluidized bed bioreactor, biological contractor reactor etc. Immobilized microbial systems greatly improved bioreactor efficiency for dye decolorization, for instance, increasing process stability and tolerance to shock loadings, allowing higher treatment capacity per unit biomass, generating relatively less biological sludge, repeated and long term applications (Siddiqui et al., 2009). Selecting an appropriate reactor is essential in improving the economy and efficiency of immobilized cell process. There is need of bioreactor system that can sustain production of high level of enzymes for long period together with controlled growth of fungi.

The objective of the present study was to develop a biotreatment process using immobilized R. oryzae to treat textile dye effluent containing azo dyes in a Laboratory Column Bioreactor. The dye removal efficiency was monitored in terms of decolorization (%), detoxification (COD reduction - %) and laccase expression (EU/ml) by both immobilized and free fungi.

**Materials and Methods**

**Immobilization Support Material**

Scotch-Brite™ was used as immobilization support material (80% polyester and 20% nylon, color green).
Inoculum Preparation for Bioreactor (Spore Suspension)

*R. oryzae* was grown on PDA plate at 37°C for 4 days. The fungal spores were scratched and picked up with a loop from mature colony of fungi. The loop containing fungal spores was then dipped and mixed in 100 ml sterile distilled water containing 0.05% Tween 80 solution. After vigorous shaking, 1 ml of inoculum was poured on the Neubauer’s Chamber and was observed under microscope for spore counting. This inoculum was used for further experimentation.

Immobilization of *R. oryzae* on Scotch-Brite

Small pieces Scotch-Brite size 3 x 3 cm (as immobilized support material) were equally cut and weighed. These pieces were thoroughly washed with distilled water and sterilized in an autoclave (NOVA model OS AC 3) prior to use. The pieces were then placed in methanol overnight, washed twice with distilled water and dried in hot air oven at 50°C, until the moisture had been removed from the pieces. The pieces of Scotch-Brite (3) were taken in one sterile petri plate and soft agar was added onto the surface of pieces (0.5 ml) with an auto pipette. The same procedure was followed for all the pieces of Scotch – Brite. After this, 300 μl of inoculum containing $10^8$ spores / ml prepared as above was added to the pieces. The plates were incubated at 37°C for 48h for proper immobilization of fungal spores.

These steps were performed in order to maintain equal size, shape, weight and equal addition of spore suspension to each piece.

Fig. 8.1 shows Scotch-Brite with immobilized *R. oryzae* and Scotch-Brite without immobilized fungi.
Configuration and operation of Bioreactor

A laboratory Column Reactor built from fibre and plastic was a modified model of 1 L having 55 cm total length, 4.5 cm column width and was divided into two parts- the upper part being removable and sealed with 2 stoppers fabricated with the same material. The stoppers were equipped with tubular ports for the liquid and air inlets and outlets. A simple liquid distributor was fitted to the upper stopper of the reactor. The column was filled with 37 immobilized pieces of *R. oryzae* with 800 ml decolorization medium containing dye effluent (1:10) for decolorization whereas, for laccase production 800 ml Asther’s medium was used. The conditions as optimized in Chapter IV and V were used for decolorization and laccase expression respectively. Column was connected at the upper side to the feed tank and the outlet was connected to a tubing that was attached to sample collection tank. The system was operated at room temperature and pH was maintained at 4.5 for decolorization of effluent and pH 5 for laccase production (optimized as in Chapters IV and V respectively). Effect

Fig. 8.1 A Scotch-Brite without immobilized *R. oryzae*  
B Scotch-Bright with immobilized *R. oryzae*
of recycling with the same immobilized fungi on decolorization, COD reduction and laccase expression were examined twice, as recycling is an important parameter in any industrial sector in terms of time and cost. The samples were withdrawn at every 24h interval up to a period of 96h for examining decolorization (%), COD reduction (%) and laccase expression (EU/ml). A control column was also run in which pieces were examined for their ability to adsorb the dye to ascertain whether decolorization is by adsorption or is an enzymatic mechanism. The experiments were performed in triplicates in order to avoid errors.

**Free Mycelia**

To examine the influence of immobilization over free mycelia on decolorization, COD reduction and laccase expression, 10^8 spores/ml of *R. oryzae* were inoculated in 800 ml decolorization medium and Asther’s medium for decolorization of textile dye effluent and laccase expression respectively, in a Column Bioreactor. The system was operated at room temperature and pH was maintained 4.5 (Chapter IV) for decolorization of effluent and pH 5 for laccase expression (Chapter V). Effect of recycling on decolorization of effluent was also examined. The samples were withdrawn at every 24h interval upto 96h for examining decolorization (%) COD reduction (%) and laccase expression (EU/ml).

**Statistical Analysis**

Data obtained during experiments was statistically analyzed using SPSS 17.0. Probability \( (p) \) less than 0.05 and 0.01 was considered significant and highly significant respectively. The results were expressed in terms of Means and Standard error.
Results and Discussion

Appended Tables 8.1 and 8.2 shows decolorization (%), COD reduction (%) and laccase expression (EU/ml) and effect of recycling with immobilized and free mycelia of R. oryzae in a column bioreactor after 24 and 96h, when maximum activity had been achieved.

Fig. 8.2 shows decolorization (%), COD reduction and laccase expression (EU/ml) with immobilized and free mycelia of R. oryzae after 24h and 96h respectively, the period when maximum activities had been attained.

The results (Fig. 8.2, Table 8.1) indicated that with immobilized R. oryzae, decolorization achieved was 90%, COD reduction 85% and laccase expression was 10.34 EU/ml at 24h, after which there was no further change in decolorization, COD reduction and laccase expression up to 96h. As compared to immobilized fungi, with free mycelia of R. oryzae, decolorization, COD reduction and laccase expression increased from 24h reaching maxima at 96h. At 96h, decolorization achieved was 60%, COD reduction 55% and laccase expression was 1.21EU/ml. Thus, immobilization and using a column bioreactor led to 1.5 times increase in decolorization, 1.54 times increase in COD reduction and 8.54 times increase in laccase activity with the retention time from 96h to 24h.

The high decolorization, COD reduction and laccase yield levels with immobilized fungi on Scotch – Brite compared to free biomass may be attributed to the static immobilized fungal cultures on Scotch-Brite which allows the contact area between cells and oxygen transfer to be increased with the effect of shear stress.

The increased surface area of fungal biomass on Scotch-Brite tends to reduce the mass transfer limitations compared to mycelia, which increases easy access to the substrate utilization. In addition, oxygen transfer is necessary for production of
lignolytic enzymes, but mechanically agitated cultures are known to have an inhibition effect on production of enzymes due to shear stress by mycelia in submerged cultures.

Fig. 8.3 shows the results of recycling for two repeated cycles with immobilized and free mycelia of R. oryzae for decolorization (%), COD reduction (%) and laccase expression (EU/ml), at 24h and 96h respectively, the period where maximum activities had been attained. The results are expressed as mean of two cycles.

The results of recycling for two repeated cycles as per Fig 8.3, Table 8.2, indicated decolorization of 82%, COD reduction of 71% and 6.51 EU/ml laccase activity at 24h with immobilized fungi after which there was no further change in the activities examined. However, with free mycelia maximum decolorization achieved was 45%, COD reduction 55% and 0.910 EU/ml laccase activity at 96h. Thus, with recycling experiments, decolorization rate, COD reduction and laccase expression were slightly lower.

The reduction in the ability for decolorization, COD reduction and laccase expression during recycling could be associated with the formation of by-products, saturation of the biomass and an uncontrolled growth of the mycelium. Under these conditions, the systems can have anaerobic or microaerophilic atmosphere that could cause death of fungus which in the present study is an aerobic isolate (Castillo – Carvajal et al., 2012).

By running a control column it was observed that there was no change in color of the mycelia and the mycelial extract did not give any increase in the absorbance at 465 nm, indicating that decolorization was an enzymatic mechanism.
Fig. 8.2 Decolorization (D - %), COD reduction (%) and laccase expression (EU/ml) with immobilized and free mycelia of *R. oryzae*

Fig. 8.3 The effect of recycling on decolorization (D - %), COD reduction (%) and laccase expression (EU/ml) with immobilized and free mycelia of *R. oryzae*
A $p$ value obtained represents the probability significance level i.e. $p < 0.05$ less significant, $p < 0.01$ significant, $p < 0.001$ highly significant (Tables 8.1 and 8.2).

Tavčar et al., 2006 have reported degradation of azo dye RO 16 with immobilized *Irpex lacteus* in different bioreactors with 90% decolorization. Moreover, he reported that the ability of fungal cultures to decolorize dyes can be correlated to the activities of extracellular peroxidases and laccases present in the culture liquid. He reported that MnP and laccases of *I. lacteus* are implicated in decolorization of RO 16, an observation that corroborates the present findings. He also found that in static cultures of *I. lacteus* 8000 fold amount of laccases was found to be associated with fungal mycelium compared to the free fraction dissolved in culture liquid. No mycelial bound MnP was found in static cultures. It can be hypothesized that the mycelium-associated enzyme participates in the decolorization process.

Kunamneni, 2000 reported that *Myceliophthora thermophila* laccase was covalently immobilized on polymethacrylate-based polymers (Sepabeads EC-EP3 and Dilbeads NK) activated with epoxy groups. The enzyme immobilized on Sepabeads EC-EP3 exhibited notable activity (203 U/g). In addition, the immobilized laccase also showed good operational stability, maintaining 84% of its initial activity after 17 cycles of oxidation of ABTS. The immobilized biocatalyst was applied to the decolorization of six synthetic dyes. Immobilized laccase decolorized 41% methyl green in a fixed-bed reactor after five cycles. The features of these biocatalysts are very attractive for their application on the decolorization of dyes in the textile industry in batch and continuous fixed-bed bioreactors.

Champagne, 2009 reported the use of laccase immobilized on controlled porosity carrier (CPC) silica beads for the decolorization of Reactive blue 19 (an
anthraquinone dye) by *Trametes versicolor*. Decolorization of Reactive blue 19 by immobilized laccase was 90% as compared to free mycelia within 48h.

Cui, 2009 have reported the use of Bioreactors for enhanced laccase production from *Coriolopsis polyzona*. Maximum laccase produced was 6.3 g/L in 6 days with use of 150L Stirred tank bioreactor proving the efficacy of bioreactor. Rivela et al., 2000 found that MnP and LiP were produced maximally in a Immersion bioreactor with *P. chrysosporium* immobilized on nylon sponge. He reported that MnP was produced maximally on 10th day (1593 U L^-1), whereas LiP activity was highest on sixth day (229 U L^-1).

Prasad et al., 2005 have reported laccase production using *Pleurotus ostreatus* 1804 immobilized on PUF cubes in packed bed bioreactors. Maximum laccase activity of 312.6U was observed with immobilized mycelia whereas with free mycelia activity was 272.2U. Thus, enhanced laccase yield produced was due to immobilization. Driessel and Christov, 2001 have reported the decolorization abilities of *R. pusillus* and *C. versicolor* in Rotating Biological Contractor. The relative high decolorization results i.e. 32-55% decolorization were achieved within 0.67-6 h that may be due to a rapid adsorption of color by the biomass.

The results obtained clearly shows that using Column Bioreactor and an immobilized fungi, it is possible to obtain decolorization of 90% and enhanced laccase activity 8.54 times without any operational problem. The activities obtained were due in part to the design of the reactor, which provided suitable production conditions including a low shear environment. The reactor permitted good attachment of fungus to the carrier as well as a proper oxygen and nutrient diffusion into the bioreactor. Scotch-Brite showed the best characteristic to be suitable support because it has a desirable porosity and saturation volume that allows the immobilization of microorganisms, as well as improving the pollutant removal. Carbon and nitrogen sources could be added to maintain the primary
metabolism of the fungi and increase the color removal, in addition, the enzymatic production increases in treatments with immobilized biomass.

The study clearly reveals the potential of lignolytic fungi growing on a solid phase to degrade recalcitrant xenobiotics and organopollutants dissolved in water media and effluents. Current work has appreciably validated the role of *R. oryzae* in the decolorization of first stage textile dye effluent containing azo dyes. Both immobilized and free mycelia were used for decolorization experiments proving the efficiency of immobilized fungi over free mycelia. Apparently, decolorization in the present study seems to be due to production of enzyme laccase.

From the economical point of view, in general the process should be fast and effective. There are several descriptions of degradation kinetics with isolated enzymes and a few with whole mycelia, but for industrialization of fungal bioremediation, more attention should be paid to the degradation kinetic studies. Practically there is no data about bioremediation cost, so it would be interesting to compare this promising technology with other alternative processes for the treatment of effluents. More improvement of decolorization performance through the optimization of culture conditions and laccase production in bioreactor needs further investigation. This is just a first step towards green technology.