6.1 Introduction

Fungal fermentations in the commercial production of a wide range of secondary metabolites have been extensively exploited in recent times. Fungi are morphologically complex organisms which differ in structure at different times in their life cycle, differ in form between surface and submerged growth, and also differ in the nature of the growth medium, physical environment, and physical (temperature, pH, mechanical forces, etc.) culture conditions (Kossen, 2000). During submerged fungal fermentation, dispersed mycelial filaments (hyphae) to densely interwoven mycelial masses were observed (Papagianni, 2004), with the morphology playing a fundamental role in determining the overall process productivity. In addition, the manipulation of fungal morphology resulted in an increased metabolite yield (Schugerl et al., 1997). Mycelial pellets are naturally aggregated mycelia, which grow in a compact form around the core. The main disadvantage with pellet culture is the internal mass transport resistance imposed due to the diffusion of O\textsubscript{2} and other nutrients present, as well as that of biosynthetic activity (Wittler et al., 1986). Moreover, there are no realistic means by which the structure of the pellets can be controlled to reduce the diffusion resistance in their internal structure, which often impairs their biosynthetic efficiency. In this regard, the recognition of the fundamental role of fungal morphology in determining the performance in mycelial fermentation has led to a search for alternatives for engineering the structure of these microorganisms into more desirable forms, e.g., cell immobilization using various methodologies, and the application of cell immobilization techniques to such beneficial microbes appears valuable for use in biotechnology.

There are essentially four different procedures available for cell immobilization: adsorption, entrapment in gels or polymers, covalent coupling, and cross-linking to...
insoluble matrices (Brouers, 1989). A variety of matrices, such as agar carragenin, calcium alginate gels, polyacrylamide, etc. have been used, but the nature of the cell to be immobilized, the nature of the substrates and products formed, and the culture conditions are major factors for the choice of the matrix and the immobilization procedure. Adsorption to surfaces and encapsulation within gels or porous materials (a particular type of physical entrapment) have been the most widely studied methods for the immobilization of microbes. These techniques represent a particular form of cellular adhesion based on the ability of certain microorganisms to fix themselves to solid surfaces by means of the secretion of polymucoosaccharides (Moonmangmee et al., 2002). When the pore size of the matrix is small, bearing in mind the dimensions of the cell, adsorption occurs only at the surface, as in the case of diatomaceous earth, clays, and other related materials.

However, when the carrier has pores that are large relative to the dimensions of the cell, it is possible to find adhesion within the pores. This situation occurs in materials such as active carbon, polyurethane foam, and sintered glass. Adsorption techniques reduce the problems associated with oxygen diffusion and do not possess the same the drawbacks of scale-up experienced with encapsulation matrices. Literature reports are available on the studies to enhance secondary metabolite production using the immobilized approach (Nakamura et al., 1999; Ory et al., 2004). Adsorption to surface and encapsulation within gel or porous materials were also reported in the literature for immobilization (Ory et al., 2004). These methods represent a particular form of cellular adhesion based on the ability of certain microorganisms to fix themselves to solid surfaces by means of the secretion of polymucoosaccharides (Moonmangmee et al., 2002). Once the initial adhesion is achieved, trap-net formation stabilizes the formation of aggregation in mycelial microorganisms. This non-specific cell cohesion phenomenon is common to a variety of biological systems. The application of these aggregates to metabolite production by natural aggregation processes or by artificial immobilization has increased rapidly (Nakamura et al., 1999).
Encapsulation of enzymes in alginate gels is characterised by the very mild conditions in which the immobilisation procedure is carried out and by its low cost and ease of use. Moreover, it is possible to immobilise several enzymes at the same time. Alginate has been used as a matrix for the immobilisation of lipase, glucose oxidase, tannase, tyrosinase and coimmobilisation of glucose oxidase and catalase (Betigeri and Neau, 2002; Blandino et al., 2001; Boadi and Neufeld, 2001; Munjal and Sawhney, 2002; Blandino, 2003; Foster, 2003). Recently, Foster et al. have reported the first encapsulation of *Agrobacterium tumefaciens* extract in alginate capsules to synthesise glycine (Foster, 2003).

The aim of the present work was to study the immobilization of growing cells of *Pleurotus florida* in Polyurethane foam (PUF) and calcium alginate gel capsules, to determine the factors affecting the production of extracellular laccase by the immobilized system and to compare the efficiency of immobilized system.

6.2 Materials and methods

6.2.1 Fungal strain

Cultures of *P. florida* were maintained on Potato dextrose agar extract agar plates at 4 °C.

6.2.2 Cultivation medium

Optimized medium component that was previously shown to support a high laccase activity (Chapter 3) was used in this study. The medium contained glucose (5.61 g.l\(^{-1}\)), ammonium tartrate (5.72 g.l\(^{-1}\)) and sodium acetate (6.31 g.l\(^{-1}\)) and the inducer veratryl alcohol (Vasconcelos et al., 2000 and Mansur et al., 1997) which showed high laccase activity (Chapter 2) was added at a concentration of 16mM and it was adjusted to pH 6.0. Veratryl alcohol was aseptically added to the medium 24 h after inoculation due to the potential toxicity of this compound (Vasconcelos et al., 2000).
6.2.3 Inoculum preparation

The fungal inoculum was prepared by growing *P. florida* on Potato dextrose agar plates (PDA) and transfer approximately ten pieces (2mm x 2mm) of colonized agar to 100 ml of Potato dextrose medium in a 500 ml conical flask and incubating it for ten days at 25°C. After ten days the mycelial mat on the broth surface was homogenized and the suspension of fragmented mycelium was transferred aseptically to the 5-L bioreactors and used for further immobilization. (As mentioned in chapter 4).

6.2.4 Cell Immobilization on Polyurethane foam (PUF)

Polyurethane foam (PUF) cubes (1×1×1 cm) were used as supporting material for the immobilization of fungal aggregated mycelium as biofilm. Prior to use, the PUF cubes were pretreated by boiling for 20 min at 80°C and washed with distilled water. The cubes were then placed in methanol overnight, washed twice with distilled water, and dried in a hot air oven at 50°C until the moisture had been completely removed from the cubes. For the immobilization of *P. florida* on PUF cubes, 5 g of pretreated PUF cubes were placed in a 250 ml Erlenmeyer flask containing potato dextrose broth. After sterilization (121°C, 15 lbs, 15 min), the flasks were inoculated with 0.6 ml of homogenized mycelia (0.1330 dry weight of mycelia g/l) under sterile conditions. *P. florida* was found to grow profusely on PUF cubes after 5 days of incubation. The growth medium was then removed and the immobilized cubes were thoroughly washed with autoclaved saline water under sterile conditions. Experiments were carried out for laccase production by transferring the immobilized cubes into the 100 ml production medium (standardized media) under sterile conditions and incubating for a fermentation period of 192 h.

6.2.5 Cell Immobilization on alginate capsules

Immobilization was carried out under sterile conditions. Unless otherwise stated about 20 mg of wet cells were suspended in 12.5 ml of 2% (w/v) sodium alginate solution (0.16% (w/v) wet weight in gel). The mixture obtained was extruded drop-wise through a syringe into a 25 ml of 3.5% (w/v) CaCl₂ solution. Alginate drops were solidified upon contact with CaCl₂, forming capsules and thus entrapping the *P. florida* cells. The
capsules (mean volume and diameter 35μl and 4 mm, respectively) were allowed to 
harden for 30 min and then were washed with sterile saline solution (0.9% (w/v) NaCl) to 
remove excess of Ca²⁺ and cells.

6.2.6 Optimization of the capsule characteristics

Various amounts of sodium alginate (1–5%, w/v) were added to the gel mixture to 
study the effect of sodium alginate concentration on the gel capsule permeability. The 
gelation of the mixture was induced by a 3.5% (w/v) CaCl₂ solution.

In order to investigate the effect of CaCl₂ concentration on the rigidity of the 
beads the 2% (w/v) sodium alginate solution was extruded drop-wise into varying CaCl₂ 
concentrations from 1 to 5% (w/v).

Different cell loading in the capsules was maintained by incorporating different 
amounts of cells (0.16, 0.32, 0.64, 1.28, 1.92, 2.56 and 3.2% (w/v) wet weight in gel) in 
the 12.5 ml aqueous sodium alginate solution.

The size of the pellets (10, 20, 35 and 50 μl) was varied by altering the diameter 
of the syringe needle used.

6.3 Results and discussion

6.3.1 Production of laccase in batch system using PUF

Batch systems are a convenient method of growing microorganisms in submerged 
cultures under aerobic conditions by agitation, as it is a small scale equivalent of the 
stirred tank bioreactor. Laccase was constitutively produced during primary metabolism 
of P. florida. However, production levels were very much dependent on the applied 
culture conditions. The effect of immobilized biomass concentration on laccase yield was 
investigated by varying different levels of inoculum size in the production medium 
(Fig.6.1). It can be observed from the provided figure that an increase in the biomass 
concentration of up to 1.38 (wet weight of mycelia g/l), the laccase yield also showed 
increasing yield (12.10 to 14.5 U/ml). Subsequent increases to the biomass concentration
suppressed the laccase yield. It may be reasoned that fungal mycelia exceeding the optimum biomass results in the growth of mycelia rather than metabolite synthesis. A biomass concentration of 1.38 wet weight of mycelia g/l was found to be optimum for the maximum laccase yield.

One of the operation parameters, pH, influences the metabolic activity of the organism, playing an important role in the optimization protocol of any fermentation process (Greasham and Inamine, 1986). Laccase production by immobilized *P. florida* on PUF cubes was studied at different culture pH values from 4.0 to 8.0. Laccase activity was observed at all of the studied culture pH levels (Fig. 6.2). When the culture pH was increased from 4.0 to 5.5, the yield of laccase was found to increase from 8.0 to 15.2 U/ml during the 6 day fermentation period. Any subsequent increase in pH showed a reduction in laccase yield (6.025 U/ml at pH 8.0), which may be attributed to the poor mycelial growth within an alkaline pH range. The optimal culture pH for the laccase production of *P. florida* was found to be 5.5.

### 6.3.2 Influence of immobilization compared to free mycelia on laccase expression

Lacasse expression studies using free mycelia of *P. florida* have been reported elsewhere (chapter 2). The maximum laccase activity of immobilized mycelia in shake flasks was 15.2 U/ml, whereas the maximum laccase activity of free mycelia was 12.1 U/ml. The high yield in laccase levels in immobilized fungi compared to free biomass may be attributed to the static immobilized fungal culture on PUF cubes, which allows the contact area between cells and oxygen to be increased without the effect of shear stress. The increased surface area of fungal biomass of PUF cubes tends to reduce the mass transfer limitations compared to free mycelia, which increases easy access to the substrate utilization. In addition, oxygen is necessary for the production of ligninolytic enzymes by white rot fungi, but mechanically agitated cultures are known to have an inhibitory effect on the production of these enzymes due to shear stress suffered by mycelia in submerged culture. Additionally, the difference observed was not only in the laccase yield, but also in the biomass growth. It was observed more in the free biomass incubated flask (8.25 wet weight of mycelia g/l) than in the immobilized PUF cube flasks.
(3.98 wet weight of mycelia g/l). This indicates that most of that substrate was utilized for biomass growth instead of laccase yield.

6.3.3 Production of laccase in batch system using alginate capsules

Growth and production of laccase were followed up in batch cultures for 10 days both in free and in immobilized cell cultures of *P. florida*. In free cultures rapid cell growth was observed in the first 6th day of cultivation. Immobilized cells (by alginate entrapment), on the other hand, exhibited different kinetics (Fig. 6.3). The rapid increase in biomass entrapped in the gel capsules was continued for 10th day, reaching a cell concentration of 2.56 mg/ml in the alginate gel (data not shown). Some cells were released from the capsules into the medium. However, this represented only a small part of the entrapped biomass, since the concentration of the released cells in the culture liquid was restricted to 0.2 mg/ml.

The data indicated that the productivity of laccase by the immobilized cells was significantly greater than that of the freely suspended cells. Alginate entrapment technique led to 13.5 U/ml in the laccase yield in comparison to free cell cultures which is 12.1 U/ml (Fig. 6.3).

6.3.4 Optimization of parameters for immobilization of *P. florida* in alginate capsules

Cell immobilization in calcium alginate gel capsules offers the advantage of improving some of the capsule characteristics, such as thickness and percentage of cell leakage by changing certain conditions like sodium alginate and CaCl$_2$ concentration. The effect of the concentration of sodium alginate solution used for the preparation of capsules on laccase production and cell leakage. Higher sodium alginate concentrations had an adverse effect on laccase production. Although increase in the biopolymer concentration from 2 to 5% (w/v) improved the durability of the capsules and reduced cell leakage by 7%, it resulted in 25% lower enzyme yield. This could be ascribed to the reduction in diffusion of nutrients and oxygen into the gel or to limitation of laccase
release out of it due to its high rigidity. On the other hand, gel capsules prepared from 1\% (w/v) biopolymer were fragile and difficult to handle and released significant amount of cells into the culture medium. The highest laccase production was obtained with capsules prepared from 2\% (w/v) sodium alginate (13.75 U/ml).

The mechanical strength of alginate capsules appeared to be also highly dependent on the CaCl$_2$ concentration of the gelation solution. Increase in the CaCl$_2$ concentration from 1 to 3.5\% (w/v) resulted in 23\% limitation of cell leakage from the capsules, which was followed by a 40\% increase in laccase yield. It should be stressed that an equivalent increase in the sodium alginate concentration led to reduction of cell leakage by only 17\%, indicating that the use of concentrated CaCl$_2$ solutions had a higher effect on the efficiency of the immobilized system.

6.4 Conclusion

The immobilization of \textit{P. florida} cells increased the laccase production both in PUF and alginate capsules. However the PUF was found favour more production of laccase enzyme than alginate.
Fig 6.1 . Effect of immobilized mycelia (PUF) on laccase production

Fig 6.2 . Effect of pH on laccase production
Fig 6.3. Effect of immobilized mycelia (alginate capsules) on laccase production