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Laccase Production Using *Pleurotus ostreatus* 1804 Immobilized on PUF Cubes in Batch and Packed Bed Reactors: Influence of Culture Conditions

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The feasibility of laccase production by immobilization of *Pleurotus ostreatus* 1804 on polyurethane foam (PUF) cubes with respect to media composition was studied in both batch and reactor systems. Enhanced laccase yield was evidenced due to immobilization. A relatively high maximum laccase activity of 312.6 U was observed with immobilized mycelia in shake flasks compared to the maximum laccase activity of free mycelia (272.2 U). It is evident from this study that the culture conditions studied, i.e. biomass level, pH, substrate concentration, yeast extract concentration, Cu^{2+} concentration, and alcohol nature, showed significant influence on the laccase yield. Gel electrophoretic analysis showed the molecular weight of the laccase produced by immobilized *P. ostreatus* to be 66 kDa. The laccase yield was significantly higher and more rapid in the packed bed reactor than in the shake flask experiments. A maximum laccase yield of 392.9 U was observed within 144 h of the fermentation period with complete glucose depletion.

Key words: Laccase, *Pleurotus ostreatus* 1804, immobilization, packed bed reactor, culture condit conditions

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_2 and other nutrients present, as well as the 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 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 polymucosaccharides (Moonmangmee *et al*., 2002). When the pore size of the matrix is small, bearing in mind the dimensions of the

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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 polymucosaccharides (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).

In this report, we made an attempt to investigate the feasibility of immobilization of *Pleurotus ostreatus* 1804 on laccase production with the function of media composition in both batch and reactor systems.

Experimental

White rot fungi

White rot fungi *P. ostreatus*-1804 (hyper laccase-yielding strain) used in the present study was procured from the Microbial Testing and Collection Center (MTCC), Institute of Microbial Technology (IMTech), Chandigarh, India.

Laccase production media

In the present investigations, *P. ostreatus*-1804 was studied to produce laccase in submerged agitation culture in cotton-plugged 250 ml Erlenmeyer flasks containing 100 ml of production medium. *P. ostreatus*-1804 young culture was grown for eight days in potato dextrose broth and the homogenized culture was used as the initial inoculum for immobilization (Krishna Prasad *et al.*, 2005a). The production medium consisted of (g/100 ml of distilled water): 1.0 g glucose, 0.5 g yeast extract, 0.5 g urea (90 ml), and 10 ml of salt solution. The salt solution consisted of 2.0 g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, and 0.5 g KCl dissolved in 100 ml of distilled water. The pH was adjusted to 5.6 using 3M HCl prior to sterilization (15 lbs psi, 15 min, 121°C).

Immobilization

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. ostreatus* 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. ostreatus* was found to grow abundantly 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 under sterile conditions and incubating for a fermentation period of 192 h.

Batch agitation system

The production of laccase in submerged agitation culture of cotton-plugged 250 ml Erlenmeyer flasks containing 100 ml of production medium was studied using immobilization hyper laccase secreted *P. ostreatus*-1804 as an inoculum. During the study, culture conditions like immobilized inoculum level (0.34, 0.69, 1.04, 1.38, 1.73, 2.08, 2.42, 2.77, 3.11 and 3.46 g [wet weight/l]) were maintained by varying the number of immobilized PUF cubes added to the production medium; the influence of production medium aqueous phase pH was also varied (from 4 to 8 in the intervals of 0.5; 1.38 g/l of immobilized mycelia as inoculum). The effect of glucose concentration on laccase production was studied by varying the substrate concentration from 1 - 3% (w/v) at equal immobilized mycelia as the inocula in all tested flasks. In the same manner, the effect of yeast extract concentration [0.25 to 2.0% (w/v)] on laccase expression was studied and a control flask without yeast extract supplement was operated along with tested flasks in order to determine the effect of yeast extract on laccase synthesis.

The influence of copper and different alcohol concentrations on laccase production were determined by adding it to culture medium and were added to the actively growing fungal cultures after 72 h of cultivation time. Cu^{2+} at concentrations ranging from 0.25 mM to 1.5 mM was studied. A control flask without copper was also studied in order to determine the effect of copper on the laccase yield by *P. ostreatus*. The putative alcohol inducers used were ethanol, methanol, isobutyl alcohol, and veratryl alcohol. Among these, veratryl alcohol concentration was

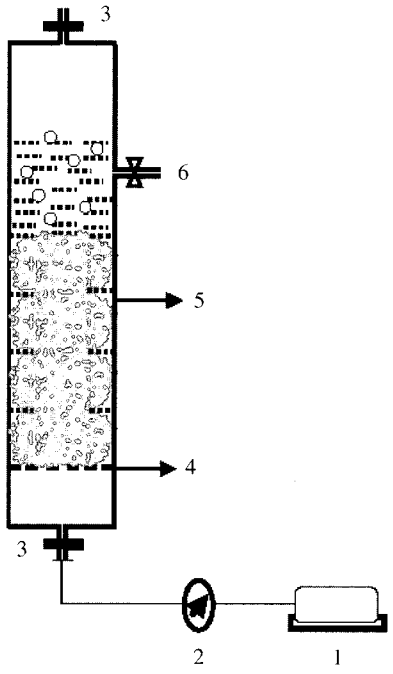
varied on the basis of mM (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0) and the remaining alcohols were taken on the basis of % w/v ratio (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0). All of these were sterilized by filtration (0.2 μm, Sortorius, India). The effect of these alcohols on substrate utilization and laccase activity by immobilized *P. ostreatus* was monitored every 48 h and a control flask without alcohol supplementation was operated along with the studied flasks.

Packed bed system

The optimized culture conditions obtained from batch studies were further studied for laccase production in a packed bed reactor. The reactor with a working volume of 280 ml was fabricated using 'Borosil' glass with an internal diameter of 4.5 cm and height of 25 cm (Fig. 1). The reactor was packed with PUF cubes (3 × 3 × 3 cm) placed on a porous plate fixed at the bottom, each with mycelia concentration of approximately 0.62 g wet weight of mycelia/l/cube. At the top, the reactor was equipped with a cone and socket (40/38 size) mechanism to feed the immobilized cubes. Air was supplied from bottom of the reactor at a rate of 125 ml/min through glass tubing (8 mm ϕ), which was integrated with the air filter (0.2 μm, Sartorius, UK). Every 24 h during the bioreactor operation, samples were collected under sterile conditions through the outlet (8 mm ϕ) arranged at the top of the reactor. Centrifuged (5000 × g, 5 min, 4°C) clear supernatant was used for the estimation of laccase activity, pH, and glucose consumption.

Analytical assay

Laccase activity was estimated by ABTS oxidation procedure (Krishna Prasad *et al.*, 2005b). Enzyme activity was expressed in U (U = μM/min/mg protein), defined in terms of the number of μM of ABTS converted/min/mg of protein. The residual glucose in the media during fermentation was measured colorimetrically (UV-Vis spectrophotometer-Beckman DU-7400, USA) using the DNS (dinitrosalicylic acid) method with glucose as standard (Ghose, 1987). The extracellular protein concentration in the fermentation broth was estimated by Lowry's method using bovine serum albumin (BSA) as standard (Lowery, 1951). The wet weight of immobilized fungal mycelium on PUF was determined each time using separate sets of fermented flasks. PUF cubes were removed from the fermentation broth at a predetermined time and were washed with distilled water. For SEM imaging, the immobilization *P. ostreatus* mycelia on PUF cubes were fixed with 5% (v/v) glutaraldehyde in 100 mM phosphate buffer for 1 h at 4°C and were subsequently washed with distilled water and dehydrated using acetone (from 10 to 100% series). The dried cubes (40°C for 6 h) were coated with a gold layer (Hitachi HUS-5GB, Japan) and observed under a scanning electron microscope (Hitachi-S 520, Japan).



1. Air sparger
2. Air flow controller
3. Air filter (0.2 μm)
4. Teflon porous plate
5. PUE cube with immonilized mycelia
6. Sampling port

Fig. 1. Schematic representation of packed bed bioreactor with immobilized *P. ostreatus 1804* mycelia on PUF cubes.

SDS gel electrophoresis

To determine the expression of laccase and its molecular weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 10% polyacrylamide gel containing 0.1% SDS, as described by Chefetz *et al* (1998). Samples (10 μg of protein) were treated prior to loading onto the gel with 0.5% SDS and 5% β-mercaptoethanol, and were boiled at 95°C for 5 min. The protein was then visualized by staining with silver nitrate, after which it was compared to low range molecular markers (Sigma, USA).

Results and Discussion

Production of laccase in batch system

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. ostreatus*. 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. 2). It can be observed from the provided figure that an increase in the biomass concentration of up

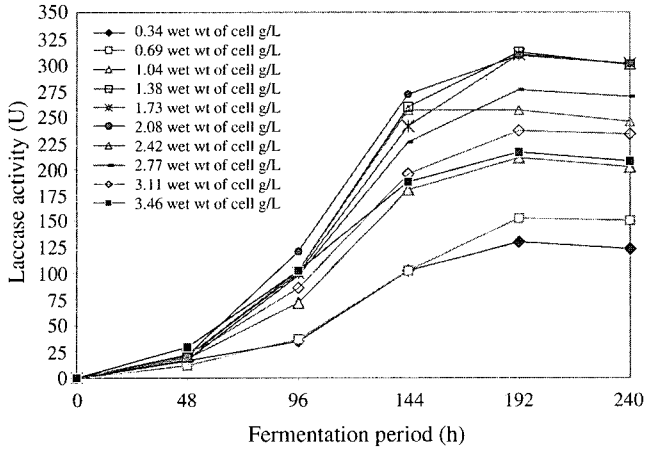


Fig. 2. Effect of immobilized mycelia on laccase production.

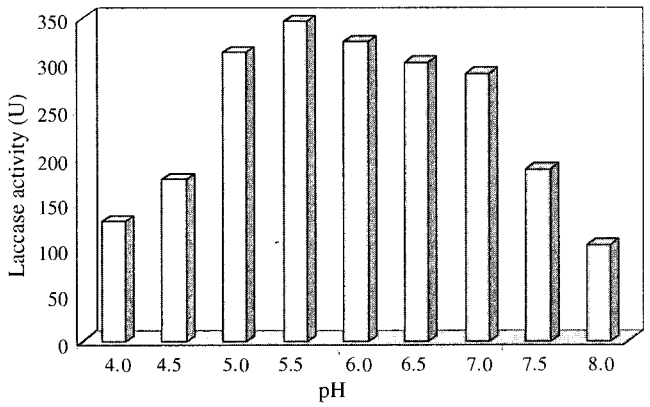


Fig. 3. Effect of pH on laccase production.

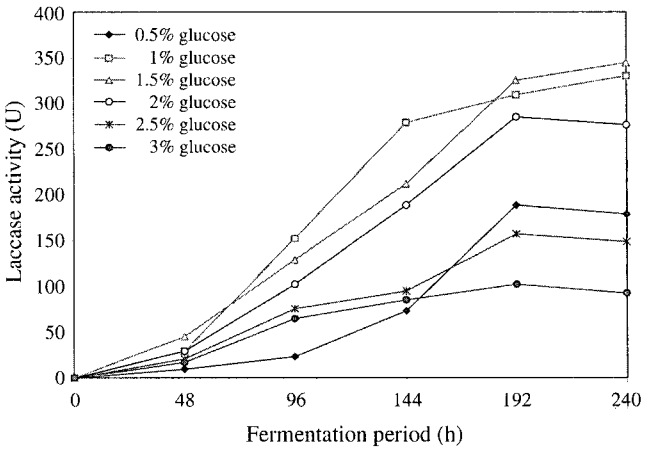


Fig. 4. Effect of glucose concentration on laccase production.

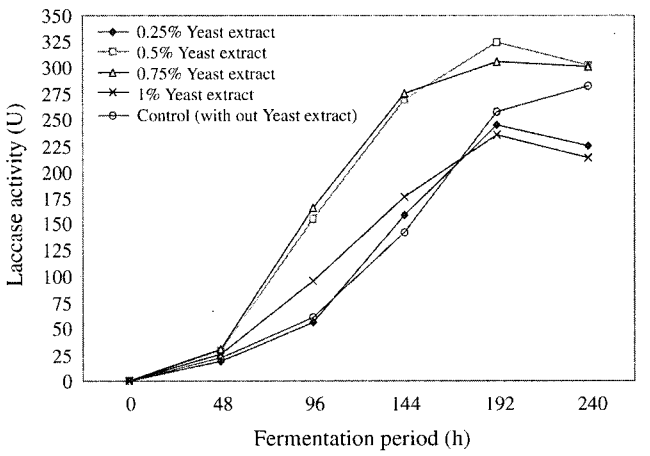


Fig. 5. Effect of yeast extract concentration on laccase production.

to 1.38 (wet weight of mycelia g/l), the laccase yield also showed increasing yield (123.6 to 299.6 U). 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. ostreatus* 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. 3). When the culture pH was increased from 4.0 to 5.5, the yield of laccase was found to increase from 129.5 U to 345.8 U during the 192 h fermentation period. Any subsequent increase in pH showed a reduction in laccase yield (104.5 U 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. ostreatus* was found to be 5.5.

Laccase formation was strongly influenced by the concentration of substrate used in the production medium. In general, substrates that were efficiently and rapidly utilized by the organism result in high levels of laccase activity. Increasing glucose concentration from 0.5% to 1.5% resulted in higher laccase activity (from 189.2 U to 325.2 U) (Fig. 4). Further increases in glucose concentration up to 3% did not show positive influence on the laccase activity. Bollarg and Leonowicz (1984) reported that higher glucose levels in the culture media showed repressed laccase activity. Supplementation of yeast extract (0.5%) showed enhanced laccase yield (322.1 U) compared to the control experiments (281.9 U; without yeast extract) after the 192 h period of fermentation (Fig. 5).

Addition of Cu^{2+} to the production medium stimulated laccase production by white rot fungi to the greatest extent. The laccase activity in production medium was examined (after 72 h of incubation) after supplementation with different concentrations of Cu^{2+} from 0.5 mM to 3 mM. Experimental results revealed that the presence of Cu^{2+} significantly influenced laccase synthesis (Fig. 6).

With an increase in Cu^{2+} concentration (from 0.5 mM to 1.5 mM), an increase in laccase activity (420.3 U at 1.5 mM) was observed compared to the control flask without Cu^{2+} supplementation (324.1 U). The expression of laccase is regulated at the level of the gene transcription by copper, although the exact mechanisms by which this trace element activates transcription have not yet been determined (Karahanian *et al.*, 1998; Palmieri, 2000). Any subsequent increase in Cu^{2+} concentration (above 1.5 mM) retarded the laccase yield, which may be attributed to growth inhibition due to the high concentration of Cu^{2+}.

The presence of aromatic compounds in production media has significant positive influence on the laccase yield. However, this phenomenon is found to mainly depend on the type of aromatic compound present, the concentration, and the time that the alcohol was added to the production medium. In the present study, different alcohols, such as ethanol, methanol, isobutyl alcohol, and veratryl alcohol, were studied to understand their relative influences on laccase expression. Fig. 7 shows the laccase expression after 192 h with the function of various alcohols in production media. The laccase yield was maximal in the case of veratryl alcohol supplemented production medium at a concentration of 50 mM (420.1 U) when compared to control (309.2 U). Laccase production was found to be significantly promoted in the presence of ethanol (356.9 U) and methanol (321.1 U) compared to control at 2.5% (w/v) and 2% concentration, respectively. In the case of isobutyl alcohol, cell growth, substrate utilization, and laccase activity were severely effected at 3% concentration. However, it was negligible at a lower concentration (0.5%) and laccase activity (291.2 U) was close to that of the control flask. The high yield in the case of veratryl alcohol may be attributed to the inducible mechanism of this alcohol at that concentration (50 mM). At higher concentrations, however, the yield was less and it may have been too toxic in nature towards the fungal growth. Veratryl alcohol is also known to stimulate the production of laccases in some basidiomycetes (Liebeskind *et al.*, 1990; Mester *et al.*, 1995).

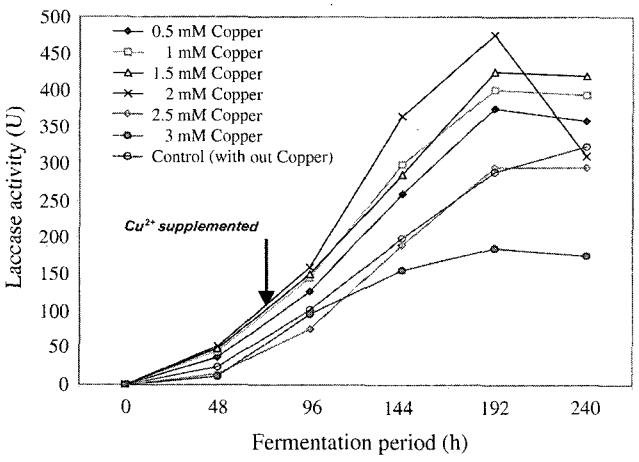


Fig. 6. Effect of Cu^{2+} on laccase production.

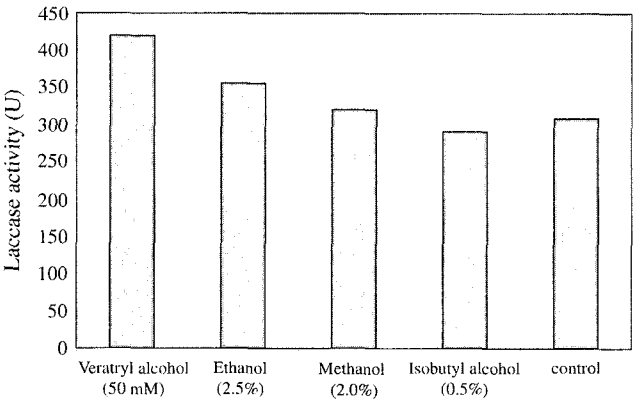


Fig 7. Effect of different alcohols on laccase yield.

Influence of immobilization compared to free mycelia on laccase expression

Lacasse expression studies using free mycelia of *P. ostreatus* have been reported elsewhere by the authors (Krishna Prasad *et al.*, 2005b). The maximum laccase activity of immobilized mycelia in shake flasks was 312.6 U, whereas the maximum laccase activity of free mycelia was 272.2 U. 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 root fungi, but mechanically agitated cultures are known to have an inhibitory effect on the production of these enzymes due

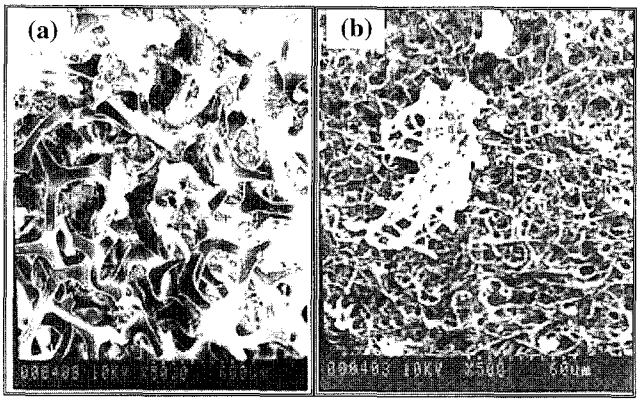


Fig. 8. SEM image of *P. ostreatus* 1804 mycelia immobilized PUF cubes (a, PUF cubes; b, *P. ostreatus* 1804 mycelia immobilized PUF cubes).

Fig. 9. SDS-PAGE showing changes in the laccase activity in fermentation broths of immobilized *Pleurotus ostreatus* 1804 mycelia grown under different concentrations of copper and alcohol supplements: lane M indicates molecular weight standard, lane 1 corresponds to sample obtained from culture fluid supplemented with 1.5 mM Cu^{2+}, and lanes 2 - 5 are samples obtained from culture broths supplemented with different alcohols; Lane 2, 50 mM Veratryl alcohol; Lane 3, 2.5% Ethanol; Lane 4, 2% Methanol; Lane 5, 0.5% Isobutyl alcohol.

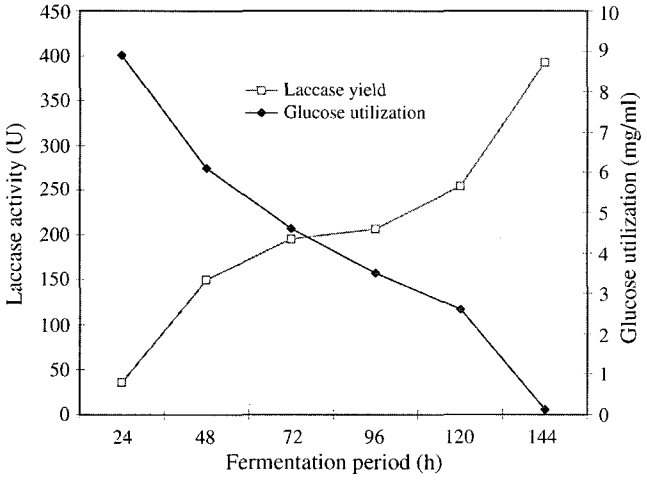


Fig. 10. Laccase production with immobilized *P. ostreatus* 1840 mycelia on PUF cubes in a packed bed bioreactor.

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. The mycelia immobilized PUF cubes SEM images are shown in Fig. 8.

Gel electrophoresis

Gel electrophoretic analysis of laccase produced by immobilized *Pleurotus ostreatus* 1804 mycelia under optimized concentrations of different alcohols and different concentrations of copper has been studied in order to visualize the laccase expression. The resulting bands have been presented in Fig. 9. It was found that isobutyl alcohol run on SDS (at 0.5% concentration) shows light bands compared to other alcohol runs. It also reveals that the molecular weight of the laccase produced by immobilized *P. ostreatus* was found to be 66 kDa.

Laccase production in a packed bed reactor

In order to study the effect of packed bed bioreactor configuration on laccase production by immobilized *P. ostreatus* on PUF cubes studied, the investigations were performed in a laboratory scale glass reactor using the optimized production conditions obtained from previously discussed batch studies (pH 5.5; inoculum level 1.38 (wet weight of mycelia g/l); carbon source 1.5; yeast extract-0.5%; copper 1.5 mM). During the reactor operation, aliquots of samples were withdrawn from the reactor every 24 h and analyzed for glucose consumption and laccase production. The laccase yield and glucose utilization rate were comparatively higher than those of the shake flask experiments (Fig. 10). The maximum laccase yield was around 392.9 U (in 144 h of fermentation period) with complete depletion of glucose.

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