Decolorization of Maxilon-Red by Kissiris Immobilized *Phanerochaete Chrysosporium* in a Trickle-Bed Bioreactor-Involvement of Ligninolytic Enzymes

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ABSTRACT: *The decolorization of Maxilon-red dye by Kissiris immobilized Phanerochaete chrysosporium in the trickle-bed reactor (TBR) using the basal nitrogen-limited growth medium was studied. The influence of the superficial liquid velocity (SLV) on the decolorizing ability of the fungus was examined at four SLVs (cm sec^{-1}): 0.05, 0.075, 0.1, and 0.15. Maximum level of the decolorizing activity was about 94 % by day 4-5 of the process when the TBR operated at SLV=0.075 to 0.1 cm sec^{-1}. At these SLVs, the maximum activities of the lignin peroxidase (LiP) and the manganese peroxidase (MnP) were (UL^{-1}): 87 and 207, respectively. In a successive decolorization of the tested dye in a continuous dye addition in the TBR when glucose level decreased to 2 gL^{-1}, significant reduction in the characteristics of the interest were observed. While by controlling the glucose concentration, these properties were improved considerably: The decolorizing ability of the Kissiris immobilized mycelia was more than 85 % after nearly 20 days of the process while the maximum activities of the LiP and the MnP were (UL^{-1}): 320 and 151, respectively. Considerable levels of the enzyme activities were detected by day 19 and after 11 times of the dye additions.*

KEY WORDS: *Ligninolytic enzymes, Kissiris-immobilized Phanerochaete chrysosporium, Decolorization, Maxilon-red synthetic dye, Trickle-bed bioreactor.*

INTRODUCTION

Widely use of synthetic dyes in the industries, such as textile dyeing, paper printing and color photography has put a constraint on these industries to seek for a proper technique to reduce the burden of the hazardous impact of the waste water generated from the industries. Over 10000 dyes are commercially available and 10-15 % of all dyestuff produced, are released directly to the environment and become part of the waste water (i.e. 7×10^5 tons dyestuff produced annually, worldwide) [1]. Conventional biological wastewater treatment systems can not be used efficiently for treating the pollutant load of wastewater of dyestuff.

Wood-rotting basidiomycetes causing white-rot are known as the most efficient microbial systems for degrading lignin in nature [2]. As it is known there are at least 12 different types of chemical linkages such as aryl, ether and carbon-carbon bonds connecting the aromatic nuclei, in lignin polymer [2]. The described linkages in lignin are not sensitive to enzymatic hydrolysis and in fact, lignin depolymerization by the oxidative mechanisms characterizes the recalcitrance of this heterogeneous polymer toward degradation by most microorganisms [3, 4]. The molecular process for this bio-degradation is brought about by action of a set of the extracellular oxidoreductases along with a H_2O_2-generating system [5,6]. Lignin peroxidase (LiP) catalyzes the H_2O_2-dependent oxidation of a wide variety of nonphenolic lignin model compounds and aromatic pollutants. Manganese peroxides (MnP) catalyses the H_2O_2-dependent oxidation of lignin and lignin derivatives and many different phenolic lignin model compounds [3].

The catalytic roles of MnP and LiP on oxidation of various organopollutants have intensified attentions toward use of these enzymes in the biotechnological applications such as in the wastewater treatments [3]. A prerequisite for large-scale application is the scale-up of the enzyme production. Production of LiP and MnP of *Phanerochaete chrysosporium* in static, submerged or immobilized liquid cultures have been reported in literature [7-9]. While, the low production rate in these attempts, has made the task of finding a suitable reactor configuration still being an attractive research subject. Use of the nonimmersed culture system provides a suitable environment for this fungus, nearly close to the conditions occurring in nature. The authors continued that these features facilitate laboratory studies on the ligninolytic enzyme production.

Attention in this subject in recent years has been directed toward the trickle-bed reactor (TBR) [10, 11]. This reactor consists of a multiphase (gas-liquid-solid) catalytic configuration in which downward movement of gas and liquid through a fixed bed of solid catalyst particles determines the behavior of the reactor. The hydrodynamics of the reactor is the key for proper operation and performance of TBR since the conversion of substrates, the yield and productivity are not only dependent on reaction kinetics, temperature and pressure but also the hydrodynamic parameters have determining roles in the reactor performance [10]. Balanced distribution of the liquid containing target nutrients to the microbe is an engineering characteristic of interest in TBR that can be provided by aerating the liquid-solid system properly.

Work on adhesion of *P. chrysosporium* on various synthetic type of solid supports showed that enhancement in quantity of immobilized mycelium was closely associated with an increased yields of LiP synthesis [1]. The researchers pointed out that immobilization can offer beneficial effects on the biosynthetic capacity and stability of the microbes' cells [1].

Successful implementation of an enzymatic process in the commercial scale heavily depends on a suitable low-cost support material, which provides a high viability of cell-immobilized system. The selection of an adequate support is also related to its availability. Polyurethane foam (PUF), polypropylene, vinylchloride, ceramic and wood chips are among several supports which have been used more often for immobilization of *P. chrysosporium* [11]. Despite of successful use of PUF and nylon web as support for the fungus, the lack of rigidity for these cell carriers made them unsuitable for use in large fermentors moreover, the uneasy works on removal of the spent mycelium significantly reduce the possibility of the using the support repeatedly. Moreover, the shape of the surface and materials are important factor in the attachment of biomass. For instance among the fungus supports named above, ceramic balls had least biomass adhesions. The fungus growth was spotty and unstable where could be readily detached from the support [11]. High surface roughness along high porosity greatly raises chance of the adequate cells adhesion [12].

Moreover, degradation of some cell supports is an unfavorable event. Nylon degradation by white-rot fungi is reported and results showed that some chemical functional groups such as -CHO, -CONH$_2$, were formed during this biodegradation [13]. It is important to realize possibility of formation of hazardous compounds when one uses the synthetic type of supports for immobilization of the microbes (or other biological systems) producing peroxidases capable of degrading the support. Rigidity of supports having high surface porosity is characteristic of the interest which increases possibility of the using the support repeatedly. Kissiris is a mineral glass foam material with high porosity formed by foam thickening volcanic lava. Kissiris as an inexpensive cell support material has been used for immobilizing *Saccharomyces cerevisiae* for ethanol production by repeated batch fermentation. While continuous ethanol production by thermotolerant *Kluyveramces marxianus* immobilized on Kissiris, has been also reported [14]. In our laboratory to study potential use of mineral Kissiris as a solid matrix for immobilization of the mycelial form of the fungus, several tests were conducted; the results were promising and discussed elsewhere [15].

To our knowledge there is no report in the literature concerning use of mineral Kissiris for the immobilization of *P. chrysosporium* in a TBR and decolorization of a synthetic dye as may relate to the production of the ligninolytic enzymes (LiP and MnP).The objective of the present work was therefore, to study the decolorizing capacity of Kissiris immobilized *P. chrysosporium* in the trickle-bed bioreactor while trends of the activities of the ligninolytic enzymes (LiP and MnP) produced by the immobilized fungus in this TBR system were monitored. Moreover, the longevity of decolorizing ability of the immobilized fungus and the presence of the enzymatic activities were confirmed using system consisting of the multiple additions of the dye.

MATERIALS AND METHODS
Microorganism cultivation and preparation of the inoculum

Phanerochaete chrysosporium (1557) was purchased from the Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ) and was maintained at low temperature 4 ºC; on 2.5 % malt extract agar slants. Subcultures were routinely made every 2 months. Inocula
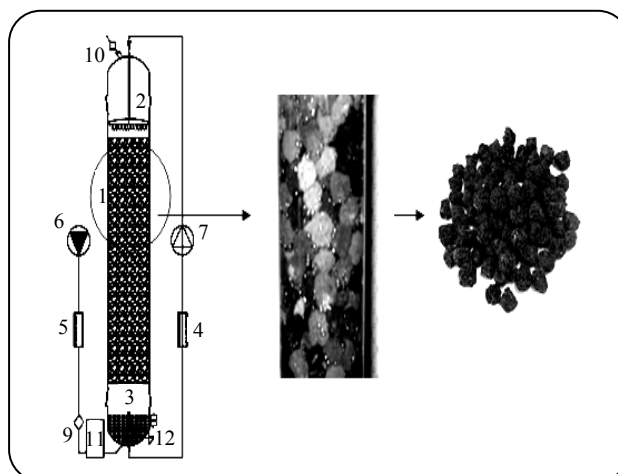

Fig. 1: Schematic representation of the trickle-bed bioreactor assembly used in the present study, 1) fixed bed; 2) liquid distribution; 3) air nozzle; 4) liquid flow meter; 5) air flow meter; 6) air compressor; 7) peristaltic pump; 8) liquid reservoir; 9,10) air filter; 11) gas humidifier; 12) sampling port.

consisted of conidial suspensions and were diluted so that the absorbance at 650 nm was 0.5, in a 1 cm path-length cuvette. The number of spores was determined using a Thoma counting-cell under the light microscope at × 1000 magnification (2.4×10^6 cells mL^{-1}). For preparation of conidial suspension, spores were taken from 2 week-old slants (stationary mode of the incubation at temperature 25 ºC).

Bioreactor configuration and operating conditions

The bench scale experiments were performed in a 5 cm (ID) glass column with 60 cm high having the round bottom portion as the liquid reservoir (capacity: ~350 ml). The column was packed with Kissiris (~500 g) in the form of spherical pieces (~1 cm in size). The liquid culture medium was circulated around an external loop using a peristaltic pump. A schematic TBR assembly used in the present study is given in Fig. 1.

The TBR was packed up to a height of 54 cm with Kissiris pieces and then sterilized in an autoclave (15 psi, 15 min). The liquid culture medium sterilized separately (9 psi, 30 min) was than introduced to the TBR. The spore suspension at level of 10 % (v/v) was added to the growth medium (nitrogen-limited medium prepared as described below) and after mixing, the TBR was inoculated with this culture mixture. Aeration to the system provided by usual laboratory air compressor (1 vvm) while 0.2 μm PTFE (P/N 4250) filter was positioned at the air inlet.

The loaded TBR was let to stand at 37 ºC for 2 days to ensure that the fungal mycelia would grow on the Kissiris pieces uniformly (the TBR was maintained at 37 ºC by circulation of temperature-controlled water). The content of the column was then empted and the solution of the tested dye was added to the reservoir. Down flow circulation of the liquid culture medium containing the Maxilon-red dye (75 ppm) was set the desired rate using a peristaltic pump, at (mL min^{-1}): 59, 88, 118, and 177. Trickling the culture medium (~350 mL) at four different superficial liquid velocities (SLV) was provided at (cm sec^{-1}): 0.05, 0.075, 0.1, and 0.15.

Following protocol was used to prepare mineral Kissiris for the fungal immobilization: Kissiris as the whole piece (~100 g) was broken to the round pieces (~1 cm diameter) using a small hammer. The Kissiris pieces were then soaked in the NaOH solution (0.1 N) for few minutes followed by soaking in the H_2SO_4 solution (0.1 N). The pieces were washed with distilled water several times until the pH of the solution was no longer acidic (~ pH 6.5). Composition of the mineral Kissiris used in the present study was approximated previously, using energy dispersive X-ray analysis (EDX) (LEO 1455 VP). The solid mineral contains silicon dioxide at about 16 (w/w %) [15].

In order to use Kissiris repeatedly, the colonized Kissiris was inactivated at the end of the each test run using muffle furnace (600 ºC for 40 min) followed by water washing. The cleaned Kissiris thereafter was used as described above.

Nitrogen-limited synthetic growth medium, as it has been used previously also was used in the present study, had following composition (liter^{-1} of distilled water): glucose 10 g; KH_2PO_4 2 g; $MgSO_4.7H_2O$ 0.5 g; $CaCl_2$ 0.1 g; $MnSO_4$ 0.03 g; NaCl 0.06 g; $FeSO_4. 7H_2O$ 6 mg; $CoCl_2$ 6 mg; $ZnSO_4.7H_2O$ 6 mg; $CuSO_4$ 6 mg; $AlK(SO_4)_2.12H_2O$ 0.6 mg; H_3BO_3 0.6 mg; $Na_2MoO_4.2H_2O$ 0.6 mg; yeast extract 12 mg; di-ammonium tartarate ($C_4H_{12}N_2O_6$) 0.2 g, thiamin 1 mg; veratryl alcohol 0.07 g and Tween 80, 0.5 g and pH of the medium was adjusted to 4.5 using 20 mM sodium acetate [15, 16]. The growth medium composition was based on the culture composition given elsewhere [15, 17] and the preliminary works in our laboratory showed that some components could be reduced or completely eliminated without any major adverse effect.

Capacity of the cell-supporting matrix in TBR can be approximated as the amount of liquid culture medium retained and drained from the fixed bed under particular defined condition (liquid hold-up measurement). In the present study, at the beginning and at the end of each test run, the following procedure was used to measure the liquid hold-up: during the each test run, constant level of liquid culture in the reservoir was marked. The peristaltic pump was stopped and trickling the liquid from the bed was collected as the liquid culture directed to the reservoir and the level of liquid in reservoir was marked again. The liquid hold up capacity of the Kissiris bed was calculated with respect to the difference of liquid volume between these two levels. The drainage was performed for about 10 min and verification was done for 30 min while no change in the volume of liquid present in reservoir was detected.

Analytical methods

The manganese peroxidase activity was measured according to the method described in the literature [18]. The complex formation of Mn(III) ions with malonate can be monitored spectrophotometrically at 270 nm (extinction coefficient of the complex = 11590 $M^{-1}cm^{-1}$) [19]. One unit activity (U) is the amount of the enzyme that catalyses the formation of one μmole/min Mn(III)-malonate complex. LiP activity was measured by the method described by *Tien* and *Kirk* 1988 while one unit enzyme activity was that amount of enzyme-catalyzed oxidation of 1 μmole of veratryl alcohol (VA) in 1 min at room temperature. The activities are average of at least two assays during each day.

The residual dye level was monitored spectro-photometrically at 530 nm which is the maximum absorbance wavelength of the tested dye (Fig. 2).

The external surface area of the Kissiris support was determined by the Brunauer - Emmett - Teller (BET) method using N_2 as adsorbate (NOVA-1000, Ver 6.10). The value obtained was 4.9642 m^2g^{-1}.

RESULTS AND DISCUSSION
Decolorization process

Maxilon-red synthetic dye at 75 ppm as the initial concentration was added at the 2nd day of the process. Fig. 2 shows the results of monitoring the visible spectrophotometry at 530 nm which is the maximum
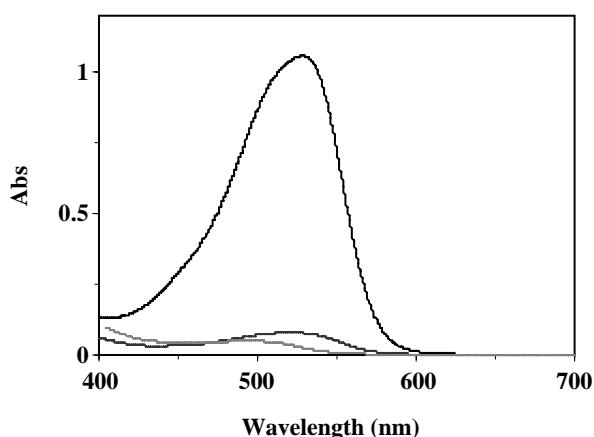
Fig. 2: Absorption spectra of the Maxilon-red during decolorization by P. chrysosporium immobilized on mineral Kissiris in the TBR, the overlays correspond to day 2, 3 and 4.
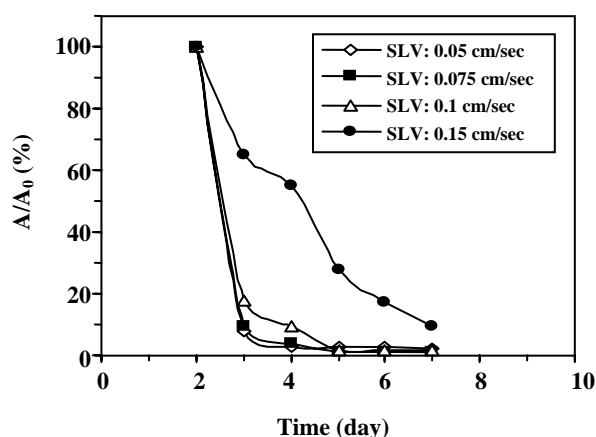

Fig. 3: Decolorization of the synthetic dye (Maxilon-red) by the Kissiris-immobilized P. chrysosporium in the TBR, at the four specified superficial liquid velocity.

absorbance was significant and the peak of the maximal absorbance was completely disappeared by day 4 of the process. Decolorization of the synthetic dyes by white rot fungi could be a useful indication of the presence of ligninolytic enzymes [19, 20]. In the present work, superficial liquid velocity was established at four levels (cm sec^{-1}): 0.05, 0.075, 0.1, and 0.15 while the initial glucose concentration was 10 g L^{-1}. The decolorization results obtained in the all four superficial liquid velocities tested are shown in Fig. 3.

Upon addition of the dye, first the biomass became highly colored an indication of the dye absorption on the immobilized mycelial matrix. After 24 hrs and at the 3rd day of the process, significant color removal was observable for the three superficial liquid velocities (cm sec^{-1}): 0.05, 0.075 and 0.1 as compared to that of the 4th test run (0.15 cm sec^{-1}). Extensive works on biosorption performance of the microorganisms have been done but the results of the present study on the colored mycelium in the beginning of the decolorization appears to due to rather simple sorption no attempt was made to study the biosorption behavior of the *P. chrysosporium* in relation to Maxilon-red color, in this work. Absorption (colored mycelia) could be as an indication of the metabolic activities of the fungus which may soon be able to handle the dye degradation as the enzymatic events. Trend of the decreasing color, was more or less similar for the three SLVs except 0.15 cm sec^{-1}.

Biomass formed more readily at low SLVs (i.e. 0.075 and 0.1 cm sec^{-1}) and it appeared that flow of the

metabolic activities of the fungus was less disturbed thus the mycelial mode of growth was in satisfactory condition. Determination of residence time distribution (RTD) before and after each of the four test runs showed that noticeable channeling occurred at the low SLV (0.05 cm sec^{-1}) (RTD data are not shown). In fact at SLV= 0.05 cm sec^{-1} significant sporulation was occurred after day 4 of the decolorization process. While biomass formation was considerably lower at the high SLV (0.15 cm sec^{-1}) and the color removal ability of the immobilized fungus, was significantly low. At low SLV the wetting of the cell support is limited and rate of supplying nutrient (i.e. glucose) could be reduced. On the other hand at high SLV, aeration is mainly through the liquid phase and it means direct contact between mycelial mass and air supply is decreased. Limitation of the transport rate of oxygen and also carbon dioxide could modify the fungal cells behavior. Liquid hold up tests at 0.15 cm sec^{-1} on the other hand, showed deterioration of the TBR performance and the tested system acted more or less like submerged fermentation condition.

Capacity of the particular support matrix to retain microbial cells is important in evaluating the performance of the reactor. The difference between amount of culture medium retained by the matrix and amount drained from the support gives an appropriate estimate of this capacity [4, 10]. The liquid hold -up test was conducted and its SLV dependency in the present TBR configuration was examined (Fig. 4). The liquid hold-up was considerably higher at the beginning of each test run before the

Table 1: Kissiris characteristics of the interest as determined in the present study (see the text for the detail).

Number of test runs	Immobilized mycelium (%) sterile non-sterile	Decolorization (%) sterile non-sterile	LiP_{max} (U / l) Sterile non-sterile	(U / l) $_{max}$ MnP sterile non-sterile
1	94 ± 2 94 ± 4	98.8 98.9	84 72	182 175
2	92 ± 4 91 ± 2	98.9 98.6	90 110	180 195
10	94 ± 3 95 ± 1	98.8 98.8	80 92	194 186

mycelial form of the growth became dominant over the Kissiris matrix. The liquid hold-up increased with increasing SLV. The hold-up was lower for the SLV tested when mycelia structure grew over the support matrix. The decreasing levels were (%): 81, 63, 50 and 21 for the SLVs tested (cm sec^{-1}): 0.05, 0.075, 0.1 and 0.15, respectively. This observation is very probable in this TBR when one considers the porous structure of Kissiris, where there were no empty spaces on the packing materials upon the cells' growth. Works on TBR by the other researchers showed that trend of liquid hold-up measurements in TBR, with biomass (*P. chrysosporium*) entrapped in a ceramic supports coated with Ca-alginate matrix, were reverse of the observations reported in the present study [10]. The authors concluded that the thickness of Ca-alginate with or without biomass was very small with respect to the packing channels. Considering the results of the present study it appears that the capacity of the porous structure of the Kissiris in retaining liquid medium with and without biomass was not comparable with the ceramic matrix used in the study described above [10, 21]. As it is stated elsewhere in the literature, a solid porous support for the fungus immobilization mimics the natural environ-ment for the microbe's growth while the mycelium may have enough strength that makes cells resistant to damages which may be caused by shear force [9].

Mineral Kissiris is rigid support and can be used repeatedly. In the present study the Kissiris pieces were used up to ten test runs and no deterioration in the cell's characteristics was observed (table 1). Use of Kissiris with or without sterility also is an important characteristic. The ability of using non-sterilized Kissiris in this TBR system was tested and the result is shown in table 1. The decolorization process was carried on readily and reduction of the color intensity values and the active growth of the fungus on the non-sterilized support both were as usual. The ligninolytic enzymes activities tested in this experiment were also satisfactory.
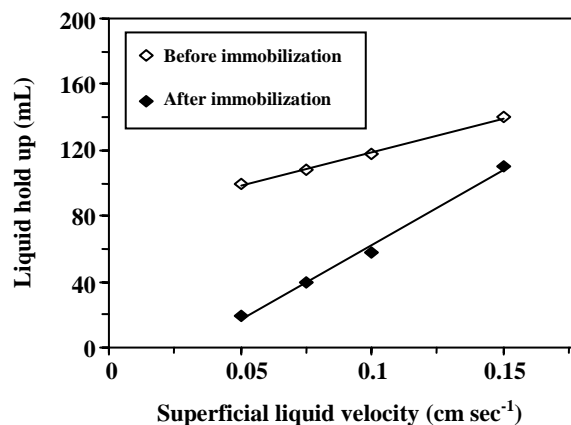
Fig. 4: Liquid hold-up against superficial liquid velocities used in the present TBR configuration.

Adhesion of *P. chrysosporium* as conidiospores or mycelium to various solid supports was studied using a thermodynamic model [12]. Amount of immobilized mycelium was found to be higher for hydrophobic than for hydrophilic cell support (polyurethane versus stainless steel) while production of LiP was stimulated in the same way. Moreover, hydrophilic character of the cells' system was under influence of the physiological state: conidiophores were more hydrophobic than mycelium. Analysis of the surface morphology of the solid supports showed that immobilization of *P. chrysosporium* was at higher level when the surface roughness of the solid carrier was increased. Importance of intraparticle porosity as well as a higher total surface area of the solid support, on the fungal immobilization was clearly described [12]. Kissiris is the light weight support (i.e. specific gravity ≈ 1 g mL^{-1}) and mineral composition of the support used in t he present study was approximated using energy dispersive X-ray analysis (EDX) and Si preferentially in the form of the oxide constitutes about 16 (wt %) of this solid rock [15].

In addition to the composition, the measured surface area of the support may play an important role. Silica gel
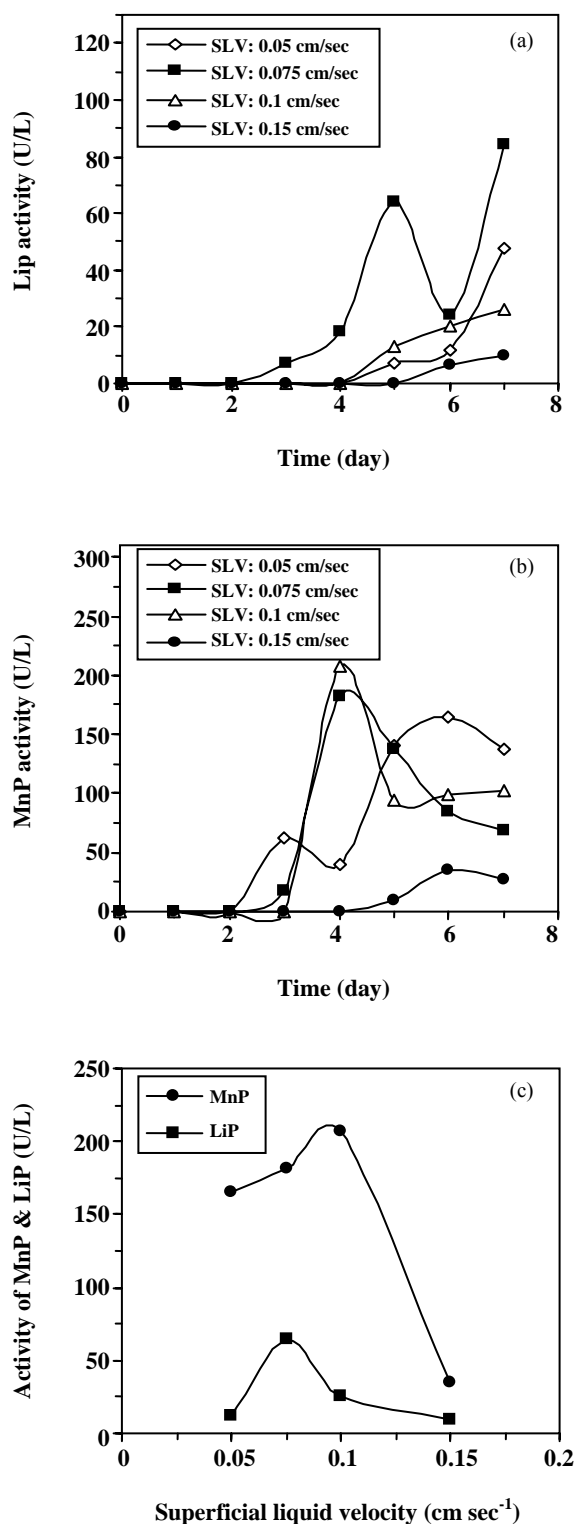
Fig. 5: Time course of the activities of the LiP (a) and MnP (b) produced by Kissiris-immobilized P. chrysosporium in the TBR system. Trend of the maximum activities of the enzyme vs. tested superficial liquid velocity is also shown (c).

(SG) with a measured specific surface area (BET) of 422 m^2g^{-1} has been used as the support for the immobilization of laccase of *Trametes versicolor* to study the decolorization of textile reactive dyes [22]. SG support was efficient in the enzymatic decolorization process, however use of reagents such as xylene, 3-chloro-propyltrimethoxysilane in the process of the modification SG, may make interested researcher reluctant to use this modified support. Moreover, the above named authors also used titanium dioxide particles with a BET surface area of 50 m^2g^{-1} bout no claim about the efficiency of this support was given by the authors. The specific surface area of the porous poly(styrene divinylbenzene) support was obtained through the works reported by the other researchers (246 m^2g^{-1}) who used *P. chrysosporium* in two forms: i.e. the immobilized spores and immobilized pellets [23]. Higher LiP activity was obtained with the spores while the degradation rate of 2-chlorophenol was considerably higher as compared to that of the freely suspended mycelial pellets. Moreover, controlling the fungal pellet size by the limitations of the physical properties of the PUF has been shown to have a determining role in the prevention of clumping which is important in optimization of the LiP production [24]. The irregular shape and size of the pores of the Kissiris pieces put restriction on the size of the fungal mycelia which plays role in the ligninolytic enzymes secretion. The mineral Kissiris used in the present study had surface area at much lower level as compared to that of the above named cell carriers (4.9642 m^2g). There was no need to chemically modify the highly porous Kissiris support used in the present study while the ability of the Kissiris immobilized mycelia, in producing LiP and MnP was considerable and role of these enzymes in the decolorization of the tested dye was evident. There is a need to know the possible relationship(s) between the surface areas of the Kissiris available for the mycelia after 20 days of the decolorization for instance, and the molecular structure of the dye which progressively may encounter the occupied active sites of the support.

Fig. 6 shows that initial pH of the medium in the TBR with the Kissiris-immobilized fungus did not change considerably after about 18-20 days (pH changed between 4 to 5.5) while the glucose level was not controlled during this decolorization process. Works on the nylon web-immobilized *P. chrysosporium* in the packed-bed aerated bioreactor showed that the initial pH
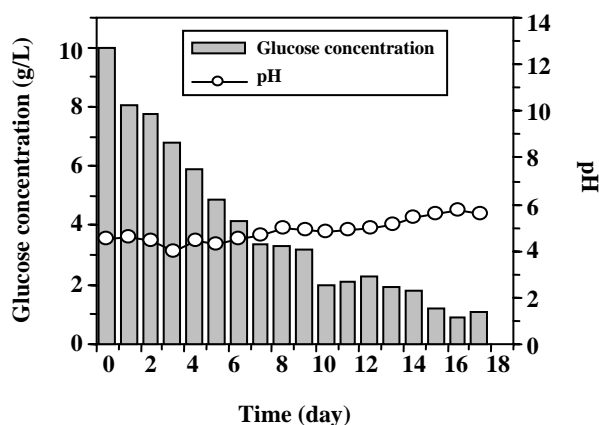
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Fig. 6: Glucose consumption by Kissiris-immobilized P. chrysosporium in the TBR. The pH profile obtained during the decolorization process is also shown.

of the growth medium was slightly higher as compared to that of the shaking culture [9]. Some decomposition of the support could be possible during the sterilization cycle as the author pointed out. As it can be seen from Fig. 6 glucose consumption by the Kissiris-immobilized *P. chrysosporium* in the TBR system was not significantly changed and by day 10 of the decolorization process, about 80 % of the glucose consumed. This rate of the glucose consumption, i.e. 0.033 g L^{-1} hr^{-1} then the rate changed to 0.0208 g L^{-1} hr^{-1} during day 10 to 14 of the process thereafter, little amount of the glucose remained in the TBR system (see Fig. 6).

Consumption of the glucose by *P. chrysosporium* immobilized on PUF and nylon sponge in a laboratory scale bioreactor was (g L^{-1} hr^{-1}): 0.023 and ~0.02, respectively [25]. In a separate study when the researcher used the packed-bed aerated bioreactor, the rate of the glucose consumption by free and nylon-web immobilized *P. chrysosporium* were (g L^{-1} hr^{-1}): 0.066 and 0.095, respectively. The results of the glucose consumption rate in the present study is close to those results reported above [25] although the mineral Kissiris has advantages over the synthetic supports including nylon sponge or PUF. Moreover, although spores and pellets are attached easily on the nylon carrier and growth occurs throughout the carrier [9] these carriers are not reusable (see table 1).

Enzyme production

Works of Glenn and Gold in 1983 showed that decolorization of the several polymeric dyes by *P. chrysosporium* occurred under conditions similar to

those needed for degrading lignin and suppressive effect of nitrogen at high concentrations and requirement of atmospheric oxygen, were among most studied factors [13, 20, 26]. Through these years, the lignin degrading ability of the white-rot fungi was extended to variety of toxic organic chemicals from wastewaters and solid wastes. Syntheses of LiP and MnP, as two major oxidative enzymes, by *P. chrysosporium* occur during secondary phase of the fungus growth [5]. Complexity of the mechanisms involved in these biodegradation and decolorization process is recognized. Attempts are directed toward collecting informative data on the production of the fungal peroxidases in a suitable reactor configuration and relating role of the enzymes secretion to the decolorization of the wastewaters such as textile dyes.

Growth of *P. chrysosporium* in a commercial type of bioreactor (2 L, 250 rpm) as reported elsewhere, was with pellets formation and no ligninolytic enzyme were secreted [27]. Although, when the silicon tubing (wrapping around four stainless steel sticks to form a spiral with an appropriate size) was used inside the reactor, the fungus grew well on this tubing in the form of mycelial mates and no pellets formation while ligninolytic enzymes were produced after four days [27]. Fig. 5 shows the activities of LiP and MnP during the maxilon-red dye decolorization. Maximal MnP activity at about 207 U L^{-1} was obtained at SLV=0.1 cm sec^{-1} on day 4 of the process. At the same time the residual dye concentration was at its lowest level at SLVs=0.075-0.1 cm sec^{-1} (Fig. 3). The level of the MnP activity at SLV=0.1 cm sec^{-1} was decreased on day 5 of the process and thereafter remained nearly constant up to day 7 of the process. The level of the activity for LiP at SLV=0.075 cm sec^{-1} 64 U L^{-1} on day 5 of the process and after decreasing about 60 % by day 6, it raised again and reached to 87 U L^{-1} on day 7 of the process (see Fig. 5). Those researchers who worked on the TBR using calcium alginate coating a ceramic support for the immobilization of the *P. chrysosporium* found that growth of the fungus was under influence of SLV and by conditioning the physiological state, the uniformity of the biomass growth could be obtained and this resulted in higher LiP activities [10, 28].

In the present study, the fluctuation in LiP activities at SLV=0.075 cm sec^{-1} was observed. Studies on the primary and secondary extracellular proteases showed that proteases formed during primary phase of the fungus

metabolism was at highest level on day 2 and could inactivate LiP activity. Although the proteases produced during secondary phase of the growth did not inactivate LiP [29]. The authors discussed their results on the basis of possible role of proteases formed during idiophasic growth in releasing of extracellular LiP from the fungal hyphae. Fig. 5(c) shows the maximum LiP and MnP activities versus four levels of the SLV tested in the present study.

The ability of the Kissiris-immobilized *P. chrysosporium* in producing MnP was higher at all four SLVs. This increased level of activity was pronounced more at SLV=0.1 cm sec^{-1}. The study conducted on the synthetic dyes (poly R-478 and crystal violet) decolorization by ligninolytic enzymes of *P. chryso-sporium* produced in a fixed-bed bioreactor containing cubes of fibrous nylon sponge as the cell supports [29]. The maximum levels of activities for LiP and MnP in the first batch were 1000 and 1200 U L^{-1}, respectively while in the second successive batch, the MnP and LiP activities were 200 and 150 U L^{-1}, respectively. The LiP activity was fluctuated in the first batch and reaching to a maximum at < 150 U L^{-1} on day 4 of the process. It decreased on day 6 thereafter it raised to 225 U L^{-1}. This fluctuation in the activity was not observed in the second batch [29]. The researchers studied MnP production by *P. chrysosporium* immobilized on PUF in a pulsed packed-bed bioreactor. Effect of Mn(II) concentration in the feed affected the activity and the highest level (~110 U L^{-1}) was obtained when Mn(II) levels were at 5000 μM. The lower level of MnP activity was at about 30-60 U L^{-1} which was obtained at Mn(II) concentration < 5000 μM and when the levels of Mn(II) increased to 10000 μM, the activity of MnP was repressed to 20 U L^{-1}. The authors pointed out the repressive effect of high concentrations of Mn(II) on the MnP activity. On the other hand, enhancing effect of Mn deficiency in nitrogen-limited cultures of *P. chrysosporium* on LiP production has been known for sometime [26].

Our previous report on Kissiris-immobilized *P. chrysosporium* showed that Mn(II) enhanced the MnP activity [15]. This effect found to be related to increased levels of veratryl alcohol produced endogenously and expressing protective role on LiP, from being inactivated by H$_2$O$_2$. The findings presented by that report led authors to state that upon exposure of the cultures to air

under non-immersed liquid conditions, availability of O$_2$ to the cultures increased and higher levels of Mn needed to suppress LiP production compared with the manganese concentrations required in shallow stationary cultures. The TBR configuration used in the present study was found to act favorably in the production of LiP and the results are in agreement with other findings [28].

Various configuration of bioreactors have been used to study LiP production by *P. chrysosporium* and the LiP was lowest (~102 U L^{-1}) in stirred tank while the activity was highest (~1220 U L^{-1}) for the TBR as reported by the other researchers [28]. Capacity of *P. chrysosporium* to produce LiP and MnP enzymes is well demonstrated (table 2). Use of synthetic type of the supports is predominant in the cell immobilization studies particularly works on white-rot fungi. The drawbacks of the using those supports are mentioned above. Based on the results of the present study, we believe that using the Kissiris pieces for the fungus immobilization in the TBR system is interesting and encouraging. Of course more works are needed to evaluate the influence of the some factors such as manganese concentration, Tween, on the TBR operation relevant to both the textile dye decolorization (i.e. wastewater treatment) and the production of the LiP and MnP.

Successive decolorization in the TBR configuration with reference to the glucose concentration

Levels of glucose used in the decolorization studies with white-rot fungi are generally about 15-20 g L^{-1} [29]. Effect of initial glucose concentration for decolorization of textile dyestuff by *Coriolus versicolor* in a rotating biological contactor was studied [29]. Efficiency of the decolorization was highest (77 %) when initial glucose levels for growth phase and decolorization phase where 10 and 5 g L^{-1} respectively. The data also showed that decreasing concentration of glucose from 5 to 2 g L^{-1} in decolorization phase, was not effective for the process and the fungus did not maintain its decolorizing activity. Initial glucose concentration in the present study was 10 g L^{-1} which decreased gradually and reached to about 5 g L^{-1} by day 5 of the decolorization (Fig. 6).

The level of the glucose was about 2 g L^{-1} on day 10 of the process and the trend of pH changes which was nearly constant, is also shown in Fig. 6. Successive decolorization of the dye by the original immobilized

***Table 2: Results of the some reports on the levels of the activities of LiP and MnP (U L^{-1}) produced by
P. chrysosporium in different fermentors.***

Fermentor type	Ligninolytic enzymes		Reference
	LiP	MnP	
Bioreactor filled with: PUF , NS	478 571		[25]
Fixed-bed bioreactor filled with Ns: - first batch - second batch	1000 150	1200 200	[30]
Rotating drum bioreactor using: NS	360	1350	[21]
Air-lift bioreactor: - low Mn(II) - high Mn(II)	760	700	[4]
Packed-bed aerated bioreactor	730		[9]
Rotating disc fermentor	100		[6]
Bioreactor using: -free pellets -poly(styrene divinylbenzen)	307 602		[23]
Solid-stat (Immersion) bioreactor using: NS	356	987	[29]
Bioreactor filled with: -PUF -Polypropylene -Vinylchloride -Ceramic -Wood cheeps	280 810 540 670 1100		[12]
TBR filled with calcium alginate coating a ceramic supports	1220		[10, 26]
TBR filled with Kissiris supports	320	207	present work

NS: nylon sponge, PUF: polyurethane foam.

mycelia was investigated in the next step. The maxilon-red dye was added repeatedly in 9 numbers of times on days 2, 6, 7, 8, 9.5, 11, 13, 14.5 and 16 and each time the dye was added to obtain the constant level at 75 ppm. The result is shown in Fig. 7.

The decolorization activity of the immobilized mycelia decreased gradually from the 4th addition of the dye (i.e. on 9.5 days of the process). The decolorizing ability was dropped to about 20 % by 7th addition of the dye and in day 17.5 more than 92 % of the dye remained in the system. The concentration of the glucose was about 2 g L^{-1} from day 10 up to day 14 of the process (Fig. 6).

The activities of the LiP and MnP are given in Fig. 7. The highest levels of the MnP and LiP during the successive dye decolorization were (U L^{-1}): 182 and 335, respectively (see Fig. 7). The sudden drop in the activities of both enzymes by day 11 of the process was noticeable. In the second step of this part of the present study and aiming to confirm the necessity of keeping glucose concentration at nearly constant level (>2 g L^{-1}), the multiple dye additions were carried out along the glucose addition. The level of the glucose in this system was controlled (the result is shown in Fig. 8).The dye was added repeatedly and the decolorizing ability of the original immobilized mycelia was significant even after 11 times of dye addition. More than 85 % of the dye was decolorized by day 19.5. The trends of the changes in the activities of the ligninolytic enzymes tested in this study are shown in Fig. 8. The highest level of LiP activity was about 320 U L^{-1} on day 8 of the repeated batch of the dye addition similar to that activity when the glucose level was not controlled (see Fig. 7). But the LiP activity remained at relatively high levels even after 11 times of the dye additions. Simultaneously the MnP activity remained at high levels and this was about 127 U L^{-1} by day 19 of the successive dye decolorization process (see Fig. 8).

LiP and MnP are glycoproteins containing up to 20-30 % sugar and in fact, LiP produced under the carbon-limited medium is more labile than the enzyme synthesized under the nitrogen-limited environment [8]. Protective role of the carbohydrate in the LiP molecule
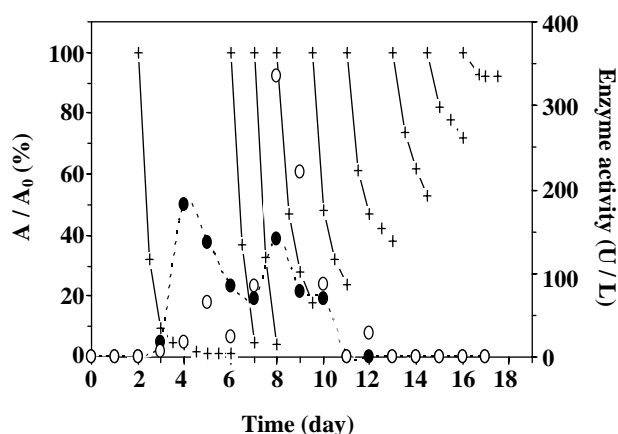
Fig. 7: Successive decolorization of the Maxilon-red dye in a continuous dye addition in the TBR using Kissiris-immobilized P. chrysosporium (+) (initial glucose concentration was 10 g L^{-1} and the level of glucose in the system was not controlled). Time course of the activities of the LiP (o) and MnP (•) in this multiple dye addition is also shown
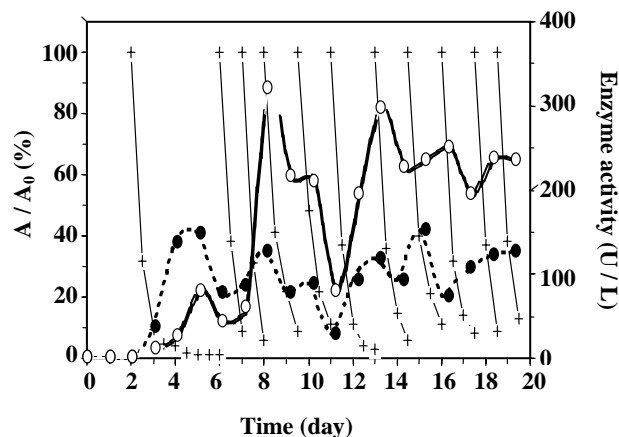

Fig. 8: Successive decolorization of the Maxilon-red dye in a continuous dye addition in the TBR using Kissiris-immobilized P. chrysosporium (+) (Initial glucose concentration was 10 g L^{-1} and the level of glucose in the system was kept nearly constant at about 2 g L^{-1}). Time course of the activities of the LiP (o) and MnP (•) in this multiple dye addition is also shown.

has been discussed. The findings of the present study showed that controlling the glucose concentration in the nitrogen-limited medium was critical factor in the enzymatic performance of the Kissiris-immobilized *P. chrysosporium.*

CONCLUSIONS

1- Mineral Kissiris having highly porous structure was used for the immobilization of the white-rot fungus *P. chrysosporium* in the TBR system.

2- Kissiris-immobilized *P. chrysosporium* had significant decolorizing ability. The color of the tested dye (i.e. maxilon-red dye solution at the 75 ppm concentration) was completely decolorized by day 4 to 5 of the process.

Superficial liquid velocity at 0.075 to 0.1 cm sec^{-1} was appropriate to observe the characteristics of the interest in this TBR system. Considerable sporulation occurred at SLV=0.05 cm sec^{-1} while TBR at the high SLV (i.e. 0.15 cm sec^{-1} in this study) was operated more or less like a fermentor under submerged condition.

3- This system of the bio-decolorizer exhibited high ligninolytic enzymes activities (LiP and MnP). These activities were found to be related to the decolorizing ability of the immobilized fungus. In fact, the Kissiris-immobilized fungus could become an interesting

alternative in the production of the fungal enzymes such as LiP and MnP (simultaneous production of the LiP and MnP).

4- The spores were used for immobilization.

5- The nitrogen-limited growth medium similar to that introduced in the relevant literature was used in this TBR system while the initial level of the glucose used was 10 g L^{-1}.

6- Successive decolorization was operated by the multiple additions of the dye. While the decolorizing ability of these bio-decolorizers was decreased after 9.5 days of the operation corresponded to the four times of the dye additions. The decreasing trends of the LiP and MnP were noticeable also.

The repeated batch of the decolorization operated satisfactorily when the concentration of the glucose as the major nutrient in the flow of the growth medium in the TBR was kept nearly constant at 2 g L^{-1}. The operation lasted for about 20 days with 11 times of the dye additions.

7- The reuse of the Kissiris pieces was possible simply by incinerating the colonized pieces in a muffle furnace while no deterioration in the metabolic behavior of the fungus upon its immobilization on the used Kissiris pieces was observed. Moreover, the Kissiris pieces could be used without sterilization. The characteristic of the

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fungus immobilized on the non-sterile Kissiris pieces did not change. These are important properties for the natural support which also do not need any pre-treatment. The use of the Kissiris in the scale-up processes regarding the production of the fungal enzymes in the TBR system is an interesting subject. Preliminary works in this laboratory showed that the LiP and MnP in the culture fluids (free of biomass) were active and could be used in-vitro, to treat the colored solutions.

REFERENCES

[1] Fu, Y., Viraraghavan, T., Fungal Decolorization of Dye Wastewater: A Review, *Bioresourse Technology* **79**, 251 (2001).

[2] Gold, M.H., Alic, M., Molecular Biology of the Lignin-Degrading Basidomycete *Phanerochaete chrysosporium*, *Microbiological Reviews*, **57**(3), 605 (1993).

[3] Aust, S., Degradation of Environmental Pollutants by *Phanerochaete chrysosporium*, *Microbial Ecology*, **20**, 197 (1990).

[4] Bonnarme, P., Jeffries, T.W. Selective Production of Extracellular Peroxidases from *Phanerochaete chrysosporium* in an Airlift Bioreactor, *J. Fermentation and Bioengineering,* **70**(3), 158 (1990).

[5] Kirk, T., Fraell, R., Enzymatic Combustion: The Microbial Degradation of Lignin, *Annual Reviews Microbiology*, **41,** 465 (1987).

[6] Kirk, T.K., Croan, S., Tien, M., Murtagh, K.E., Farrell RL Production of Multiple Ligninases by *Phanerochaete chrysosporium*: Effect of Selected Growth Conditions and Use of a Mutant Strain, *Enzyme and Microbial Technology,* **8**, 27 (1986).

[7] Kirkpatrick, N., Palmer, J.M. Semi-Continuous Ligninase Production Using Foam-Immobilized *Phanerochaete chrysosporium, Applied Microbiology and Biotechnology,* **27**, 129 (1987).

[8] Leisola, M., Waldner, R. Production, Characterization and Mechanism of Lignin Peroxidase. In: Zadrazil. F., Reiniger P. (Eds) Treatment of Lignocellulosic with White Rot Fungi Proceedings of a Workshop Held in Braunschweig (1988).

[9] Linko, S. Production and Characterization of Extracellular Ligninperoxidase from Immobilized *Phanerochaete chrysosporium* in 10-L Bioreactor, *Enzyme and Microbial Technology,* **10**, 410 (1988).

[10] Bosco, F., Ruggeri, B., Rassi, G. Performance of a Trickle-Bed Reactor (TBR) for Exoenzymes Production of *Phanerochaete chrysosporium*: Influence of Superficial Liquid Velocity, *Chemical Engineering Science,* **54**, 3163 (1999).

[11] Shim, S., Kawamoto, K. Enzyme Production Activity of *Phanerochaete chrysosporium* and Degradation of Pentachlorophenol in a Bioreactor, *Water Research,* **36**, 4445 (2002).

[12] Asther, M., Bellon-Fontaine, M.N., Capedvila C., Corrieu, G., A Thermodynamic Model to Predict *Phanerochaete chrysosporium* INA-12 Adhesion to Various Solid Carriers in Relation to Lignin Peroxidase Production, *Biotechnology and Bioengineering,* **35**, 477 (1990).

[13] Deguchi, T., Kakezawa, M., Nishida, T., Nylon Degradation by Lignin-Degrading Fungi, *Applied and Environmental Microbiology,* **63**(1), 329 (1997).

[14] Nigam, P., Banat, I.M., Singh, D., McHale, A.P., Marchant, R., Continuous Ethanol Production by Thermotolerant *Kluyveromyces marxianus* IBM3 Immobilized on Mineral Kissiris at 45 ºC, *World Journal of Microbiology and Biotechnology,* **13**, 283 (1997).

[15] Karimi, A., Vahabzadeh, F., Bonakdarpour, B., Use of Kissiris Immobilized *Phanerochaete chrysosporium* for Synthetic Dye Decolorization - Involvement of Manganese Peroxidase, *World Journal of Microbiology and Biotechnology,* **22**, 1251 (2006).

[16] Vahabzadeh, F., Mehranian, M., Satari, A., Color Removal Ability of *Phanerochaete chrysosporium* in Relation to Lignin Peroxidase and Manganese Peroxidase Produced in Molasses Wastewater, *World Journal of Microbiology and Biotechnology,* **20**, 859 (2004).

[17] Tien, M., Kirk, K. Lignin Peroxidase of *Phanerochaete Chrysosporium*, *Methods in Enzymology,* **161**, 238 (1988).

[18] Gandolfi-Boer, C., Obici, L., Souza, C., Perelta, R., Decolorization of Synthetic Dyes by Solid State Cultures of *Lentinula (Lentinus) edodes* producing Manganese Peroxidase as the Main Ligninolytic Enzyme, *Bioresourse Technology,* **94**, 107 (1994).

[19] Wariishi, H., Valli, K., Gold, M.H. Manganese (II) Oxidation by Manganese Peroxidase from the Basidomycete *Phanerochaete chrysosporium*, *The Journal of Biological Chemistry,* **267**, 23688 (1992).

[20] Platt, M.W., Hadry Chet, I., The Decolorization of the Polymeric dye Poly-Blue (Polyvinylamine Sulfonate-Anthroquinone) by Lignin Degrading Fungi, *Applied Microbiology and Biotechnology,* **21**, 394 (1985).

[21] Dominguez, A., Rivela, I., Rodriguez Couto, S., Sanroman, M.A. Design of New Rotating Drum Bioreactor for Ligninolytic Enzyme *Production by Phanerochaete Chrysosporium* Grown on an Inert Support, *Process Biochemistry,* **37**, 549 (2001).

[22] Peralta-Zomora, P., Pereira, C.M., Tiburtius, RL., Moraes, S.G., Rosa, M.A., Minussi, R.C., Duran, N., Decolorization of Reactive Dyes by Immobilized Laccase, *Applied Catalysis B: Environmental,* **42**, 131 (2003).

[23] Ruckenstein, E., Wang, X. Production of Lignin Peroxidase by *Phanerochaete chrysosporium* Immobilized on Porous Poly (Styrene-Divinyl-benzene) Carrier and its Application to the Degrading of 2- Chlorophenol, *Biotechnology and Bioengineering,* **44**, 79 (1994).

[24] Capdevila, C., Corrieu, G., Asther, M., A Feed-Harvest Culturing Method to Improve Lignin Peroxidase Production by *Phanerochaete chryso-sporium* INA-12 Immobilized on Polyurethane Foam, *Journal Fermentation Bioengineering,* **68** (1), 60 (1989).

[25] Rodriguez, S., Longo, M. A., Camesella, C., Sanroman, A., Production of Manganese Peroxidase and Laccase in Laboratory-scale Bioreactors by *Phanerochaete chrysosporium*, *Bioprocess Engineering,* **20**, 531 (1999).

[26] Rothschild, V., Levkowitz, A., Hadar, Y., Desoretz, C., Manganese Deficiency Can Replace High Oxygen Levels Needed for Lignin Peroxidase Formation by *Phanerochaete chrysosporium*, *Applied and Environmental Microbiology,* **65**(2), 483 (1999).

[27] Cornwell, K., Tinland-Butez, M., Tardone, P. J., Cabasso, I., Hammel, K.E., Lignin Degradation and Lignin Peroxidase Production in Cultures of *Phanerochaete chrysosporium* Immobilized on Porous Ceramic Supports, *Enzyme and Microbial Technology,* **12**, 916 (1990).

[28] Bosco, F., Ruggeri, B., Sassi, G. Experimental Identification of a Scalable Reactor Configuration for Lignin Peroxidase Production by *Phanerochaete chrysosporium*, *Journal of Biotechnology,* **52**, 21 (1996).

[29] Rivela, I., Rodriguez Couto, S., Sanroman, A., Extracellular Ligninolytic Enzyme Production by *Phanerochaete chrysosporium* in a New Solid-State Bioreactor, *Biotechnology Letters,* **22**, 1443 (2000).

[30] Moldes, D., Rodrigues Couto, S., Cameselle, C., Sanroman, M.A., Study of the Degradation of Dyes by MnP of *Phanerochaete chrysosporium* Produced in Fixed-Bed Bioreactor, *Chemosphere,* **51**, 295 (2003).