K. Balan, P. Pratheebaa, T. Palvannan

APPLICATION OF THE LACCASE, PRODUCED ON COCONUT FLESH BY *PLEUROTUS FLORIDA* FOR DYE DECOLORIZATION

Periyar University, Tamil Nadu, India

We investigated the ability of Pleurotus florida to produce laccase on coconut flesh as a solid substrate fermentation. The decolorization of two structurally different dyes such as azo (Reactive Blue 198) and triphenylmethane dye (Malachite Green) were analysed. The decolorization of Reactive blue 198 and Malachite Green at 8 hrs was 93% and 63% respectiely. The untreated and treated dye was characterized by UV-Vis and fourier transform infrared (FTIR) spectroscopy scan. FTIR analysis pointed out the involvement of alkene (C = C) and carboxylic (C – O) groups in the decolorization process. The toxicity with respect to Allium cepa root inhibition was measured to demonstrate the potential of laccase in the detoxication and bioremediation process.

Keywords: *Allium cepa,* dye decolorization, FTIR-UV, laccase, *Pleurotus florida*.

Introduction

Synthetic dyes are extensively used in the textile industry. Due to inefficiencies of industrial dyeing process, 10 – 15% of dyes are lost in the effluents of textile units, rendering them highly coloured [1, 2]. It is estimated that 280,000 tons of textile dyes are discharged in such industrial effluents every year worldwide [3]. Large quantities of these dyes are released daily into the environment from various indust-ries. The discharge of waste waters containing recalcitrant residues into river and lakes leads to higher biological oxygen demand thus posing a serious threat to native aquatic life. The available waste water treatment systems are unable to completely remove the recalcitrant dyes and other organic residues from such effluents [4, 5].

White-rot fungi produce several types of oxidative enzymes, which are useful for remediation of environmental pollutants [6 –8]. Laccase (benzenediol: oxygen oxido reductase, EC 1.10.3.2) is one of such enzymes, which is also

found in plants and bacteria [9, 10]. Solid-state fermentation (SSF) is defined as any fermentation process occurring in absence or near absence of free liquid, using an inert substrate or a natural substrate as a solid support [11]. The former only functions as an attachment place for the microorganism, where as the latter also acts as a carbon source, which considerably reduces the production costs [12]. SSF is advantageous in obtaining concentrated metabolites and subsequent purification procedures are economical [13].

Coconut flesh has been selected to perform the present study due to its content in sugars easily metabolized by microorganisms, vitamins and minerals and it has the physical integrity to serve as a supporting material [14]. The aim of present study was to investigate the plausible of coconut flesh as a raw material for the production of laccase by *Pleurotus florida* under SSF condition. For azo and triphenyl dye decolorization and colour removal from a dye industry effluent were examined.

Experimental

Microorganism. The white rot fungus *Pleurotus florida* NCIM 1243 was obtained from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The organism was maintained through fortnightly transfer at 25°C on potato dextrose agar plates and kept at 4°C until used.

Dyes and chemicals. Malachite Green (MG), Reactive blue 198 (RB) were purchased from Qualigens chemicals, India and 2,22-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was purchased from "Sigma-Aldrich Chemical Corporation" (USA) and all other chemicals were of analytical grade.

Laccase production. Coconut flesh was used as a support-substrate for laccase production by *Pleurotus florida* under SSF conditions. Chemical composition of the coconut flesh (values per 100 g) [14]:

Compound (g)			
Water	46.990	Carbohydrate	15.230
Protein	3.330	Fibre	9
Total lipid	33.490		
Minerals (mg)			
Calcium	14	Sodium	20
Iron	2.430	Zinc	1.100
Magnesium	32	Copper	0.435
Phosphorus	113	Manganese	1.500
Potassium	356	Selenium (mcg)	10.100

Vitamins (mg)

Vitamin C.....3.300	Pantothenic acid.....0.300
Thiamine..... 0.066	Vitamin B-6.....0.054
Riboflavin.....0.020	Folate (mcg).....26.400
Niacin..... 0.540	

Coconut flesh was pretreated as follows: they were first soaked for an hour in 30 ml of 83.17 mM KOH (10 g of fresh support) to neutralize organic acids [15]. Then, they were thoroughly washed with distilled water and dried at moderate temperature. Prior to use, the flesh was autoclaved at 121°C for 20 min. The cultures were performed in cotton plugged *Erlenmeyer flasks* (250 ml) containing 3g of grated coconut flesh and 20 ml of culture medium. Inoculation was carried out directly in the *Erlenmeyer flasks*. Three agar plugs (diameter, 7 mm), from an actively growing fungus on potato dextrose agar, per *Erlenmeyer* were used as inoculum. The *Erlenmeyer flasks* were incubated statically under an air atmosphere at 30°C and in complete darkness. The composition of the culture medium was prepared according to Rodriguez Couto et al. (2006) [16].

Enzyme extraction and partial purification. Enzyme extraction and partial purification was performed according to a protocol described by Sathishkumar, et al. (2010) [17].

Laccase assay and protein determination. Laccase activity was determined with ABTS as the substrate[18]. The reaction mixture contained 20 mM ABTS, 20 mM sodium acetate buffer (pH 5.6) and the culture filtrate. ABTS was monitored by an absorbance increase at 420 nm ($\varepsilon 420 = 36{,}000 M^{-1} cm^{-1}$) at 30°C. One unit of laccase activity was defined as the amount of enzyme that oxidised 1μmol of ABTS per min. The activities were expressed in Uml^{-1}. Protein was estimated by Lowry et al. [19].

Laccase dye decolorization. The decolorization of industrial dye RB 198 and MG were investigated. Stock solutions of dye (RB 198 and MG) – 500 ppm were stored in the dark at room temperature and the desired concentrations of were obtained by further dilutions. The reaction mixtures contained sodium acetate buffer (pH 5.6), laccase, and dye in a final volume of 5 ml. All the reactions were incubated at room temperature, without shaking and kept in dark. The residual dye concentration was measured spectrophotometrically and was associated with decrease in the absorbance at the peak of maximum visible wavelength (590 nm for RB 198 and 620 nm for MG) and calculated by measuring the area under the plot. Dye decolorization was calculated by means of the formula

$$D = \frac{A_{ini} - A_{obs}}{A_{ini}},$$

where D is the (%) decolorization of RB 198 and MG, A_{ini} is the area under the curve of the absorption spectrum at 590 and 620 nm at time zero and A_{obs} is the area under the curve of the absorption spectrum at 590 and 620 nm at a determined time [20].

UV-Vis and fourier transform infrared (FTIR) spectral analysis. Decolorization was monitored by UV-Vis spectroscopic analysis, where as biodegradation was monitored using FTIR spectroscopy. Decolorization of each dye was followed by monitoring changes in its absorption spectrum (190 – 700 nm) using a shimadzu UV-Vis spectrophotometer and comparing the results, to those of the respective controls. FTIR analysis was carried out using perkin elmer spectrophotometer and changes in % transmission at different wave lengths were observed. The analysis of extracted metabolites was done on perkin elmer, spectrum one instrument and compared with control dye in themed IR region of 4000 – 400 cm^{-1}.

Allium cepa linn assay. The *Allium cepa* test provides a rapid screening procedure for chemicals and environmental agents which may represent environmental hazards. Root growth inhibition assay was performed as a 96 hr semi-static exposure test [21]. Healthy equal sizes of common onions were obtained from Salem local market of Tamil Nadu, India.

The dried outer scales were carefully removed leaving the ring of the root primodial intact. *A. cepa* was exposed for 96 hr at various concentrations of RB 198 and MG whereas laccase treated RB 198 and MG. The base of each onion bulbs was placed on RB198 and MG samples inside a 30 ml beaker and kept away from the sunlight for 4 days after which the root length was measured. Growth inhibition was estimated as EC50 (the effective concentration of a chemical producing 50% of the total effect) [21].

Results and discussion

Production and partial purification. The production of coconut in the world is around 50,000 millions of kg per year. Part of this production (damaged nuts, those without milk and germinating nuts) is rejected by the coconut processing industries. The composition of coconut flesh is very appropriate for the production of laccase.

Hence, its utilization as a raw material to produce laccase by *Pleurotus florida* operating in solid-state conditions was tested. The extracellular enzyme produced by *Pleurotus florida* on coconut flesh are shown in Fig. 1.

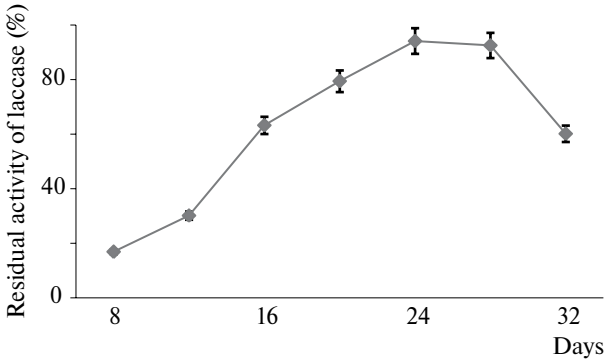

Fig. 1. Laccase production by solid substrate culture Pleurotus florida grown on coconut flesh.

The laccase production started on 8th day and increased to a maximum activity 5.78 Ug^{-1} on the 24^{th} day there after gradually decreased. The present report is similar to that of Susana Rodriguez Couto et al. [14] on laccase production from *Trametes hirsuta* in coconut flesh. The high values of laccase detected in the present work are likely due to the content in soluble phenolic compounds of coconut flesh, which are known to stimulate laccase production [22]. In addition, they are also may be due to coconut maintaining high glucose values along cultivation, which is an essential factor to keep high laccase levels by this fungus as it was shown in Moldes et al. (2003) [23]. On the whole, it points out the enormous potential of coconut flesh as a raw material for laccase production.

The specific activity of partially purified laccase was found to be 124.4 Umg^{-1} of protein. The percentage of final yield was 53.3% and its partially purified fold was 10.6 respectively. When compared to other reports the purification yield was increased in our approach [24, 25].

UV-Vis spectroscopic analysis and dye decolorization. Decolorization was monitored by UV-Vis spectroscopic analysis, decolorization of dyes using laccase was followed by monitoring, changes in their absorption spectrum (190 – 700 nm) and comparing the results, to those of respective controls. UV-Vis spectrum of presented in Fig. 2 RB 198 and MG dyes, showed decreased in optical density. UV-Vis absorption, peaks decrease approximately in proportion to each other, where as in biodegradation, either the major visible light absorbance peak disappears completely, or a new peak appears [26].

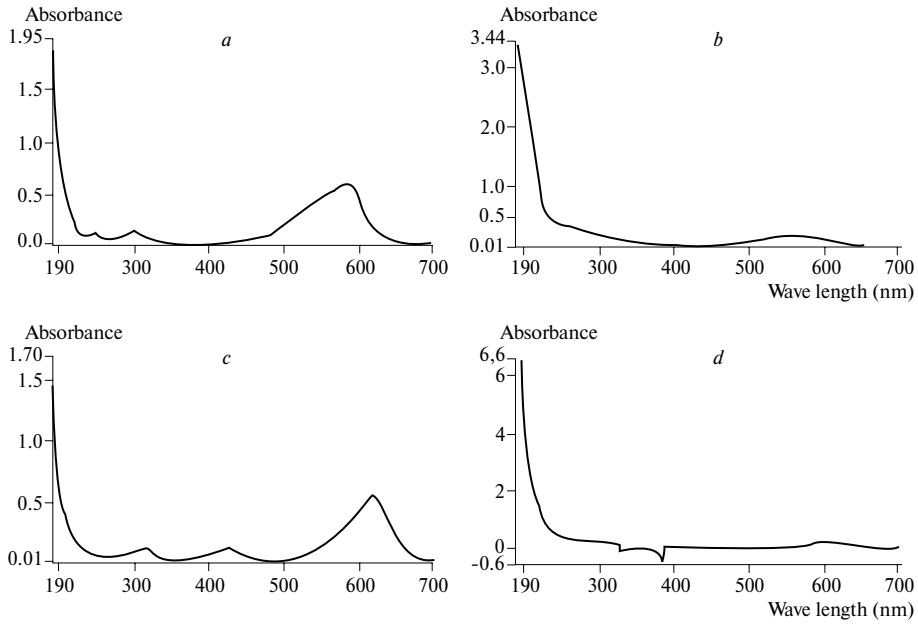

Fig. 2. UV-Vis absorption spectrum of (100 ppm): a – RB 198 dye, b – RB 198 dye with laccase, c – MG dye, d – MG dye with laccase decolorization by Pleurotus florida laccase.

RB 198 dye was used in the industry to dye wool, silk and leather industery. The RB 198 was effectively decolorized by laccase (Fig. 3). The rate of decolorization at 2 hr was 20% followed by after 4 hr (40%), and 8 hr (93%) respectively. The observed reactive blue dye turned from deep blue to light black, reaching a decolorization. The present report with *Pleurotus florida* compared favorably with those of Soares et al. [27] on dye decolorization.

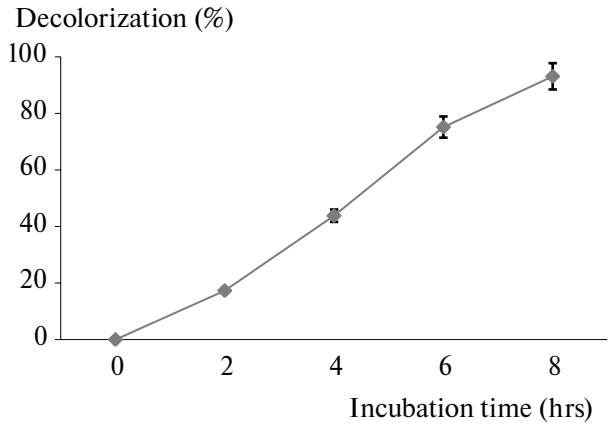

Fig. 3. Decolorization of RB 198 by Pleurtous florida.

The colour removals versus time curves for MG are shown in (Fig. 4). MG was decolorized about 20% in 4 hr reaching a decolorization of after 63% at 8 hr. The dye was transformed from green color to light yellow color and then turned colorless. As a result, the decolorization rate was slower than that of reactive blue. It has been reported that highly substituted triphenylmethane dyes required longer time to be decolorized or could only be decolorized to a certain extent [28].

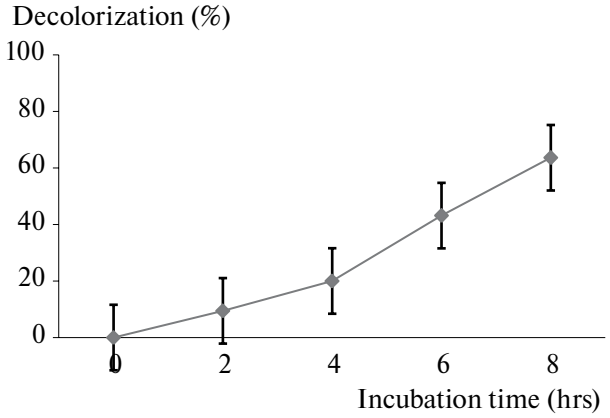

Fig. 4. Decolorization of MG by Pleurotus florida.

FTIR analysis. Spectrums of RB 198 are given in Fig. 5 (*a*) and (*b*). The FTIR spectroscopic analysis pointed out broad absorption bands of C – H groups of nanosubstituted alkyenes at 3405 cm^{-1} in control RB 198. After the laccase treatment peak had shifted the low wave number 3388 cm^{-1} that the O – H stretching. In untreated peaks are seen at 2081 cm^{-1}, 1638 cm^{-1} and 1413 cm^{-1} and this may be due to C ≡ C, C ≡ N and C = C (monosubstutited alkenes that is vinyl group) stretching frequency. After laccase treated slight change was observed in the corresponding peaks.Hence these corresponding groups are involved in decolorization process. The peaks are found at 1277 cm^{-1}, 1119 cm^{-1} and 1018 cm^{-1} this may be due to C – O (carboxyl band), C – C stretching frequency. After laccase treatment there was a significant change observed suggesting C – O and C – C groups are involved in the decolorization process of reactive blue. In addition peak at 658 cm^{-1} due to in reactive blue dye disappeared after laccase treatment, hence corresponding peak at 659 cm^{-1} slight changed.

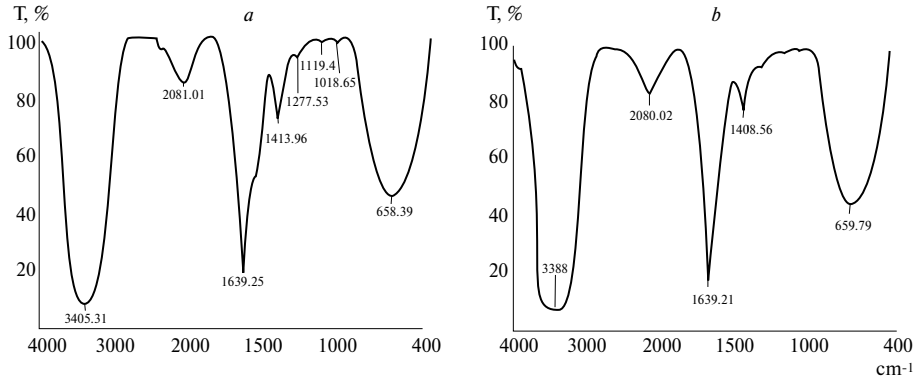

Fig. 5. The FTIR spectra of RB 198 untreated (a) and RB 198 treated (b).

The FTIR spectra Fig. 6 (*a*) and (*b*) of the untreated dye (MG) and laccase treated with different substitutions showed clear traces of modification. In the spectra of the untreated dye (MG) a broad band due to hydrogen bonded hydroxyl (O – H) group appeared at 3393 cm^{-1} after laccase treatment the peak had shifted to higher wave number 3411 cm^{-1} the C – H stretching group involved in the decolorization process. In untreated dye are seen at 2081 cm^{-1}, 1639 cm^{-1} and 1407 cm^{-1} this may be due to NH_3, C = C and O – H stretching.

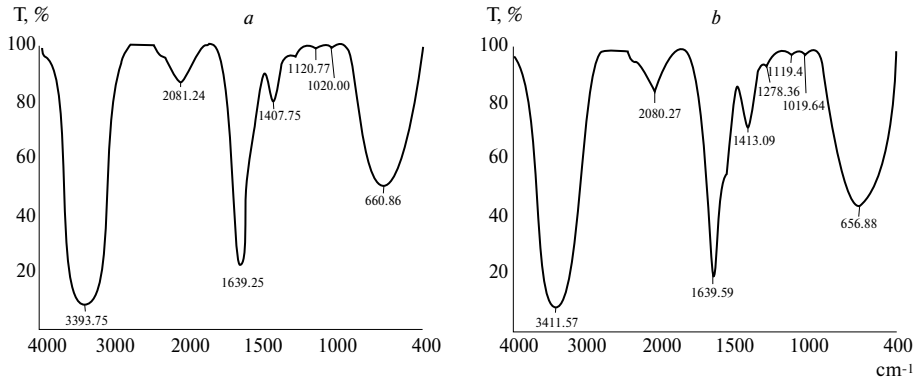

Fig. 6. The FTIR spectra of MG untreated (a) and of MG treated (b).

After laccase treatment a slight change was observed in the corresponding peaks. Hence these corresponding peaks may be involved in the decolorization process. The peaks are found at 1020 cm^{-1} and 660 cm^{-1} this due to C – C, phenols stretching frequency, by the following laccase in treatment new peak was appeared at 1278 cm^{-1} and 1119 cm^{-1} that the C – O stretching frequency this group are involved decolorization process.

Toxicity assessment using Allium test. The estimated EC50 of *A. cepa* exposed to effluent dye (RB 198 and MG) with laccase was 98 ppm and 72 ppm (Fig. 7 (*a*) and (*b*)). Very little growth was observed in the *A. cepa* exposed to effluent dye (concentration greater than 100 ppm). The results obtained from laccase decolorization revealed a concentration of dye dependent decrease in root length, as the concentration increases from 25 – 125 ppm the root length significantly decreased ($p<0.05$) when compared with the control. This result was compare to the other reports [29, 30].

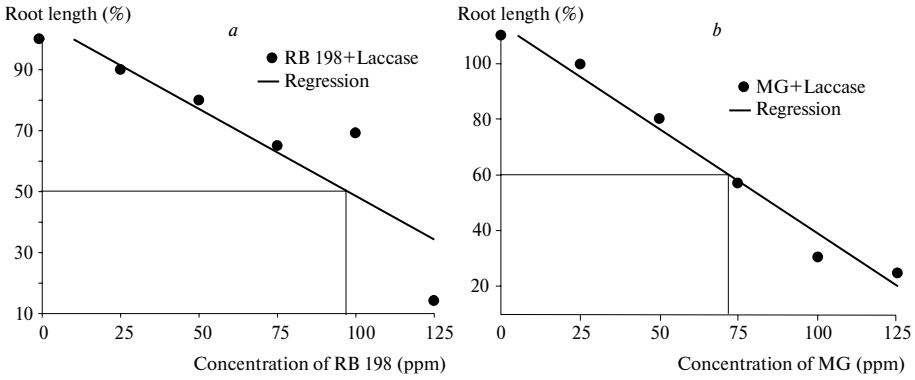

Fig. 7. Growth inhibition of A. cepa roots exposed to RB 198 (a) and MG (b) decolorised by Pleurotus florida laccase.

Conclusions

The results implement that laccase was produced by enormous of coconut flesh as a cheapest source.

. The results obtained, it can be asserted enormous of coconut flesh to produce laccase by *Pleurotus florida* under solid state conditions.

. The laccase produced presented a highly decolourising ability, for azo and triphenylmethane dyes.

. Further structural characterization of the dye decolorization was achieved using UV-Vis spectral scan and FTIR spectroscopy.

. This will enable proper biochemical analysis of industrial effluent in order to identify the constituent that is really toxic and its prompt removal from the effluent before discharge.

Acknowledgements

The Corresponding author (Principal Investigator – Project No.: BT/PR8973/GBD/27/57/2006 Dt 14/08/07) is grateful to Government of India, Ministry of Science and Technology, Department of Biotechnology for financial support.

References

[1] *Vaidya A.A., Date K.V.* // Colourage. 1982. **14**. P. 3 – 10.

[2] *Boer C.G., Obici L., Souza CGMde., Piralta R.M.* // Biores. Technol. 2004. **94**. P. 107 – 112.

[3] *Maas R., Chaudhari S.* // Proc. Biochem. 2005. **40**. P. 699 – 705.

[4] *Reddy C.A.* // Current Opinion Biotechnol. 1995. **6**. P. 320 – 328.

[5] *Mcmullan G., Meehan C., Connely A., Kirby N., Robinson T., Nigam P., Banat I.M., Merchant R., Smith, W.F.* // Appl. and. Environ. Microbiol. 2001. **56**. P. 81 – 87.

[6] *Call H.P., Mucke I.* // J. Biotechnol. 1997. **53**. P. 163 – 202.

[7] *Colombo J.C., Cabello M., Arambarri A.M.* // Environ. Pollut. 1996. **94**. P. 355 – 362.

[8] *Dietrich D., Hickey W.J., Lamar R.* // Appl. and Environ. Microbiol. 1995. **61**. P. 3904 – 3909.

[9] *Diamantidis G., Effose A., Potier P., Bally R.* // Soil Biol. Biochem. 2000. **32**. P. 919 – 927.

[10] *Mayer A.M., Staples R.C.* // Phytochemistry. 2002. **60**. P. 551 – 565.

[11] *Pandey A., Selvakumar P., Soccol C.R., Nigam P.* // Current Sci. 1999. **77**. P.149 – 162.

[12] *Murthy R.M.V., Karanth N.G., RaghavaRao K.S.M.S.* // Adv. Appl. Microbiol. 1993. **38**. P. 99 – 147.

[13] *Ashakumary L., Selvakumar P.S., Pandey A.* // Wiley Eastern Limited: Newage Int. Publish. 1994. P. 33.

[14] *Couto S.R., Sanroman A.M.* // J. Food Eng. 2005. **71**. P. 208 – 213.

[15] *Stredansky M., Conti E.* // Proc. Biochem. 1999. **34**. P. 581 – 587.

[16] *Rodriguez Couto S., Rodriguez A., Paterson R.R.M., Lima N., Teixeira J.A.* // Lett. in Appl. Microbiol. 2006. **42**. P. 612 – 616.

[17] *Sathishkumar P., Murugesan K., Palvannan T.* // J. Basic Microbiol. 2010. **50**. P. 360.

[18] *Wolfenden B.S., Wilson R.L.* // J. Chem. Soc. Perkin. Trans. 1982. P. 805 – 812.

[19] *Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J.* // J. Biol. Chem. 1951. **193**. P. 265 – 275.

[20] *Lopez M.J., Guisado G., Vargas-Garcia M.C., Suarez-Estrella, F., Moreno J.* // Enzyme Microbiol. Technol. 2006. **40.** P. 42 – 45.

[21] *Mamta Kumara A., Mukherjee N., Chandrasekaran* // Sci. Total Environ. 2009. **407.** P. 5243 – 5246.

[22] *Tychanowicz G.K., Zilly A., Marques de Souza C.G., Peralta R.M.* // Proc. Biochem. 2004. **7**. P. 855 – 859.

[23] *Moldes D., Gallego P.P., Rodriguez Couto S., Sanroman A.* // Biotechnol. Lett. 2003. **25**. P. 491 – 495.

[24] *Nirmalendu Das Tapas, Chakraborty Mina Mukherjee* // J. Basic Microbiol. 2001. **41**. P. 261 – 267.

[25] *Lei Lu, Min Zhao, Bei-Bei Zhang, Shu-Yu, Yu Xi-Jun, Bian Wei Wang, Yan Wang.* //Appl. Microbiol. Biotechnol. 2007. **74**. P. 1232 – 1239.

[26] *Crestini C., Argyropoulos D.S.* // Bioorg. Med. Chem. 1998. **6**. P. 2161 – 2169.

[27] *Asad S., Amoozegar M.A. Pourbabaee A.A., Sarbolouki M.N., Dastgheib S.M.M.* //Biores. Technol. 2007. **98**. P. 2082 – 2088.

[28] *Soares G.M.B., Pessoa de Amorim M.T., Costa-Ferreira M.* // J. Biotechnol. 2001. **89**. P. 123 – 129.

[29] *Sandra Radic., Drazenka Stipanicev., Valerija Vujcic., Marijanovic rajcic., Sinise Sirac., Branka Pevalek-kozline* // Sci. Total Environ. 2010. **408**. P. 1228 – 1233.

[30] *Olusegan Samuel B., Fidelia Osuala I., Peter Odeigah G.C.* //African J. Environ. Sci. and Technol. 2010. **4**. P. 21 – 27.

Received 12.12.2011