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ENHANCEMENT OF LIGNOCELLULOLYTIC ENZYME PRODUCTION BY CO CULTURE OF COMPATIBLE FUNGI

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ABSTRACT

In the industrial biotechnology, production of lignocellulolytic enzyme is generally carried out by single fungal strain but experimental evidence suggests that co-culture of ligninolytic fungi and filamentous micro-fungi results in increased enzyme activity. Present investigation was carried out to assess the efficiency of lignocellulolytic enzyme production by mono culture of *Trichoderma reesei, Irpex lacteus* and *Pycnoporus sanguineus* and mixed/co cultures of *T. reesei* with *P. sanguineus* and *I. lacteus*. Amongst mono cultures studied *T. reesei* and *I. lacteus* showed the absence of LiP and cellulase enzyme activity. The amount of enzymes produced by the co cultures was higher than that of its mono cultures and the incubation period for the maximum enzyme production was also significantly reduced.

***Key words: Trichoderma reesei*, *Irpex lacteus*, *Pycnoporus sanguineus*, Enzymes, Co-culture**

INTRODUCTION

Large amount of lignocellulosic materials are used by different industries like paper-pulp industries, timber industries and many agro- industries. Lignocellulolytic enzymes-producing fungi are widespread and include species from the ascomycetes (e.g. *Trichoderma reesei*) and basidiomycetes such as white-rot (e.g. *Phanerochaete chrysosporium*) and brown-rot fungi (e.g. *Fomitopsis palustris*).

The application of xylanases in prebleaching of pulps is gaining importance as an alternative to toxic chlorine-containing chemicals [1, 2, 3] where xylanases offer an attractive and commercially viable option to eliminate chlorine in bleaching, and reduce chlorinated organic compounds in bleach plant effluents, reduce the kappa number (residual lignin content in the pulp), and increase the brightness of the pulp [4, 5]. Cellulase-free xylanase preparations have been tested successfully in industrial applications such as the prebleaching of kraft pulp in the pulp and paper industry.

Trichoderma is one of the best known fungus which produces enzymes with high xylanolytic activity. In the natural environment, Trichoderma are ubiquitously present and widely used in commercial applications as a bio control agent of plant diseases [6].

The non specific enzyme systems of the white rot fungi makes them useful in the bio technological applications in paper and pulp industry like bio pulping and bio bleaching and to degrade the aromatic dyes and recalcitrant compounds like aromatic hydrocarbons and pollutants [7]. Majority of information available so far concerns the lignin-degrading enzyme systems of Phanerochaete chrysosporium and Trametes versicolor.

Since biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is to search for efficient xylanase producers, and the efficient production of lignocellulolytic enzymes from mixed/co culture of fungal isolates which enhance the enzyme production [8]. Two biopotential fungi compatible/synergistic of which one or both are selective in lignin degradation and producing xylanase enzyme would help in increasing the efficiency of enzyme production.

The present study was carried out to evaluate the potential of an ascomycetous fungus Trichoderma reesei in mono culture and mixed culture of the same with two basidiomycetous fungi for efficient production of lignocellulolytic enzymes. Amongst the fungi utilized, Trichoderma reesei (TR) is known to produce cellulase free xylanase while Irpex lacteus (IL), and Pycnoporus sanguineus (PYS) are selective for the delignification process.

MATERIAL AND METHODS

Fungal cultures and maintenance

On the basis of paired interaction tests performed in the laboratory Trichoderma reesei, Irpex lacteus and Pycnoporus sanguineus were selected for co culture experiment [9]. Pure culture of Trichoderma reesei was procured from Microbial Type Culture Collection (MTCC), Chandigadh while the cultures of Irpex lacteus and Pycnoporus sanguineus were obtained from Forest Research Institute, Dehradun. All the cultures were maintained on Malt Extract Agar (MEA) medium at 4 $(+1)^0$C in Seed Anatomy laboratory of Department of Botany, The Maharaja Sayajirao University of Baroda, Gujarat, India.

Preparation of fungal enzyme sample

Enzyme assays were performed by taking aliquots from 100 ml of 3% Malt extract broth (MEB) medium. The medium was sterilized by autoclaving at 121^0 C temperature and 15 psi pressure for 20 minutes. After cooling, the medium was inoculated with 9 mm disc of 10 days old fungal culture in aseptic condition and for

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co-culture/dual culture 9 mm agar disc of both the selected fungal mycelium were inoculated in the Erlenmeyer flask of MEB medium. The culture was incubated at 25 $(\pm 1)^0$ C for the desired incubation period. After completion of incubation period the cultures were removed, mycelium was homogenized in a Waring laboratory blender and filtered through whatman paper No. 1 disc. The obtained enzyme supernatant was used to evaluate the activity of enzymes.

Enzyme assay

Lignin peroxidase (LiP) and Manganese-dependent peroxidase (MnP)

Lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) activities were measured according to the procedure of Castillo et. al. [10] using 0.167 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) as substrate which interact with 2.37 mM 3-(dimethylamino) benzoic acid (DMAB) producing a purple coloured reaction. Substrates were prepared on 0.1 M succinic-lactic acid buffer at pH 4.5. Reaction mixture contained 417.5 µl MBTH, 417.5 µl DMAB, 100 µl MnSO4, 50 µl supernatant (enzyme) and 15 µl H2O2 to measure the enzyme activity. To distinguish between magnesium dependent and independent peroxidases, reaction was performed in the presence and absence of 3 mM MnSO4, 4 mM H2O2, respectively to initialise the reaction. Reactions were carried out at 37°C and monitored spectrophotometrically at 590 nm. The enzyme activity was calculated using extinction coefficient ε = 53000 M-1cm-1.

Laccase

Laccase activity was assayed using 2, 2'-azino-bis (3-ethylbenziazoline-6-sulphonic acid) (ABTS) as a substrate [11]. Reactions were carried out with 500 µM ABTS in 50 mM Sodium acetate buffer at pH 4.5. A 20 µl aliquot of enzyme solution was added to 580 µl of the ABTS. Absorbance at 420 nm were measured and the enzyme activity was calculated using extinction coefficient (ε = 36, 000 M-1cm-1).

Aryl alcohol oxidase (AAO)

Aryl alcohol oxidase (AAO) activity was estimated using Veratryl alcohol as substrate [12]. The reaction mixture contained 5 mM veratryl alcohol in a 0.1 mM sodium phosphate buffer pH 6 and 50 µl enzyme solution. Reaction was performed at 30°C and visualized spectrophotometrically at 310 nm and the enzyme activity was calculated using extinction coefficient (ε = 9300 M-1cm-1).

Xylanase

The amount of xylanase produced was measured by using 1% birch wood xylan as the substrate [13]. Xylanase activity was assayed in 3.0 ml of a reaction mixture containing 0.1 ml of crude extracellular enzyme sample, 0.5 ml of 1% birch wood xylan (prepared in 0.05 M Na-citrate buffer, pH 5.3). The mixture was incubated at 55°C for 30 min. The reaction was stopped by the addition of 0.6 ml of 3, 5- dinitrosalicylic acid (DNSA) and 1.8 ml of distilled water was added to the tubes to make the reaction mixture 3 ml. The contents were boiled for 15 min [14]. After cooling, the color developed was read at 540 nm. The amount of reducing sugars liberated was quantified using xylose as standard. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmol of xylose in 1 min under assay conditions [15].

Cellulase

Cellulase (CMCase) activity was determined by mixing 0.5 ml of 1% (w/v) CMC (prepared in 0.05 M acetate buffer pH 5.3) with 0.1 ml of crude extracellular enzyme solution and incubating at 50°C for 30 min [16]. The reaction was stopped by the addition of 0.6 ml of 3, 5-dinitrosalicylic acid (DNSA) [15] and 1.8 ml of distilled water was added to the tubes to make the reaction mixture 3 ml. The contents were boiled for 15 min. The colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase is defined as the amount of enzyme that liberates 1 µmol of glucose equivalents per minute under the assay conditions [17].

RESULT AND DISCUSSION

In the present study evaluation and comparison of the lignocellulolytic enzymes such as Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), Laccase, Aryl alcohol oxidase (AAO), Xylanase and Cellulase in monocultures and co cultures of fungi at different incubation periods were carried out.

The optimum incubation period for maximum production of the enzyme was also observed. There was a variation in the enzyme activity of mono culture and co culture. Graphical representation of the enzymatic activity is shown in Fig 1.

In mono cultures TR, PYS and IL showed maximum activity of all the enzymes except xylanase on 20th, 25th and 30th day of incubation period respectively. Xylanase activity was found to be maximum on the 20th day of incubation period after which there was a decline in the activity.

In the co cultures of the fungal isolates all the enzymes were produced maximum on the 20th day except xylanase which showed a maximum production on 15th day by TR and PYS while co culture of TR and IL showed maximum xylanase activity on the 20th day. TR and IL showed absence of LiP and Cellulase activity in mono culture and in their co culture. LiP activity was produced in the range of 0.15 U/L to 1.82 U/L. Monoculture of PYS produced 1.74U/L LiP enzyme activity (Fig.1 A). MnP activity was produced in the range of 0.38 U/L to 1.51 U/L. Amongst the fungi tested in mono culture the lowest MnP activity was produced by TR (0.38U/L) followed by IL (0.69U/L) while PYS produced 1.46 U/L (Fig.1 B).

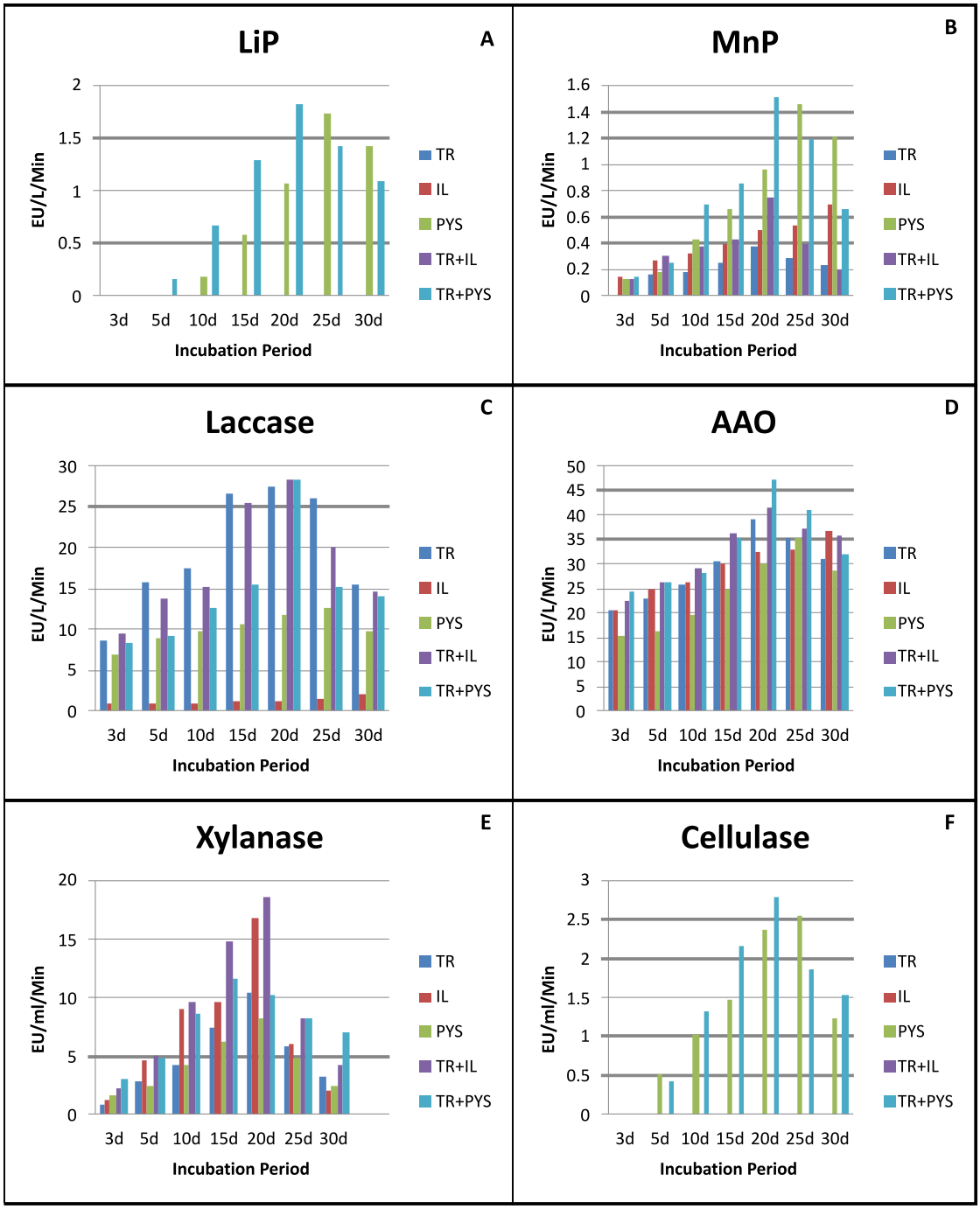

Fig. 1 Enzymes produced by monocultures and cocultures at different incubation period
TR- *Trichoderma reesei*, IL- *Irpex lacteus* PYS- *Pycnoporus sanguineus*

Highest Laccase activity was produced by TR (27.36 U/L) followed by PYS 12.65U/L and lowest Laccase activity was represented by the mono culture of IL which showed 1.94U/L (Fig.1 C). Maximum AAO activity amongst the monoculture was found in TR (38.86 U/L) followed by IL (36.95) and PYS (35.48U/L) (Fig.1 D). The maximum xylanase enzyme activity was found in IL (16.87U/ml) followed by TR (10.49 U/ml) and lowest in PYS (8.29U/ml) (Fig.1 E). Cellulase activity was found in monoculture of PYS was (2.56U/ml) (Fig.1 F).

Amongst the co cultures tested TR+PYS showed maximum LiP activity of 1.82U/L on the 20th day of incubation period after which a decline was observed. Co culture of TR+ IL produced 0.75 U/L and TR+PYS produced 1.51 U/L MnP activity. The highest Laccase activity was produced by co culture of TR+PYS (28.29U/L) followed by TR+IL (28.24 U/L). Co culture of TR+PYS showed maximum AAO activity 47.02U/L followed by TR+IL which showed 41.35 U/L. Maximum Xylanase activity was observed in TR+IL 18.72U/ml

which was followed by TR+PYS 11.61U/ml. Cellulase activity produced by TR+PYS was (2.78U/ml).

According to Hatakka [18] three groups of white rot fungi according to their enzymes systems are (i) the LiP-MnP group, (ii) MnP-laccase group (iii) LiP-laccase group, whereas Tuor et al., [19] suggested two more groups (iv) LiP MnP- Laccase group and (v) only laccase, no peroxidase group. According to these groups, TR and IL belongs to group II which produce MnP and Laccase group and not producing LiP enzyme, PYS belongs to group IV which produced all the three enzymes Lip, MnP and Laccase.

Both hydrolytic and oxidizing enzymes produced by fungi are excreted into the lignocellulosic materials [20] which depolymerise the lignocellulosic polymers into lower molecular weight compounds which can be assimilated by the fungus. In the production of laccase and manganese peroxidase, malt extract-based medium is attributed because the malt extract provides the complete pool of amino acids required for synthesis of enzyme [21].

Based on the different growth rate of fungus, increase in the biomass and enzyme activity was influenced by the period of incubation. Gradual increase in the fungal biomass and enzyme activity were noticed as the incubation period increases. Once enzyme activity reached maximum clear decline was obtained. It could be expected that an increase in the biomass in co cultures was mainly because T. reesei was able to grow fast when it was inoculated with Irpex lacteus and Pycnoporus sanguineus cultures, indicating that nutrients in co-cultures were nonlimiting for T. reesei growth. Growth of T. reesei was found to be more rapid compared to the other two white rot fungi IL and PYS. The mechanism used to enhance the enzymatic activity of white rot fungi during co-culture with Trichoderma spp. is not known. Baldrian [22] has suggested that the increased activity of laccase especially by coculture could be a response against Trichoderma reesei attack due to the synthesis of certain lytic enzymes in the mycoparasitism process. As per some authors it suggests that laccase production under Trichoderma attack is a defense response by white rot fungi. The explanation for PYS produced highest enzyme activities in monoculture on 25th day but in coculture on 20th day because of synergistic effect of both the fungi to work together in the form of complex and competition for the nutrient consumption from the media leads to oxidative stress which results in the higher enzyme production in the coculture within shorter incubation period compared to mono culture.

Baldrian [22] reported significant increase in the activity of laccase in the co-culture of Trichoderma harzianum and Pleurotus ostreatus on 12th day of incubation time as compared to 14th day in mono culture of P. ostreatus. Differences in laccase production between cultures inoculated with harzianum could not be explained as both the fungal isolates produced laccase enzyme. Result of the present study indicated the fact that in the co culture the activity of all the enzymes are more and required less incubation period for the maximum production of enzymes compared to its mono culture.

Dhillon et al. [23] studied cellulase and xylanase production by using different agro industrial wastes in co culture of Aspergillus niger and Trichoderma reesei. The result of the enzyme activity was highest when wheat bran was used which appeared to be the appropriate substrate for the cellulase enzyme activity. A. niger produced 48.22 IU/gds and T. reesei produced 68.57IU/gds while co culture of T .reesei and A. niger showed 117.11 IU/gds which was higher than that of mono culture. In the present study T. reesei and I. lacteus did not show cellulase enzyme activity while P. sanguineus produced 2.56U/ml activity. Co culture of T.reesei and P. sanguineus showed 2.78U/ml cellulase activity which clearly indicated that the production of cellulase was more in co culture than that of the mono culture.

Xylanase produced by T. reesei, I. lacteus and P. sanguineus was 10.49 U/ml, 16.87 U/ml 8.29 U/ml respectively and the co cultures of T .reesei and I. lacteus and T .reesei and P. sanguineus showed 18.72 U/ml and 11.61 U/ml respectively. This result was found to be in accordance with [23] in which xylanase produced by T. reesei was 2467.53IU/gds and A. niger was 2604.06 IU/gds while co culture of T. reesei and A. niger showed 2710.62IU/gds clearly depicting that the xylanase production was more in the co culture as compared to its mono culture.

Haq et al. [24] also reported that xylanase produced by T. viride was 68.6U/ml and A. niger produced 154.56 U/ml while their co culture produced 189.7 U/ml Xylanase which also indicated that the production of xylanase enzyme was higher in mixed cultures of fungi. Similar results were obtained in the present study where both the co cultures T. reesei with I. lacteus and T.reesei with P.sanguineus showed higher xylanase production than all the three individual mono cultures. Present study leads us to conclude that the co culture of two compatible fungal isolates proved to be an excellent source for the production of lignocellulosic enzymes, because mixed cultures have the capacity to utilize the nutrition media more efficiently as the source of energy than that of the mono culture or pure culture.

REFERENCES

[1] Pawar, S., Mathur, R.M., Kulkarni, A.G. (2002) Control of AOX discharges in pulp and paper industry-the role of new fiberline. Ippta Convention Issue 57.

[2] Ragauskas, A.J., Poll, K.M., Cesternino, A.J. (1994) Effects of xylanase pretreatment procedures on non chlorine bleaching. *Enz MicrobTech,* **16:** 492-495.

[3] Srinivasan, M. C., Rele, M.V. (1999) Microbial xylanases for paper industry. *Curr Sci,* **77:**137-142.

[4] Roncero, M.B., Torres, A.L., Colom, J.F., Vidal, T. (2000) Effects of xylanase treatment on fiber morphology in total chlorine free bleaching (TCF) of *Eucalyptus* pulp. *Process Biochem,* **36:** 45-50.

[5] Techapun, C., Poosaran, N., Watanabe, M. and Sasaki, K. (2003) Thermostable and alkaline-tolerant microbial cellulase-free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocesses: A review. *Process Biochem,* **38:** 1327-1340.

[6] Harman, G.E. (2006) Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology,* **96:** 190–194.

[7] Akhtar, M., Blanchette, R., Myers, G., Kirk, T.K. (1998) 'An overview of biomechanical pulping research'. In: Young, R.A. and Akhtar, M. (ed.) *Environmentally friendly technologies for the pulp and paper industry.* John Wiley and Sons, New York, USA, pp. 309-340.

[8] Elisashvili, V., Parlar, H., Kachlishvili, E., Chichua, D., Kvesitadze, G. (2001) Ligninolytic activity of basidiomycetes grown under submerged and solid-state fermentation on plant raw material (sawdust of grapevine cuttings). *Advances in Food Science,* **23:** 117–123.

[9] Pandya, B. and Albert, S. (2014) Evaluation of *Trichoderma reesei* as a compatible partner with some white rot fungi for potential bio-bleaching in paper industry. *Annals of Biological Research,* **5(4):** 43-51.

[10] Castillo, M.P., StenstrÖm, J. and Ander, P. (1994) Determination of manganese peroxidase activity with 3-methyl-2-benzothiazolione hydrazone and 3-(dimethylamino) benzoic acid. *Anal biochem,* **28:** 399-404.

[11] Niku-paavola, M.L., Karhunen, E., Salola, P. and Raunio, V. (1988) Ligninolytic enzymes of the white rot fungus *Phlebia radiate. Biochem J,* **254:** 877-884.

[12] Guillen, F., Martinez, A.T. and Martinez, M.J. (1990) Production of hydrogen peroxide by aryl alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii. Appl Microbiol Biotechnol,* **32:** 465-469.

[13] Bailey, M.J., Biely, P., Poutanen, K. (1992) Interlaboratory testing of methods for assay of xylanase activity. *J Biotechnol,* **23:** 257–270.

[14] Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for the determination of reducing sugars. *J Anal Chem,* **31:** 426-428.

[15] Khan, A.W., Tremblay, D., LeDuy, A. (1986) Assay of xylanase and xylosidase activities in bacterial and fungal cultures. *Enz Microb Tech,* **8(6):** 373-377.

[16] Casimir, S.J., Davis, S., Fiechter, A., Gysin, B., Murray, E., Perrolaz, J.J., Zimmermann, W.S. (1996) Pulp bleaching with thermostable xylanase of *Thermomonospora fusca.* US Patent.

[17] Mandels, M., Medeiros, J.E., Andreotti, R.E., Bissett, F.H. (1981). Enzymatic hydrolysis of cellulose: Evaluation of cellulase culture filtrates under use conditions. *Biotechnol and Bioeng,* **23(9):** 2009-2026.

[18] Hataka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol Rev,* **13:** 125-135.

[19] Tour, U., Winterhalter, K., Fiechter, A. (1995) Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay. *J Biotechnol,* **41:** 1–17

[20] Wood, T. M. (1985) Properties of cellulolytic enzyme systems. *Biochem Soc Trans,* **13:** 407-410.

[21] Arora, D.S., Sandhu, D.K. (1985) Laccase production and wood degradation by a white rot fungus *Daedalea flavida. Enz MicrobTech,* **7:** 405–408.

[22] Baldrian, P. (2004) Increase of laccase activity during interspecific interactions of White rot fungi. *FEMS Microbiol Ecol,* **50:** 245–253.

[23] Dhillon, G.S., Oberoi, H.S., Kaur, S., Bansal, S., Brar, S.K. (2011) Value-addition of agricultural wastes for augmented cellulase and xylanase production through solid state tray fermentation employing mixed-culture of fungi. *Ind CropsProd,* **34:** 1160–1167.

[24] Haq, I.L., Javed, M.M., Khan, T.S. (2006) An innovative approach for hyperproduction of cellulolytic and hemicellulolytic enzymes by consortium of *Aspergillus niger* MSK-7 and *Trichoderma viride* MSK-10. *African j Biotechnol,* **5 (8):** 609–614.