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Enhancement of Ligninolytic & Xylanolytic Enzyme Activities in *Trichoderma reesei* co-Cultured with two White Rot Fungi

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Abstract

A wide range of enzyme applications have been identified in pulp and paper industry, especially xylanase & laccase which is used in pre-bleaching of pulp. The use of biological agent is becoming an increasingly important alternative to chemical agent in pulping and bleaching process of paper industry. *Trichoderma reesei* produce alkaline xylanase, which is an enzyme very important for brightening of paper in the pulp and paper industry. White rot basidiomycetes fungi secrete extracellular enzyme laccase which plays an important role in the delignification & brightening of pulp and also in the removal of lipophilic extractives responsible for pitch deposition from both wood & non- wood paper pulp. Experimental evidence suggests that co-culture of ligninolytic and filamentous micro fungi results in increased enzyme activities. In the present study co-culture of *Trichoderma reesei* with two white-rot fungi *Pleurotus sajorkaju* and *Phanerochaete chrysosporium* has been compared with monocultures for enzyme activities. Results revealed that co-cultures of *T. reesei* with *P. sajorkaju* and *P. chrysosporium* showed an increase in the activities with a reduction in the incubation period. *T. reesei* with *P. sajorkaju* proved to be a better co-culture for all the ligninolytic enzyme activities but *T. reesei* with *P. chrysosporium* showed significantly higher activity than *T. reesei* and *P. sajorkaju*. Thus using this co-culture technique in paper and pulp industries can enhance the enzyme activity.

Keywords: Bio pulping, fungal degradation, enzymatic treatment, *Trichoderma reesei,* White rot fungi

INTRODUCTION

Enzymatic treatment in the pulp and paper industry has been an increasingly interesting

topic during the current years. Decay fungi play a major role in the process of decomposition since, alone among microorganisms, they have evolved the means to decompose large volumes of wood completely [1].

Decay fungi produce three major types of enzymes such as Ligninolytic, Xylanolytic and Pectinolytic. Ligninolytic enzymes are involved in the degradation of the complex and recalcitrant polymer lignin. The white rot fungi produce an array of extracellular oxidative enzymes that synergistically and efficiently degrade lignin. The major groups of ligninolytic enzymesinclude lignin peroxidases (LiP), manganese peroxidase (MnP), versatile peroxidase and laccases. Lignin peroxidases (LiP) have the unique ability to catalyze oxidative cleavage of C-C bonds and ether bonds in non-phenolic anomatic substrates of high redox potential [2]. Manganese peroxidase (MnP) oxidize MnI to MnII, which facilitates the degradation of phenolic compound or inturn, oxidizes a second mediator for the breakdown of non-phenolic compounds. MnP is glycoprotein dependent on H_2O_2 that requires Mn^{2+} to oxidize monoaromatic phenols and aromatic dyes. Xylanolytic enzymes catalyze the hydrolysis of xylan, the major constituent of hemicelluloses. Which is the second abundant molecule in plant cell. Xylan is a complex process that requires the coordination of several xylanolytic enzymes which hydrolyze xylan and arabinoxylan polymers. The enzyme group includes endo-1,4-xylanase which attack the main chain of xylans and D-xylose [3]. Endo-1,4-β-xylanase and xylan 1,4-β-xylosidase are mainly responsible for the hydrolysis of β-1,4-xylans. Phanerochaete chrysosporium is considered to be the model.

Ascomycetes filamentous fungi, *Trichoderma reesei* has the capacity to secrete large amounts of xylanolytic enzymes [4], which is an enzyme very important for brightening of paper in the pulp and paper industry [5]. T. reesei could produce alkaline xylanase which could be used to reduce the use of bleaching chemicals for pretending process and degradation of hemicellulose so as to break the linkage of lignin- carbohydrate complexes [6].

White rot fungus because of its specialized ability to degrade the abundant aromatic polymer lignin leaving white cellulose untouched. White rot fungi *pleurotus sajorkaju* is also known to be adaptable and to utilize many lignocellulase residue for extensive growth & fruit body formation. The co-cultivation of fungi has recently been described as a promising strategy to induce the production of novel metabolites through possible gene activation. When compared to monocultures, co-cultures of fungi may lead to better substrate utilization, increased productivity, increased adaptability to changing conditions and increased resistance to contamination by unwanted microbes. Nutritional limitations may be overcome in synergetic interactions between compatible partners.

Previous compatible studies conducted by paired interaction in the test laboratory revealed *T. reesei* to be compatible with *P. sajorkaju* & *P. chrysosporium* and could be

experimented for co-culturing for enhanced production of enzyme activity [5]. In the present study two major enzymes activity are analyzed**;** i.e. Ligninolytic enzymes (Lip, MnO, Lacc, AAO) andHydrolytic enzymes (Cellulase, Xylanase) in *T. reesei* and its co-culture with *P. sajorkaju* & *P. chrysosporium.*

MATERIALS AND METHODS

Source of Fungal isolates

The funagal isolates of *Trichoderma reesei* (TR) and *Phanerochaete chrysosporium* (PC) were procured from Microbial type culture collection (MTTC), Chandigadh. *Pleurotus sajorkaju* was procured from Forest Research Institute, Dehradun and then maintained on Potato Dextrose Agar (PDA) at 4(±1)□C in Seed Anatomy laboratory of Department of Botany at The Maharaja Sayajirao University of Baroda, Gujarat, India.

Fungi and Maintenance

All the fungal cultures were sub-cultured from the provided slant on the PDA Medium. Potato dextrose agar (PDA) medium was prepared by taking 10 gm Dextrose, 10 gm Agar, 500 ml distilled water and 100 gm potato and autoclaved at 121 □ C. All the culture isolates were maintained on PDA slants and plates at 4 □ ± C. For the further experiments the cultures of PDA plates were used.

Before using the culture compatibility of the fungi were confirmed by paired interaction method. The compatibility of two test fungi was analyzed by observing the interaction between the two test fungi at the zone of contact as per Porter (1924) [7].

Preparation of media

The Malt extract broth (MEB) medium was prepared by taking 100 ml distilled water and 3% malt extract powder in each culture bottle and then was autoclaved at 121 □ C for 20 min. For co-cultivation of fungi in culture bottles, two discs of 9mm in diameter were cut by using a sterile cork borer from PDA culture plate containing fungal lawn. Mycelial discs were removed from the margins of actively growing 10-days-old cultures and added into the culture bottles containing 100 ml of MEB medium. In monoculture single disc of 9mm in diameter was inoculated in culture bottles. Culture bottles were incubated at 25 ± 1□ C for the desired incubation period. The culture bottles were taken out and then filtered with whatman paper No.1.

Preparation of Enzyme

The MEB medium was prepared by taking 100 ml distilled water and 3% malt extract

powder in each culture bottle and then was autoclaved at 121 □ C for 20 min. For co-cultivation of fungi in culture bottles, two discs of 9mm in diameter were cut by using a sterile cork borer from PDA culture plate containing fungal lawn. Mycelial discs were removed from the margins of actively growing 10-days-old cultures and added into the culture bottles containing 100 ml of MEB medium. In monoculture single disc of 9mm in diameter was inoculated in culture bottles. Culture bottles were incubated at 25 ± 1□ C for the desired incubation period. The culture bottles were taken out and then filtered with whatman paper No.1.

Enzyme Assays

All the enzyme activity was assayed from the culture filtrate. **Lignin peroxidase** activity was determined by using 3-methyl-2-benzothiazolinone hydrozone (MBTH), 3-dimethylamino benzoic acid (DMAB), culture filtrate and H_2O_2. The reaction was initiated by addition of H_2O_2. Absorbance was recorded at 590 nm in spectrophotometer [8].

Managanese peroxidase activity was determined by reaction mixture contained 3-methyl-2-benzothiazolinone hydrozone (MBTH), 3-dimethylamino benzolic acid (DMAB), $MnSo_4$, culture filtrate and H_2O_2. The reaction was initiated by addition of H_2O_2. Absorbance was recorded at 590 nm [9].

Laccase activity was determined by using ABTS [2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] as a substrate. It was measured spectrophotometrically by monitoring the oxidation products of ABTS [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)], buffered with sodium tartrate at 418 nm [10].

Aryl alcohol oxidase activity was determined by using Vertryl alcohol (VA) as a substrate. The mixture containing Vertryl alcohol (VA) and culture filtrate was read at 310 nm in spectrophotometer [11].

Xylanase activity was assayed in a reaction mixture containing culture filtrate, 1 % beech wood xylan and citrate buffer. The mixture was incubated at 55□ C for 30 min. The reaction was stopped by the addition of 3, 5-dinitrosalicyclic acid (DNS) and the contents were boiled for 5 min. After cooling, the color developed was read at 540 nm [3].

Cellulase activity was determined by mixing 1% Carboxymethyl cellulose (CMC) (prepared in Na-acetate buffer) with culture filtrate and incubating at 55 □ C for 30 min. The reaction was stopped by the addition of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 5 min. The color developed was read at 540 nm [3].

RESULTS AND DISCUSSION

White rot fungi can offer a biological and environmentally friendly means of reducing environmental pollution by ligninolytic enzymes such as laccase, manganese peroxidase and lignin peroxidase which degrade a wide range of environmental pollutants. Most of the fungal enzymes in industrial biotechnology today are produced by process involving single microbial strains. However constitutive extracellular enzymes from white rot fungi are produced in small amounts, so their use in industrial applications has been limited due to low productivity and high economic cost. Chemical induction of laccase and MnP by the addition of compounds related to lignin derivatives, phenolic and aromatic compounds, copper, ethanol and contaminated water has been studied by many authors. However inducers are expensive and in some cases toxic. Therefore the search for economical and safe laccase & MnP production methods has been one of the main enzyme research topics in the past few decades.

The aim of present study was to check compatibility of *T. reesei* (TR) with *Phanerochaete chrysosporium* (PC) and *Pleurotus sajorkaju* (PS) then to study ligninolytic and hydrolytic enzyme activity of their monocultures and co-cultures at room temperature.

Paired interaction test of TR with PC and PS showed mutual intermingling type of interaction which indicates that both the fungal isolates are compatible with TR as shown in Fig.1.

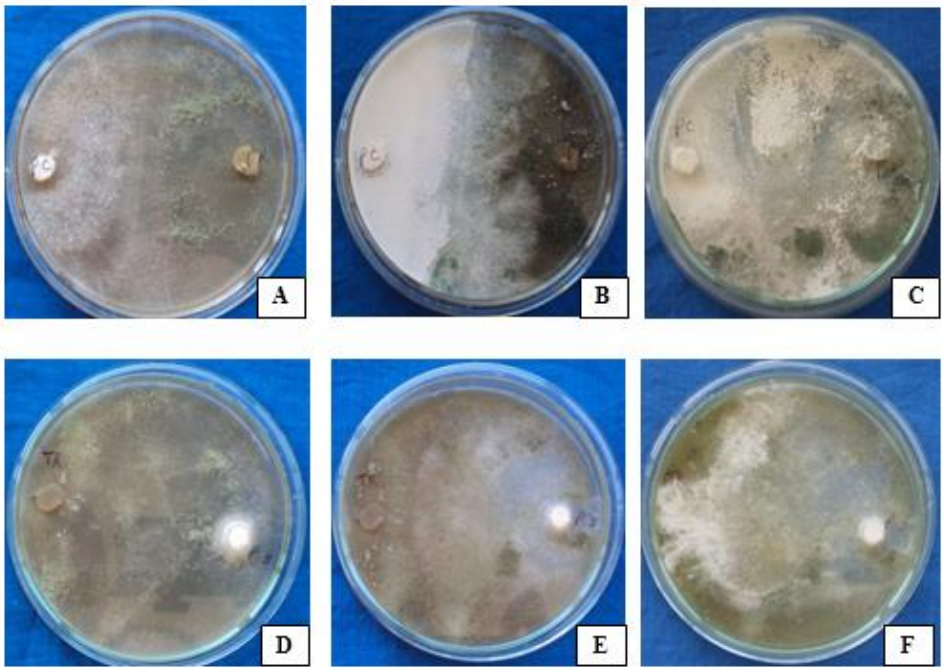


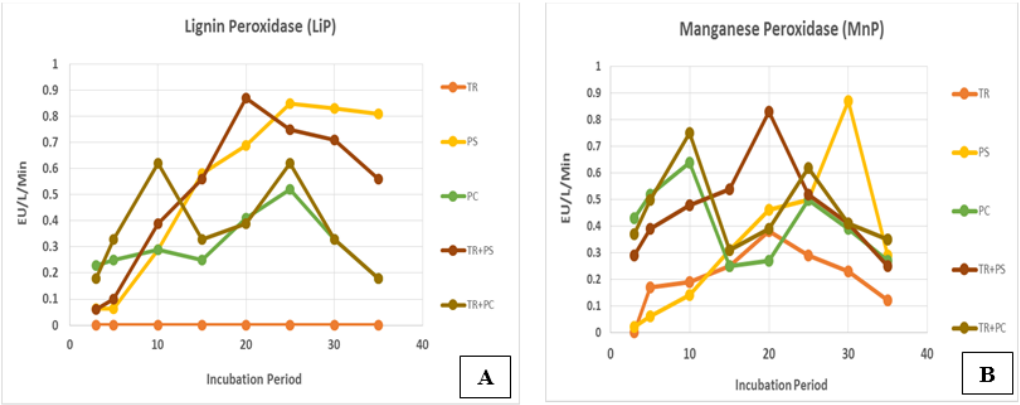
Figure 1: Results of paired interaction test

A, B, C: 3rd, 6th and 9th day incubated co-culture of T. reesei with P. chrysosporium

D, E, F: 3rd, 6th and 9th day incubated co-culture of T. reesei with P. sajorkaju

Using co-cultures appears to become successful than using single microorganism cultures, because of the potential to utilize synergisms between the metabolic pathways of the strains involved in the co-culture. For any co-culture experiments to be successful, it indicates that the two strains of the microorganism in the co-culture has to be compatible.

Results of enzyme activities showed that it was affected by the number of days of inoculation. Monoculture of *T. reesei* (TR) showed an absence of LiP activity, while *P. sajorkaju* (PS) and *P. chrysosporium* (PC) showed maximum activity on the 25th day (PS-0.85 EU/L/Min., PC-0.52 EU/L/Min.) of incubation period and thereafter a decline in the activity. PS showed activity more than PC. Co-cultures TR+PS showed maximum activity on the 20th day (0.87 EU/L/Min.) while in TR+PC the activity was maximum on the 25th day (0.62 EU/L/Min.) of incubation. The activity was slightly increased compared to the monoculture of PC as shown in Table.2 & Fig.2(A). Significant increase in peroxidase activity over the respective monocultures was observed in the combination *Pleurotus flabellatus* and *Chaetosphaeridium globosum* [11].



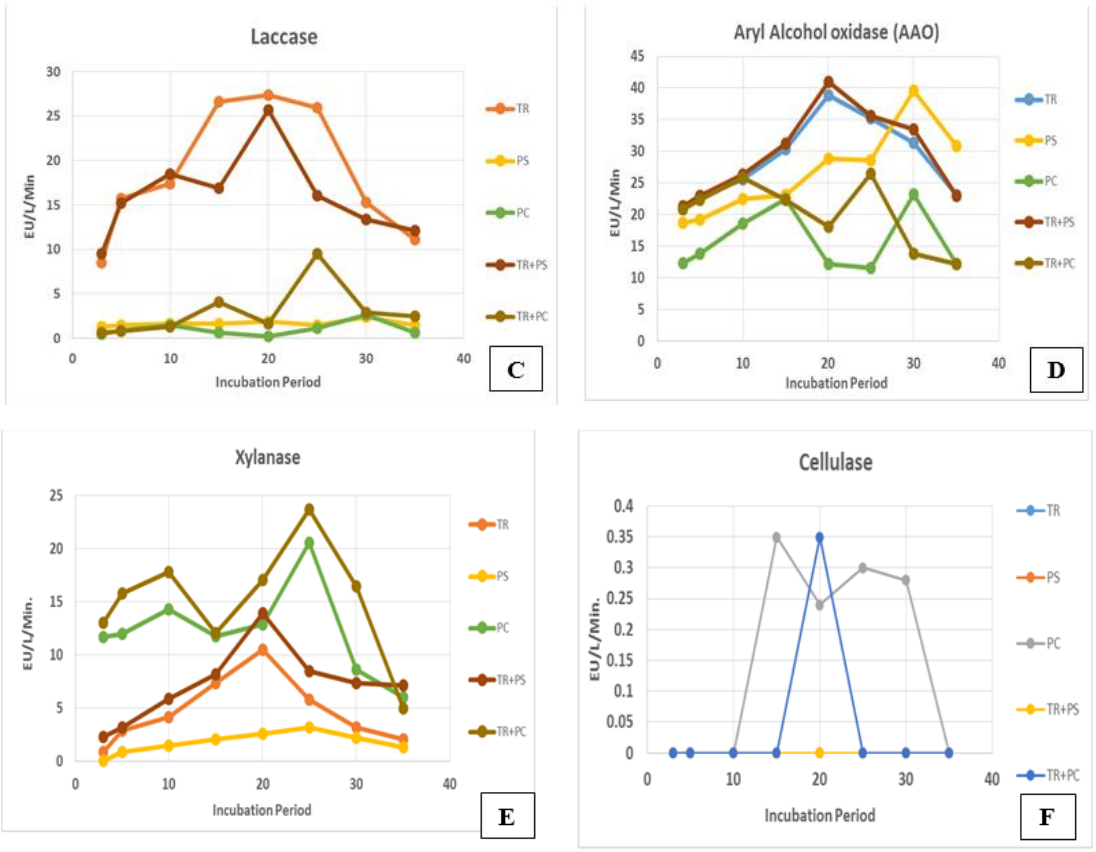


Figure 2: Different enzyme activities of monoculture and co-cultures at different incubation periods

A: Lignin peroxidase , B: Manganese peroxidase, C: Laccase , D: Aryl Alcohol oxidase (AAO), E: Xylanase, F: Cellulase

With regard to MnP activity monocultures TR, PS and PC showed maximum activity on the 20th, 30th and 10th day respectively. Monocultures of PS showed the maximum activity (0.87 ± 0.06 EU/L/Min.) which was detected on the 30th day of incubation while in PC it was maximum on the 10th day of incubation (0.64 ± 0.09 EU/L/Min.). MnP activity was found to be higher in co-cultures of TR+PS detected to be maximum on the 20th day (0.83 ± 0.09 EU/L/Min.) of incubation a period of 10 days ahead of the activity detected in the PS monocultures as shown in Table.1. & Fig.2 (B).

Lakovlve and stenlid (2000) reported that laccase activity increased in interspecific interactions of two white rot fungi or a white rot fungus and a biocontrol species *Trichoderma* [12]. Here laccase activity was observed in monocultures of TR which was detected on 20th day (27.36 ± 0.31 EU/L/Min.). While in monocultures of PS and PC highest activity was observed later on 30th day (PS- 2.4 ±0.55 EU/L/Min., 2.6 ± 0.29 EU/L/Min.) of incubation period. Laccase activity was found highest at an earlier

period of incubation in both the combinations. Co-cultures of TR+PS (26.89 ± 0.38 EU/L/Min.) showed increased enzyme activity as compared to monocultures of PS which was observed on 15th day of incubation period. While in co-culture of TR+PC laccase activity was lesser than co-culture of TR+PS and it was highest on 25th day (9.5 ± 0.43 EU/L/Min) of incubation period as shown in Table.1 & Fig.2 (C).

Aryl alcohol oxidase (AAO) enzyme activity was found maximum in monocultures of PS as compared to monocultures of TR and PC. AAO activity was increasing till the 20th, 30th and 15th day of incubation period than it started decreasing gradually in monocultures of TR (38.86 ± 0.11 EU/L/Min.), PS (39.56 ± 0.75 EU/L/Min.) and PC (22.39 ± 0.81 EU/L/ Min.) respectively. Co-cultures of TR+PS showed maximum AAO activity than co-cultures of TR+PC. Highest AAO activity was found on 20th day (41.04 ± 0.91 EU/L/Min.) of incubation period as shown in Table.1 & Fig.2 (D).

In Deshpande *et al.* (2008) study xylanase production was improved 2-3 fold in the co-culture of *T. reesei* and *A. niger* than their respective monocultures [13]. Here in this study also xylanse activity has increased in both the co-cultures than their monocultures as shown in Table.1 & Fig.2 (E). Monocultures of PS showed maximum xylanase activity than monocultures of TR and PC. The xylanase activity boosted on 20th day in monocultures of TR (10.49 ± 0.37 EU/L/Min.) and it was highest on 25th day in PS (3.21 ± 0.23 EU/L/Min.) and PC (20.59 ± 0.29 EU/L/Min.) monocultures. Co-cultures of TR+PC showed maximum as compared to co-culture of TR+PS. In co-cultures of TR+PS (23.71 ± 0.17 EU/L/Min.) maximum activity was found on 20th day which was earlier than co-cultures of TR+PC, as it was maximum on 25th day (13.91 ± 0.17 EU/L/Min.).

Ahamed and Vermette (2008) reported that co-cultivation of *T. reesei* and *Aspergillus niger* can enhance the production of cellulase enzyme. Marcel *et al*., 1999 study showed the 63% increment in cellulase enzyme production in the co-cultivation o f *T. reesei* and *A. niger* than their pure cultures [14]. Here in this study, cellulase activity has been found in monocultures of PC and its co-cultures with TR, but it is very negligible which can be considered as zero enzyme activity as shown in Table.1. & Fig.2 (F).

Table 1: Enzyme activities of monocultures and co-cultures at different incubation period

Days		3rd	5th	10th	15th	20th	25th	30th	35th
LiP	TR	0	0	0	0	0	0	0	0
	PS	0.06 ± 0.11	0.06 ± 0.06	0.29 ± 0.13	0.59 ± 0.1	0.69 ± 0.17	**0.86 ± 0.16**	0.84 ± 0.1	0.82 ± 0.19
	PC	0.23 ± 0.1	0.25 ± 0.06	0.29 ± 0.04	0.25 ± 0.06	0.42 ± 0.13	**0.52 ± 0.1**	0.33 ± 0.1	0.19 ± 0.06
	TR+PS	0.06 ± 0.06	0.1 ± 0.09	0.39 ± 0.09	0.56 ± 0.12	**0.87 ± 0.06**	0.75 ± 0.12	0.71 ± 0.09	0.56 ± 0.06
	TR+PC	0.18 ± 0.06	0.33 ± 0.09	0.62 ± 0.06	0.33 ± 0.09	0.39 ± 0.09	**0.62 ± 0.06**	0.33 ± 0.03	0.18 ± 0.6
MnP	TR	0	0.17 ± 0.04	0.19 ± 0.06	0.25 ± 0.06	**0.38 ± 0.06**	0.29 ± 0.09	0.23 ± 0.03	0.12 ± 0.03
	PS	0.02 ± 0.03	0.06 ± 0.06	0.14 ± 0.09	0.31 ± 0.06	0.46 ± 0.09	0.5 ± 0.06	**0.87 ± 0.06**	0.29 ± 0.03
	PC	0.44 ± 0.06	0.52 ± 0.09	**0.64 ± 0.09**	0.25 ± 0.06	0.27 ± 0.03	0.5 ± 0.06	0.39 ± 0.09	0.27 ± 0.09
	TR+PS	0.29 ± 0.09	0.39 ± 0.09	0.48 ± 0.07	0.54 ± 0.07	**0.83 ± 0.09**	0.52 ± 0.13	0.41 ± 0.03	0.25 ± 0.06
	TR+PC	0.37 ± 0.06	0.5 ± 0.06	**0.75 ± 0.12**	0.31 ± 0.06	0.39 ± 0.03	0.62 ± 0.06	0.41 ± 0.03	0.35 ± 0.03
Laccase	TR	8.54 ± 0.55	15.71 ± 0.47	17.43 ± 0.33	26.57 ± 0.36	**27.36 ± 0.31**	25.94 ± 0.24	15.33 ± 0.21	11.11 ± 0.46
	PS	1.3 ± 0.35	1.4 ± 0.27	1.6 ± 0.33	1.6 ± 0.36	1.89 ± 0.46	1.49 ± 0.19	**2.40 ± 0.55**	1.4 ± 0.28
	PC	0.44 ± 0.14	0.88 ± 0.24	1.46 ± 0.22	0.60 ± 0.23	0.23 ± 0.03	1.14 ± 0.28	**2.60 ± 0.29**	0.63 ± 0.30
	TR+PS	9.48 ± 0.93	15.21 ± 0.53	18.44 ± 0.6	**26.89 ± 0.38**	25.67 ±0.04	16.02 ± 0.86	13.36 ± 0.66	12.14 ± 0.71
	TR+PC	0.64 ± 0.06	0.76 ± 0.05	1.3 ± 0.05	4 ± 0.17	1.6 ± 0.07	**9.5 ± 0.43**	2.9 ± 0.18	2.43 ± 0.19
AAO	TR	20.76 ± 0.51	22.75 ± 0.21	25.53 ± 0.26	30.40 ± 0.16	**38.86 ± 0.11**	35.29 ± 0.19	31.29 ± 0.13	23.07 ± 0.75
	PS	18.65 ± 0.71	19.13 ± 0.3	22.43 ± 0.44	23.02 ± 0.59	28.82 ± 0.5	28.61 ± 0.1	**39.56 ± 0.75**	30.86 ± 0.66
	PC	12.37 ± 0.78	13.83 ± 0.8	18.59 ± 0.65	22.39 ± 0.48	12.2 ± 0.71	11.57 ± 0.74	**23.16 ± 0.81**	12.19 ± 0.71
	TR+PS	21.27 ± 0.3	22.91 ± 0.03	26.35 ± 0.71	31.16 ± 0.43	41.04 ± 0.91	**35.61 ± 0.29**	33.46 ± 0.37	22.98 ± 0.75
	TR+PC	20.83 ± 0.72	22.32 ± 0.86	25.86 ± 0.8	22.35 ± 0.75	18.1 ± 0.78	**26.47 ± 0.64**	13.84 ± 0.81	12.23 ± 0.72
Xylanase	TR	0.88 ± 0.38	2.88 ± 0.49	4.18 ± 0.77	7.39 ± 0.96	**10.49 ± 0.37**	5.81 ± 0.43	3.23 ± 0.46	2.11 ± 0.46
	PS	0.09 ± 0.03	0.92 ± 0.17	1.49 ± 0.23	2.09 ± 0.23	2.57 ± 0.19	**3.21 ± 0.23**	2.2 ± 0.26	1.36 ± 0.23
	PC	11.68 ± 0.12	11.96 ± 0.23	14.30 ± 0.12	11.79 ± 0.13	12.87 ± 0.18	**20.59 ± 0.29**	8.63 ± 0.20	6.04 ± 0.15
	TR+PS	2.33 ± 0.10	3.20 ± 0.21	5.87 ± 0.25	8.15 ± 0.19	**13.91 ± 0.17**	8.46 ± 0.20	7.36 ± 0.26	7.14 ± 0.27
	TR+PC	13 ± 0.16	15.76 ± 0.11	17.79 ± 0.14	12.03 ± 0.26	17.05 ± 0.22	**23.71 ± 0.17**	16.48 ± 0.34	4.99 ± 0.29
Cellulases	TR	0	0	0	0	0	0	0	0
	PS	0	0	0	0	0	0	0	0
	PC	0	0	0	0.35 ± 0.02	0.24 ± 0.03	0.3 ± 0.02	0.28 ± 0.04	0
	TR+PS	0	0	0	0	0	0	0	0
	TR+PC	0	0	0	0.35 ± 0.02	0	0	0	0

To conclude all the enzyme activities were found to be maximum in the 20th day of incubation period in TR monocultures, while in PS and PC it was found to be maximum on the 30th day of incubation followed by decline in the activities. Co-cultures of TR with PS and PC showed an increase in the activities with a reduction in the incubation period. TR with PS proved to be a better co-culture for all the ligninolytic enzyme activities but TR with PC showed significantly higher activity than TR and PS.

The present study demonstrates that the enzyme activities can be induced by the presence of PS and PC with TR and the optimal requirements for the enzyme induction in a co-culture system may be different from those required for a monoculture system.

CONCLUSION

Using of co-culture technique is preferable over single microorganism culture as it enhances the enzyme activity significantly indicating its potentiality to be used as biotechnological tool for industries like pulp and paper industries. The mechanism used to enhance the enzymatic activity of white rot fungi during co-culture with *Trichoderma* spp. is not known. Some authors have suggested that the increased activity of laccase especially by TR+PS could be a response against *Trichoderma reesei* attack due to the synthesis of certain lytic enzymes in the mycoparasitism process. This occurred during antagonism with white rot fungi strains and is mainly associated with host cell degradation. As per some authors it suggest that laccase production under *Trichoderma* attack is a defense response by white rot fungi.

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