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IN VITRO ACTIVITY OF CELLULOLYTIC ENZYMES *VIZ.*, EXOCELLULASE (C_1), ENDOCELLULASE AND LACCASE (C_X) BY VARIOUS MULTISPORE ISOLATES OF *PLEUROTUS* SPP.

P. Renganathan, T. Sivakumar, R. Kannan, K. Sanjeevkumar and K. R. Saravanan
Department of Plant Pathology, Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University, Annamalai Nagar 608002, Tamilnadu, India
E-mail : rengaabishek@yahoo.com

Abstract

Mushrooms have achieved significant importance in many countries due to their high nutritive and genuine medicinal values as well as an income generative venture. Blessed with varied agro-climates, Indian weather is aptly suitable for the cultivation of edible mushrooms. The *in vitro* activity of exocellulase increased with increase in days and was the maximum at 20 days after incubation. All the multispore isolates showed increased activity of exocellulase when compared to the standard parent. The activity of exocellulase (C_1) was maximum in *Pe x Po* after 20 days (0.66 mg) of incubation and it was on par with *Pc x Pe* (0.64 mg) and *Pc x Pfl* (0.62 mg). It was followed by *Pf x Po* and *P. eous* (0.60 mg). The activity of exocellulase (C_1) increased up to 20 days and there after decreased in all the isolates. Among the isolates *Pe x Po* recorded the maximum activity of the exocellulase (C_1) (0.71 mg) in paddy straw followed by the isolate *Pc x Pe* in paddy straw (0.69 mg) at 20 days after incubation. The maximum activity of endocellulase (C_x) was recorded at 20 days after inoculation in all the isolates. Among the isolates *Pe x Po* recorded the maximum endocellulase (C_x) activity (1.54 mg) in paddy straw followed by the isolate *Pc x Pe* in paddy straw (1.53 mg) at 20 days after incubation. The activity of laccase increased only up to 10 days and there after decreased in all the isolates. With regard to the substrates a similar trend as that of endocellulase (C_x) was observed in the activity of laccase too. Among the isolates, *Pe x Po* recorded the maximum activity of the laccase (0.255) in paddy straw followed by the isolate, *Pc x Pe* in paddy straw (0.251) at 10 days after incubation.

Keywords : *Pleurotus spp,* Cellulolytic Enzymes, Endocellulase and Exocellulase.

Introduction

Mushrooms have achieved significant importance in many countries due to their high nutritive and genuine medicinal values as well as an income generative venture. Blessed with varied agro-climates, Indian weather is aptly suitable for the cultivation of edible mushrooms. The entire coastal belts of India running in to thousands of kilometers is a potent place to produce low cost speciality mushrooms which could supplement the protein deficiency and malnutrition, besides bringing in a sky – rocketing export market of a kind which is incomparable to any single cell protein (SCP) product (Kohlii, 2000).

It is estimated that about 355 million tonnes of crop residue is generated annually and about 170 million is left out posing problems for disposal (Tewari and Pandey, 2002). Even if one per cent of this agricultural waste is used to produce mushrooms, India will soon become a major mushroom producing country in the world. Mushroom production is the only biotechnological means available to convert these agricultural wastes into highly valuable edible proteins. So far around 5658 species of mushroom in 230 genera have been recorded from all over the world; where as from India 850 species spread over 115 genera have been reported. Of this 850 species about 20 are being commercially cultivated (Saini and Atri, 1995).

Among these, the white button mushroom (*Agaricus bisporus*), oyster mushroom (*Pleurotus* spp.), paddy straw mushroom (*Volvariella volvacea*) and milky mushroom (*Calocybe indica*) are popular among the commercial growers in India as the techniques for their cultivation have been well developed (Vijaya Khader *et al.,* 1998). World mushroom production at present is estimated to be around 5 million tonnes/annum and is increasing @ 7 per cent/annum. The total mushroom production in India has increased from 4000 tonnes in 1955 to 30,000 tonnes in 1995 and it is estimated to be around 50,000 tonnes / annum (Tewari, 2004).

Agaricus bisporus is highly temperature specific, and its cultivation is restricted to temperate regions. But oyster mushrooms can be cultivated easily in tropical and subtropical regions. Hence, it is rightly named as "the crop of the future". *Pleurotus* spp. has the ability to degrade most of the lignocellulosic agro wastes, thus the cultivation of this mushroom is an efficient means for the conversion of agricultural wastes in to valuable edible proteins (Deepika Sud and Sharma, 2005).

The farmers and consumers have also developed preference towards *Pleurotus* spp. in recent years because of its advantages *viz.,* high nutritive value and easiness in cultivation using the farm wastes (Eswaran, 1998)

Among the thirty eight species of *Pleurotus* existing in nature, only nine species are being cultivated under artificial condition (Jandaik, 1989). Every species has its own attributes and each is known for its yield, substrate utilization and wide temp. adoption (Ravichandran, 2001). Inspite of its easy cultivation methods and adaptation to wide range of temp., the production of *Pleurotus* spp. is very less when compared to button mushroom production in India. Hence, a need was felt for up scaling the yield potential of *Pleurotus* spp. for large scale production.

In general, enhancement in mushroom production has been made by agronomic practices rather than through genetic improvement of strains (Kapoor *et al.,* 1996). The possibility of strain improvement by means of mycelial anastomosis was also reported to be promising (Kneeborne *et al.,* 1972). Also the strain improvement through mycelial fusion between the multispore cultures of *Pleurotus* spp. was tried (Geetha, 1993) and the hybrids of the fusants were reported to yield more and bear larger fruiting bodies (Pandey and Tewari, 1994).

The biology, genetics, sexuality and the cultivation technology of *Pleurotus* spp. have been fully understood and well established. The information available could be used to pool up the good qualities scattered in different species of *Pleurotus* spp., so as to identify and develop an ideal strain of *Pleurotus* sp. with desirable qualities using multispore isolates and fusion techniques.

Materials and Methods

Organism

The pure culture of *Pleurotus* spp. (*Pleurotus citrinopileatus* (Fr.) Singer, *P. djamor* (Rumph.) Boedijn, *P. eous* (Berk) Sacc*, P. flabellatus* (Berk and Br.) Sacc., *P. florida* (Eger) and *P. ostreatus* (Jacq.Fr.) Kummer) were obtained from National Centre for Mushroom Research (NCMR) Chambaghat, Solan, Himachal Pradesh. The sub cultures were maintained on oat meal agar (OMA) medium

Pc- Pleurotuscitrinopileatus

Pd- Pleurotusdjamor

Pe- Pleurotuseous

Pf- Pleurotusflabellatus

Pfl- Pleurotusflorida

Po- Pleurotusostreatus

Isolation and purification

The mushroom tissue was cut at the junction of the pileus and stipe using a sterile scalpel and surface sterilized with 95 per cent ethyl alcohol for one min. These bits were placed on OMA in sterile Petri dishes and incubated at room temp. (28 ± 2° C) for seven days. The isolates were then purified by single hyphal tip method and maintained on OMA slants.

Assay of enzymes

Assay of cellulases *in vitro*

Czapek's Dox broth was used as basal medium for the enzyme assay. Three mycelial discs (9 mm dia.) from a seven day old mushroom culture of the selected isolates was inoculated in to 100 ml of sterile broth in conical flasks and incubated for 10 days at room temp. (28 ± 2°C).

Extraction of enzymes

The broth containing the mycelial mat was filtered through Buchner funnel using Whatman No. 1 filter paper. The filtrate was centrifuged at 2000 rpm for 40 min at 6°C. The supernatant was collected and the enzyme activity was estimated (Bateman, 1964).

Production of enzymes *in vivo*

Various crop residues *viz*., paddy straw, saw dust, sugarcane trash, groundnut haulm and their combination with paddy straw (1:1) were used as substrates. The dried substrates were soaked in water for four h. except sawdust which was soaked for 24 h. The excess water was drained and the substrates were immersed in hot water for 60 min. After air drying, the substrates were used for preparing beds. Substrate samples were drawn at 10, 20 and 30 days interval and the enzyme activity was assessed.

Extraction of enzymes

After inoculation, five g of the substrate was ground with 20 ml dist. water using a pestle and mortar, filtered through muslin cloth and the filtrate was centrifuged in refrigerated centrifuge at 18,000 rpm for 20 min. at 6°C. The supernatant was collected and used as enzyme source (Maxwell and Batemen, 1967).

Assay of Celullases

Endocellulases (Cx)

The method involving dinitrosalicylic acid (DNS) was followed for determining the (Cx) β-1, 4 glucanase activity, by measuring the reducing sugar glucose. Carboxy methyl cellulose (CMC) (0.45 ml of one per cent in sodium citrate buffer at pH 5.0) was added to 0.05 ml of aliquot of the enzyme sample. The mixture was incubated for 15 min. at 55°C in a temp. controlled oven. Immediately after removing the enzyme substrate mixture from the oven, 0.5 ml of DNS reagent was added. The mixture was kept in boiling water bath for five min and then cooled to room temp. The volume was made up to 5.0 ml with de-ionized water. The absorbance of the sample was determined at 540 nm in a spectrophotometer (Miller, 1972).

Assay of exocellulase (C_1)

Filter papers (Whatman No.1) were cut into four mm dia. discs by a paper punch to ensure that each tube contained the same surface area of the substrate. The enzyme source (0.5 ml) in 0.1 M sodium citrate buffer at pH 5.8 was added to 32 mg of the filter paper. Then the assay was carried out similar to that of C_x enzyme (Miller, 1972).

Estimation of laccase *in vitro*

Activity of enzyme laccase was studied as already described for that of cellulase except for that CMC was replaced with 10 g of sawdust in the basal synthetic medium. The assay of laccase was carried out calorimetrically as described by Frochner and Eriksson (1974). The assay mixture consisting of five ml of 10 µM guaicol in 0.1 M sodium phosphate buffer at pH 6.0 was pipetted out into test tubes and equilibrated at 25°C. The enzyme source (0.1 ml) was added to the mixture and the absorbance was determined five min. after incubation. The boiled enzyme extract served as control. The activity of laccase was expressed in terms of enzyme units (1 unit = change of absorbance of 0.01 per minute).

Assay of laccase *in vivo*

The extraction of laccase was carried out following the method already described for cellulase. The assay for laccase

activity was done as per the method described for the *in vitro* assay (Frochner and Eriksson, 1974).

Production of cellulolytic and laccase enzymes *in vitro* by various multispore isolates of *Pleurotus* spp.

The results on the *in vitro* enzyme production of different multispore isolates are presented in the table 1. The *in vitro* activity of exocellulase increased with increase in days and was the maximum at 20 days after incubation. All the multispore isolates showed increased activity of exocellulase when compared to the standard parent. The activity of exocellulase (C_1) was maximum in *Pe x Po* after 20 days (0.66 mg) of incubation and it was on par with *Pc x Pe* (0.64 mg) and *Pc x Pfl* (0.62 mg). It was followed by *Pf x Po* and *P. eous* (0.60 mg).

A similar trend was as that of exocellulase was observed in the case of endocellulase (C_x) activity. The *in vitro* endocellulase (C_x) activity was the maximum in *Pe x Po* at 20 days (1.32 mg) of incubation. *Pc x Pe* ranked next by recording an enzyme activity of 1.10, 1.24, 1.30 at 10, 15 and 20 days of incubation periods, respectively. The least endocellulase (C_x) activity was recorded in the standard parent (*P. eous*). The maximum laccase activity was recorded in *Pe x Po* (0.26 mg) which was on par with *Pc x Pe* (0.25 mg) and *Pc x Pfl* (0.23 mg) at 20 days after incubation. The laccase activity increased with increase in days of incubation. The minimum laccase activity was observed with *P. eous.*

Production of exocellulase (C_1) by various multispore isolates in different substrates

The maximum activity of exocellulase (C_1) was showed by all the isolates in paddy straw substrate which was followed by paddy straw + sugarcane trash, paddy straw + groundnut haulm and paddy straw + sawdust. The activity of exocellulase (C_1) increased up to 20 days and there after decreased in all the isolates. Among the isolates *Pe x Po* recorded the maximum activity of the exocellulase (C_1) (0.71 mg) in paddy straw followed by the isolate *Pc x Pe* in paddy straw (0.69 mg) at 20 days after incubation. The least activity of exocellulase (C_1) was observed in saw dust with *Pf x Po* which recorded an activity of 0.56 mg. The standard parent recorded 0.62 mg of exocellulase (C_1) at 20 days of observation in paddy straw substrate (Table 2).

Production of endocellulase (C_x) by various multispore isolates in different substrates

All the isolates showed the maximum activity of endocellulase (C_x) in paddy straw substrate followed by paddy straw + sugarcane trash, paddy straw + groundnut haulm and paddy straw + sawdust in the decreasing order of merit. The maximum activity of endocellulase (C_x) was recorded at 20 days after inoculation in all the isolates. Among the isolates *Pe x Po* recorded the maximum endocellulase (C_x) activity (1.54 mg) in paddy straw followed by the isolate *Pc x Pe* in paddy straw (1.53 mg) at 20 days after incubation. The least activity of endocellulase (C_x) was observed with *Pf x Po* in saw dust (1.35 mg). The standard parent recorded 1.44mg of endocellulase (C_x) activity at 20 days after inoculation in paddy straw substrate (Table 3).

Production of laccase by various multispore isolates in different substrates

Laccase activity of selected multispore isolates in various substrates is presented in table 4. The activity of laccase increased only up to 10 days and there after decreased in all the isolates. With regard to the substrates a similar trend as that of endocellulase (C_x) was observed in the activity of laccase too. Among the isolates, *Pe x Po* recorded the maximum activity of the laccase (0.255) in paddy straw followed by the isolate, *Pc x Pe* in paddy straw (0.251) at 10 days after incubation. The least activity of laccase was observed in saw dust with *Pf x Po* which recorded an activity of 0.187 mg. The standard parent recorded 0.239 mg of laccase at 10 days of observation in paddy straw substrate

Discussion

Production of enzymes are of prime importance for efficient degradation of substrates and utilization of nutrients. The enzymes such as cellulase and laccase are responsible for degradation of cellulose and lignin present in the substrates. The efficiency of the enzyme production was positively correlated with the yield of mushroom.

In present study, it was found that exocellulase (C_1) and endocellulase (Cx) activity reached the peak after 20 days of inoculation *in vitro* (Table 1). Similar trend was also noticed in the activities of these enzymes in different substrates. The maximum activity of cellulase was found in paddy straw followed by paddy straw with sugarcane trash (1: 1) combination (Table 2 & 3). Positive relationships exist between the yield of sporophores and enzyme activity. Thayumanavan (1982) also reported that extra cellular cellulase and laccase activity of *P. sajor-caju* was more during the onset of sporophore formation and decreased as the yield of sporophore declined. In *Pleurotus* spp. similar activity with cellulase had been reported by several workers (Saxena and Rai, 1992b; Rajarathnam *et al.,* 1992; Rai *et al.,* 1993; Singh *et al.,* 1994; Ragunathan *et al.,* 1996; Abraham and Kurup, 1997; Sharma and Singh, 1997; Sun *et al.,* 1999). Higher activities of the cellulase complex enzymes were exhibited by *P. ostreatus* (Sharma *et al*., 1999). *P.djamor* elaborated more amount of cellulases and laccase as compared to P. *citrinopileatus* (Geetha and Sivaprakasam, 1998a).

In the present investigation, it was found that laccase activity in the substrate was at its peak during initial phase in the substrates (Table 4). Similar observation were also made by several workers (Rai and Saxena, 1990; Jin and Li, 1994; Pelaez *et al.,* 1995; Ardon *et al.,* 1996; Navarro *et al.,* 1998; Cho *et al.,* 1998). The highest amount of laccase activity on wheat straw by *P. sajor-caju* was reported by Rai *et al.* (1993). Malaya Ghosh and Nandi (1995) found that laccase activity of *P. ostreatus* and *P. sajor-caju* was the maximum after 24 days with water hyacinth as the substrate. Laccase production was the highest in *P. sajor-caju* followed by *P. menbranaceous* in composted coirpith (Theradimani *et al*., 2002). A good positive correlation existed between lignin degradation and production of laccase as found by Platt *et al.* (1985).

Table 1 : *In vitro* activity of cellulolytic enzymes by various multispore isolates of *Pleurotus* spp.

Multispore isolates	Exocellulase (C1)*			Endocellulase (Cx)*			Laccase **		
	Days after inoculation			Days after inoculation			Days after inoculation		
	10	15	20	10	15	20	10	15	20
Pc x Pe	0.48	0.58	0.64	1.10	1.24	1.30	0.17	0.20	0.25
Pc x Pfl	0.47	0.53	0.62	1.09	1.21	1.26	0.15	0.19	0.23
Pe x Po	0.50	0.64	0.66	1.11	1.26	1.32	0.19	0.23	0.26
Pf x Po	0.46	0.51	0.60	1.06	1.15	1.18	0.15	0.18	0.21
P. eous	0.45	0.50	0.60	0.99	1.01	1.03	0.14	0.16	0.19
CD (P = 0.05) Main		0.017			0.028			0.19	
Sub		0.013			0.021			0.15	
M x S		0.027			0.048			0.33	

* C_1&C_x are mg sugar released per 5 gm of substrate ** Laccase change in absorbance of 0.01per minute

Table 2 : Exocellulase (C_1) activity of various multispore isolates of *Pleurotus* spp. In different substrates

S. No	Substrates	Pc x Pe			Pc x Pfl			Pe x Po			Pf x Po			P. eous		
		Days after			Days after			Days after			Days after			Days after		
		10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
1.	Paddy straw	0.55	0.69	0.64	0.53	0.67	0.52	0.56	0.71	0.66	0.51	0.64	0.50	0.50	0.62	0.49
2.	Sugarcane trash	0.53	0.62	0.56	0.51	0.61	0.54	0.54	0.63	0.58	0.50	0.59	0.52	0.49	0.58	0.51
3.	Saw dust	0.48	0.59	0.50	0.47	0.58	0.50	0.49	0.60	0.51	0.47	0.56	0.49	0.47	0.54	0.49
4.	Groundnut haulm	0.49	0.60	0.53	0.49	0.59	0.52	0.50	0.62	0.54	0.49	0.57	0.52	0.48	0.56	0.50
5.	Paddy straw + Sugarcane trash (1:1)	0.52	0.66	0.54	0.51	0.64	0.53	0.53	0.68	0.55	0.50	0.63	0.52	0.47	0.61	0.50
6.	Paddy straw + Saw dust (1:1)	0.49	0.63	0.50	0.49	0.61	0.50	0.51	0.65	0.52	0.49	0.60	0.50	0.47	0.59	0.49
7.	Paddy straw + Groundnut haulm (1:1)	0.50	0.65	0.53	0.50	0.63	0.52	0.52	0.66	0.54	0.49	0.61	0.50	0.49	0.60	0.50
CD (P = 0.05) Main		0.009			0.012			0.014			0.016			0.018		
Sub		0.006			0.009			0.009			0.011			0.013		
M x S		0.016			0.021			0.025			0.029			0.033		

Figures are mg sugar released 5g of substrate

Table 3 : Endocellulase (C_x) activity of various multispore isolates of *Pleurotus* spp. in different substrates

S. No	Substrates	Pc x Pe			Pc x Pfl			Pe x Po			Pf x Po			P. eous		
		Days after			Days after			Days after			Days after			Days after		
		10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
1.	Paddy straw	1.25	1.53	1.27	1.25	1.51	1.25	1.27	1.54	1.29	1.24	1.48	1.23	1.20	1.44	1.19
2.	Sugarcane trash	1.20	1.47	1.23	1.20	1.46	1.22	1.21	1.48	1.25	1.20	1.43	1.20	1.19	1.39	1.20
3.	Saw dust	1.17	1.40	1.18	1.17	1.38	1.17	1.17	1.44	1.20	1.16	1.35	1.17	1.14	1.31	1.16
4.	Groundnut haulm	1.18	1.45	1.22	1.19	1.44	1.20	1.19	1.46	1.23	1.19	1.41	1.20	1.18	1.37	1.20
5.	Paddy straw + Sugarcane trash (1:1)	1.24	1.50	1.25	1.24	1.49	1.23	1.25	1.52	1.27	1.23	1.46	1.25	1.20	1.42	1.23
6.	Paddy straw + Saw dust (1:1)	1.20	1.48	1.23	1.19	1.47	1.21	1.22	1.49	1.24	1.19	1.44	1.20	1.17	1.40	1.20
7.	Paddy straw + Groundnut haulm (1:1)	1.22	1.49	1.24	1.22	1.48	1.22	1.23	1.51	1.25	1.21	1.45	1.22	1.20	1.41	1.22
CD (P = 0.05) Main		0.028			0.033			0.037			0.035			0.028		
Sub		0.019			0.022			0.025			0.024			0.019		
M x S		0.049			0.058			0.066			0.062			0.049		

Figures are mg of sugar released 5g of substrate

Table 4 : Laccase activity of various multispore isolates of *Pleurotus* spp. in different substrates

S. No	Substrates	Pc x Pe			Pc x Pfl			Pe x Po			Pf x Po			P. eous		
		Days after			Days after			Days after			Days after			Days after		
		10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
1.	Paddy straw	0.251	0.240	0.209	0.249	0.238	0.207	0.255	0.243	0.212	0.245	0.234	0.202	0.239	0.227	0.189
2.	Sugarcane trash	0.213	0.210	0.176	0.210	0.205	0.173	0.215	0.212	0.178	0.206	0.201	0.170	0.200	0.196	0.164
3.	Saw dust	0.198	0.192	0.167	0.193	0.188	0.163	0.203	0.199	0.169	0.187	0.181	0.160	0.180	0.176	0.154
4.	Groundnut haulm	0.208	0.203	0.173	0.206	0.200	0.171	0.210	0.205	0.175	0.202	0.193	0.169	0.198	0.188	0.162
5.	Paddy straw + Sugarcane trash (1:1)	0.226	0.218	0.185	0.222	0.215	0.181	0.229	0.220	0.187	0.217	0.210	0.177	0.210	0.202	0.169
6.	Paddy straw + Saw dust (1:1)	0.216	0.213	0.178	0.213	0.210	0.172	0.218	0.215	0.180	0.209	0.201	0.169	0.204	0.194	0.162
7.	Paddy straw + Groundnut haulm (1:1)	0.223	0.216	0.180	0.218	0.212	0.176	0.225	0.218	0.183	0.214	0.208	0.172	0.209	0.200	0.165
CD (P = 0.05) Main		0.009			0.012			0.013			0.014			0.015		
Sub		0.006			0.008			0.009			0.009			0.010		
M x S		0.016			0.021			0.023			0.025			0.026		

Figures are change in absorbance of 0.01per minute

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