Production and characterization of cellulolytic enzymes by *Pleurotus florida*

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Pleurotus florida, P. ostreatus and *P. sajar-caju* were screened for cellulolytic enzyme production under submerged fermentation conditions. *Pleurotus florida* produced the highest activity and it was further studied to optimize medium composition, incubation period, initial pH and incubation temperature for maximum cellulolytic enzyme production. Malt extract (0.5%), 12 days incubation and 1% CMC (carboxyl methyl cellulose) as carbon source supported maximum production of cellulases. A temperature of 35–40°C and pH 5.0 was optimum for exo- and endoglucanase production while β- glucosidase production was optimum at 30°C and pH 4.5.

Key words – Endoglucanase – exoglucanase – fermentation conditions – β-glucosidase – medium composition

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Introduction

Cellulose is the most abundant organic compound on earth and has received much attention as a substrate for the production of biofuels, single cell proteins and various other chemicals through enzymatic degradation by microbial cellulases. The conversion of cellulosic biomass to fermentable sugars requires synergistic action of three cellulolytic enzymes, namely, β-1,4 endoglucanase (EC 3.4.1.4), β-1,4 exoglucanase (EC 3.2.1.91) and β-1,4 glucosidase (EC 3.2.1.21). Various bacteria, fungi and yeast synthesize these enzymes, but the most extensively studied cellulases are those produced by efficient lignocellulose degrading fungi, particularly *Trichoderma* (Narsimha et al. 2006) and *Aspergillus* (Baig 2005). Mushrooms are an alternative and safe source of extracellular cellulolytic enzymes. Of these, *Pleurotus* spp. is most efficient in utilizing lignocellulosics (Zhang et al. 2002, Salmones et al. 2005, Albores et al. 2006). We screened three *Pleurotus* spp., namely, *P. florida*, *P. ostreatus*, and *P. sajar-caju* for cellulolytic enzymes production. *P. florida*, which produced the highest levels of enzyme activity, was studied under various cultural and nutriational parameters for enhanced production of extracellular cellulases using submerged fermentation conditions.

Materials and Methods

Cultures of *Pleurotus florida, P. ostreatus,* and *P. sajar-caju* were procured from Department of Microbiology, Punjab Agricultural University, Ludhiana. They were screened for production of cellulolytic enzymes by growing them on Czapek Dox medium at 35°C for 12 days with an initial pH of 5.0 and 1% CMC as carbon source. *P. florida* was found to be the best producer of all three components of the cellulases (Table 1), and was selected for further study. The strain was maintained and sub cultured fortnightly on potato dextrose agar (PDA) slants and stored at 4°C.

Table 1 Production of cellulases by *Pleurotus* spp. in Czapek medium.

Fungus	Enzyme activity (IUL^{-1})		
	Endoglucanase	**Exoglucanase**	**β-glucosidase**
P.ostreaus	420	82	880
P. florida	480	102	980
P. sajarcaju	450	54	856

Czapek Dox medium (Na_2HPO_4 1g, $NaNO_3$ 3g, KCl 0.5g, $MgSO_4.7H_2O$ 0.1g, $FeSO_4$ $7H_2O$ 0.001g, water 1L) was supplemented with 10–30g of carboxyl methyl cellulose in the absence or presence of 0.1–1% malt extract. Cotton plugged 250 mL Erlenmeyer flasks containing 50 mL medium were autoclaved at 121°C for 30 min, cooled to room temperature and inoculated with 1 mm square disk from the growing colony edge. The flasks were then incubated at 30°C for 15-day under stationary conditions and three flasks were drawn at each 5 days interval for enzymatic determinations in cultural media.

Glucanase activity was assayed according to the methods described by Mandels et al. (1976). For endoglucanases the reaction mixture, consisting of 1.0 mL of 0.05 M citrate buffer (pH 4.8), 1.0 mL of 1% CMC solution and 0.5 mL of culture filtrate, was incubated at 50°C. 0.5 mL samples were drawn at 0 and 30 min of incubation period for determination of reducing sugars released using dinitrosalicylic acid (DNS) method (Miller 1959). Likewise, the exoglucanase activity was determined by using 6 × 1 cm Whatman no. 1 filter paper strips, cut into small strips. The strips were incubated with 2 mL of 0.05 M citrate buffer containing 0.5 mL culture filtrate at 50°C and the reducing sugars released were determined at 0 and 60 min intervals by DNS method (Miller 1959). For β-glucosidase activity, the reaction mixture, consisting of 1 mL of 1% cellobiose solution, 0.5 mL of 0.05 M citrate buffer (pH 4.8) and 0.5 mL of enzyme, was incubated at 50°C. Reducing sugars released were measured at 0 and 15 min of incubation period using DNS method. The enzymatic activities were expressed as international units (IUL^{-1}). One unit of enzyme has been defined as the amount of enzyme that released one micromole of reducing sugars per minute under the assay conditions.

Culture conditions were optimized for production of cellulases by *Pleurotus florida* with respect to medium constituents, incubition period, incubation temperature, medium pH and concentration of carbon source. The cellulolytic enzymes produced were also characterized for their optimum pH, optimum temperature and thermostability.

Results and Discussion

Optimization of medium ingredients

Pleurotus florida was grown on Czapek Dox medium containing 1% CMC in the presence or absence of malt extract. It was found that different enzymes peaked at different fermentation periods and declined subsequently, which could be due to the inactivation and or degradation of these enzymes (Mandels & Stenberg 1976). Enzyme activity was poor in the absence of malt extract and maximum with 0.5% malt extract (Table 2). Maximum production of endoglucanase (460 IUL^{-1}) and exoglucanase (105 IUL^{-1}) with 0.5% malt extract was obtained on day 12 (Table 2). Previous reports showed a peak after 8 days of incubation using 0.5% malt extract for *P. ostreatus* (Platt et al. 1984) and *Volvariella* (Phutela et al. 1996). However, with 1.0% malt extract, an early peak at days 5 and 7 was achieved for endoglucanase (341 IUL^{-1}) and exoglucanase (85 IUL^{-1}), respectively, but the production was low when compared to that obtained with 0.5% malt extract on day 12. With lower concentrations of malt extract (0.1%), comparatively low levels of cellulolytic enzymes were produced with maxima on day 15. β-glucosidase showed maximum production with 1% malt extract (1066 IUL^{-1}) on day 5 whereas with 0.5% malt extract, a slightly lower production (1040 IUL^{-1}) was achieved on day 12. Thus, increasing malt extract concentration in production medium can shorten incubation period for maximum cellulase production. Since maximum endo- and exoglucanase can only be obtained with 0.5% malt extract, so this concentration was chosen for further experiments.

Table 2 Effect of supplementation of Czapek medium with different carbon sources (1%) and malt extract concentrations (0%, 0.1%, 0.5%, 1.0%) on cellulase activity by *Pleurotus florida* after various periods of incubation.

Carbon source 1%	Incubation period (days)	Malt extract concentration (%)			
		0	**0.1**	**0.5**	**1.0**
CMC		Endoglucanase (UL^{-1})			
	5	ND	213	234	**340**
	7	**166**	320	320	170
	10	102	313	356	192
	12	21	277	**460**	252
	15	ND	**384**	296	106
		Exoglucanase (UL^{-1})			
	5	ND	ND	8	65
	7	10	16	25	**85**
	10	5	42	82	72
	12	40	64	**105**	64
	15	**64**	**88**	41	35
		β-glucosidase(UL^{-1})			
	5	50	504	746	**1066**
	7	**304**	612	720	950
	10	262	612	840	842
	12	201	604	**1040**	822
	15	71	**752**	123	213
Cellulose		Endoglucanase (UL^{-1})			
	5	ND	2	114	146
	7	3	2	106	**213**
	10	3	3	136	210
	12	2	4	**149**	140
	15	ND	ND	128	28
		Exoglucanase (UL^{-1})			
	5	ND	ND	5	8
	7	ND	1	9	8
	10	ND	1	13	40
	12	ND	4	15	**46**
	15	ND	5	**40**	24
		β-glucosidase(UL^{-1})			
	5	ND	201	322	712
	7	ND	310	452	**854**
	10	102	217	700	605
	12	200	380	**711**	610
	15	210	**440**	422	151
Wheat straw		Endoglucanase (UL^{-1})			
	5	ND	54	87	182
	7	4	50	107	**235**
	10	4	60	120	149
	12	3	64	**128**	150
	15	ND	**106**	115	150
		Exoglucanase (UL^{-1})			
	5	ND	ND	3	62
	7	2	13	25	88
	10	2	22	102	**152**
	12	ND	ND	105	141
	15	ND	ND	**119**	104
		β-glucosidase(UL^{-1})			
	5	ND	252	402	905
	7	ND	384	510	**1025**
	10	8	427	586	100
	12	20	442	**902**	204
	15	52	**446**	412	200

Production of endo- and exoglucanases was low when cellulose (1%) was used as carbon source. However, supplementation with malt extract (0.5% or 1%) improved the cellulolytic enzymes production (Table 2). Maximum production of endo- and exoglucanases was achieved with 1% malt extract, which peaked between days 7–10 for endoglucanase (213 IUL^{-1}) and at day 12 for exoglucanase (46 IUL^{-1}). For β-glucosidase, also maximum activity was achieved with 1% malt extract concentration level at day 7 (854 IUL^{-1}). With wheat straw as an alternative carbon sources, maximum endoglucanse (235 IUL^{-1}), exoglucanase (152 IUL^{-1}) and β- glucosidase (1025 IUL^{-1}) was achieved on days 7, 10 and 7, respectively, using 1% malt extract (Table 2). Tan & Wahab (1997) detected higher cellulolytic enzymes production from *P. sajar caju* by using treated cotton waste as compared to normal cotton in culture filtration. However other workers used cotton-wheat straw mixture for inducing lignocellulytic activity of *P. Pulinonarius* in liquid culture (Masaphy & Levanon 1992).

Of the carbon sources tested, CMC gave the best enzyme production. Moreover, maximum production of glucanases was achieved with 0.5% malt extract, so for further enzyme production improvement, culture conditions were optimized with respect to incubation period, temperature and level of carbon source.

Effect of Incubation temperature

Effect of incubation temperature on cellulolytic enzyme production was studied by growing *P. florida* at different temperatures (Table 3). It was found that growth of fungus did not correlate with the production of enzymes. The optimum temperature for endoglucanase and exoglucanase production was 35–40°C. But maximum biomass production was obtained at 25°C and lowest at 40°C. Thus, high temperature promotes production of cellulolytic enzyme but not biomass production. Phutela et al. (1996) showed temperature optima of 35±2°C for cellulolytic/hemicellulolytic enzymes production by *Volvariella*. However, β-glucosidase showed an optimum activity with incubation temperature of 30°C and was lowest at 40°C. Since β- glucosidase is relatively more thermostable (Tm 72°C) as compared to endo- and exoglucanase, the lower activity of this enzyme at higher temperature cannot be attributed to its denaturation. The differential effect of temperature on the production of β-glucosidase indicates that its production might be regulated in a manner different from endoglucanase and exoglucanase. This finding corraborates the earlier results suggesting a separate control of β-glucosidase (Harchand & Singh 2001).

Effect of initial pH

To determine the optimum pH for production of cellulolytic enzymes, *P. florida* was grown at different initial pH ranging from 4.0–6.5 Maximum production for endo- and exoglucanase was 5.0 and for β-glucosidase it was 4.5 (Table 4).

Effect of CMC level

To test the effect of CMC level on cellulolytic enzyme production, three concentrations (1, 2, 3%) of CMC were used in the production medium (Table 5). Production of all the three cellulolytic enzymes increased with increase in CMC concentration and was maximum at 3% level. However, no significant change in extracellular cellulase production by *P. ostreatus* with increase concentrations of wheat straw (1–6%) has been reported (Garzillo et al. 1994).

Table 3 Effect of incubation temperature on dry biomass and cellulolytic enzyme production by *Pleurotus florida*.

Temperature (^oC)	Mycelia biomass dry weight (gl^{-1})	Cellulase activity (IUL^{-1})		
		Endoglucanase	Exoglucanase	β-glucosidase
20	2.8	142	15	956
25	6.0	149	42	983
30	5.2	260	62	1054
35	3.2	460	106	963
40	0.9	502	112	106
45	0.8	12	–	–

Table 4 Effect of pH on cellulolytic enzyme production by *Pleurotus florida* in Czapek medium fortified with 1% CMC.

Incubation pH	Enzyme activity (IUL^{-1})		
	Endoglucanase	Exoglucanase	β-glucosidase
4.0	106	16	533
4.5	341	63	853
5.0	469	98	800
5.5	320	74	106
6.0	213	24	ND

Table 5 Effect of different concentrations of CMC on cellulolytic enzyme activity in *Pleurotus florida.*

CMC concentration(%)	Enzyme activity (IUL^{-1})		
	Endoglucanase	Exoglucanase	β-glucosidase
1	452	105	1040
2	542	200	1599
3	728	224	2486

Characterization of cellulases

Enzymes produced by *P. florida* were characterized for their optimum pH, temperature and thermostability. All the three cellulolytic activities of cellulases had a broad pH range with maximum activity at pH 4.4. Likewise the optimum temperature for all the three activities was found to be 45°C. For determining thermostability of cellulolytic enzymes, the crude enzyme preparation was exposed to different temperatures ranging from 35–75°C for 15 min and then cooled in ice-cold water. The residual activity was measured under standard assay conditions. Tm, the temperature at which the enzyme activity was reduced to 50% of the original activity was determined by plotting residual activity VS exposure temperature. β-glucosidase was the most thermostable followed by endoglucanase and exoglucanase with Tm of 72°C, 66°C and 58°C, respectively.

From these results it may be concluded that *Pleurotus florida* can be exploited for the production of cellulolytic enzymes or biomass as the conditions warrant, by altering culture conditions. The differential response for production of β-glucosidase and endo- and exoglucanase towards the different culture conditions indicating separate regulatory mechanisms.

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