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Full Length Research Paper

Production and characterization of cellulolytic enzymes by *Pleurotus florida*

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Three *Pleurotus* spp., *Pleurotus florida, Pleurotus ostreatus* and *Pleurotus sajar-caju*, were screened for cellulolytic enzyme production under submerged fermentation conditions. Of these, *P. florida*, was studied for optimizing medium composition, incubation period, initial pH and incubation temperature to maximize cellulolytic enzyme production. Malt extract at 0.5%, 12 day of incubation period and 1% CMC as carbon source supported maximum production of cellulases. The optimum temperature and pH for maximum production of enzymes were 35 to 40°C and 5.0 for exo-and endoglucanases and 30°C and 4.5 for β- glucosidase.

Key words: Endoglucanase, exoglucanase, fermentation conditions, β-glucosidase, medium composition.

INTRODUCTION

Cellulose is the most abundant organic compound on earth and as such has received a greater deal of attention as a substrate for the production of biofuel, single cell protein and a variety of chemicals through enzymatic degradation by microbial cellulases. The conversion of cellulosic biomass to fermentable sugars requires synergistic action of three cellulolytic enzymes namely -1,4 endoglucanase (EC 3.4.1.4), -1,4 exoglucanase (EC 3.2.1.91) and -1,4 glucosidase (EC 3.2.1.21). Several microorganisms like bacteria, fungi and yeast have been reported to synthesize these enzymes. The most extensively studied cellulases are those produced by efficient lignocellulose degrading fungi, particularly *Trichoderma* (Narsimha et al., 2006) and *Aspergillus* spp. (Baig, 2005). Mushrooms can be exploited as an alternative and safe source of extracellular cellulolytic enzymes. Of these, *Pleurotus* spp are most efficient in utilizing lignocellulosics (Zhang et al., 2002; Salmones et al., 2005; Albores et al., 2006). We therefore first screened three *Pleurotus* spp namely *Pleurotus florida, Pleurotus ostreatus* and *Pleurotus sajar-caju* for cellulolytic enzymes production and studied in details *P. florida* for various cultural and nutritional parameters for enhanced production of extracellular cellulases using submerged fermentation conditions.

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MATERIALS AND METHODS

The three *Pleurotus* spp. procured from Department of Microbiology, Punjab Agricultural University, Ludhiana, 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 containing 1% CMC as carbon source. Based upon the activities of all the three components of the cellulases (Table 1), *P. florida* was found to be the best producer of these enzymes 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.

Czapek Dox medium used for enzyme production by *P. florida* to ferment CMC comprised (gl^{-1}): carboxyl methyl cellulose 10-30, Na_2HPO_4 1.0, $NaNO_3$ 3.0, KCl 0.5, $MgSO_4.7H_2O$ 0.1 and $FeSO_4.7H_2O$ 0.001 in the absence or presence of 0.1 to 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 disks from the growing edge of culture. The flasks were incubated at 30°C for 15 days under stationary conditions and three flasks were drawn at each 5 day interval for enzymatic determinations in culture media.

Activities of glucanases were assayed according to the methods described by Mandels et al. (1976). For endoglucanases activity, 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. Samples (0.5 ml) 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

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

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

exoglucanase activity was determined by using 6 x 1 cm Whatman no.1 filter paper strips, cut into small pieces. The cut 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 was 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 *P. florida* with respect to medium constituents, incubation 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

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

When cellulose (1%) was used as carbon source in production medium, very poor response with respect to endo- and exoglucanases production was observed. Supplementation of production media with malt extract (0.5 or 1%) concentrations, however, improved the cellulolytic enzymes production (Table 2). Maximum production of endo- and exoglucanases was achieved with 1% malt extract, which peaked between 7 to10th day of incubation period for endoglucanase (213 IUL^{-1}) and at 12th day for exoglucanase (46 IUL^{-1}). For β-glucosidase, also maximum activity was achieved with 1% malt extract concentration level at 7th day of incubation period (854 IUL^{-1}) (Table 2). With wheat straw as an alternative carbon sources, maximum endoglucanse (235 IUL^{-1}), exoglucanase (152 IUL^{-1}) and β- glucosidase (1025 IUL^{-1}) production was achieved on 7, 10 and 7th day of incubation period respectively using 1% malt extract (Table 2). Tan and 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 and Levanon, 1992).

Of all the carbon sources used, CMC gave a better 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 found to be between 35 to 40°C. But maximum biomass production was obtained at

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

Carbon source (1%)	Incubation period	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

Table 2. Contd.

		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

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

Incubation temperature (°C)	**Mycelia biomass dry weight (gl^{-1})**	**Cellulases 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	-	-

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, -glucosidase showed an optimum activity with incubation temperature of 30°C and was lowest at 40°C. Since -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 -glucosidase (Harchand and Singh, 2001).

Effect of CMC level

To test the effect of CMC level on cellulolytic enzyme production, three concentrations (1, 2 and 3%) of CMC were used in the production medium (Figure 1a). 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 increasing concentrations of wheat straw (1 to 6%) has been reported (Garzillo et al., 1994).

Effect of initial pH

To determine the optimum pH for production of cellulolytic enzymes, the fungus was grown at different initial pH ranging from 4.0 to 6.5. The maximum production for endo- and exoglucanase was 5.0 and for -glucosidase it was 4.5 (Figure 1b).

Characterization of cellulases

Enzymes produced from *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

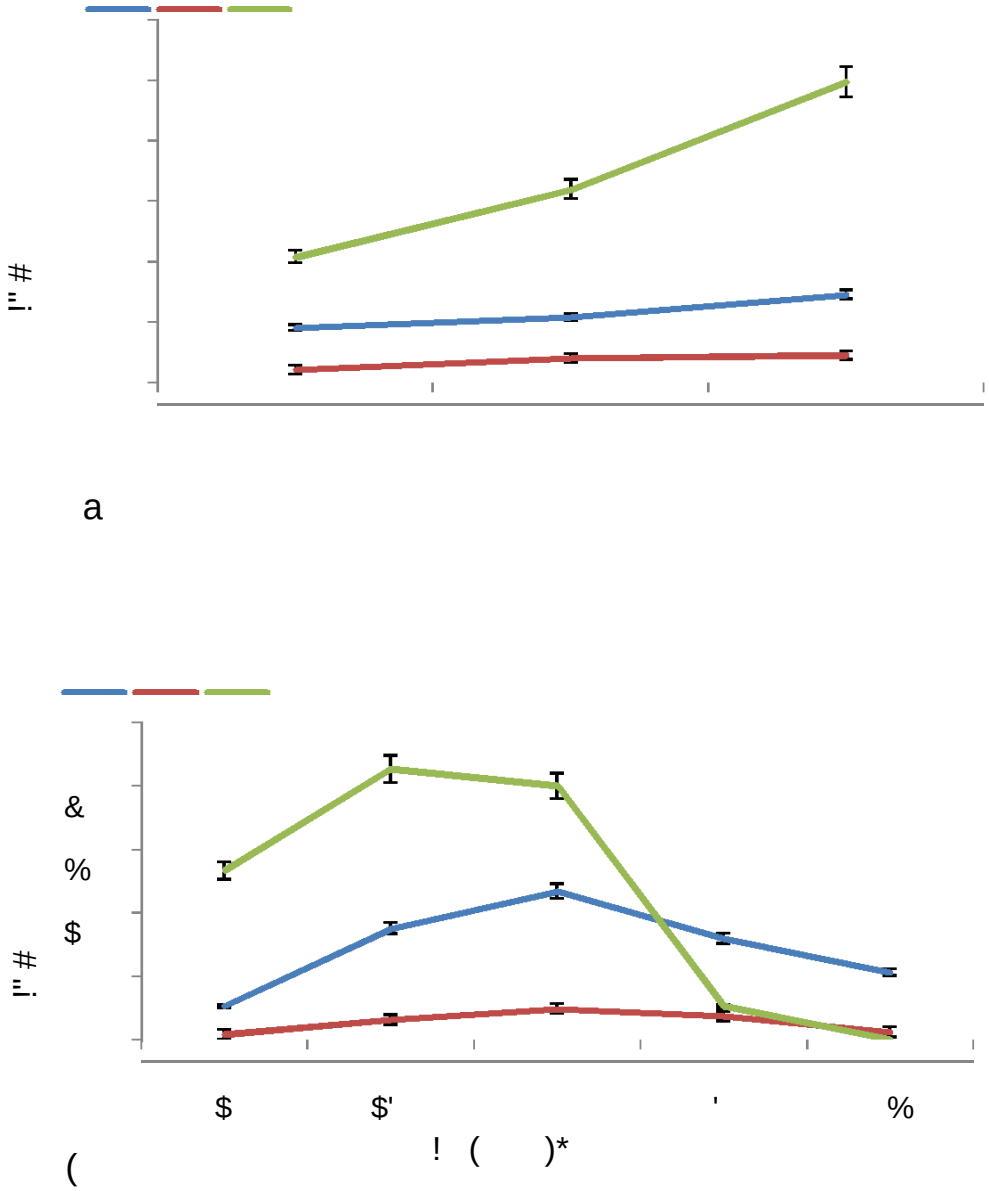

Figure 1. Effect of CMC concentration (a) and incubation pH (b) on cellulolytic enzymes production (IUL^{-1} ± SE) by *P. florida* in Czapek medium.

different temperatures ranging from 35 to 75°C for 15 min and then cooled in an 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. Of all these enzymes, -glucosidase was the most thermostable followed by endoglucanase and exoglucanase with Tm of 72, 66 and 58°C, respectively. From these results, it may be concluded that *P. 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 -glucosidase and endo- and exoglucanase towards the different culture conditions indicating separate regulatory mechanisms.

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