Cellulolytic Enzyme System of *Pleurotus ostreatus* and Its Application in Animal Feed

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Abstract: Enzymatic degradation of cellulose is an important process both in the environment and in several commercial processes. Filamentous fungi are key organisms in this process. Studies on the cellulase systems of basidiomycetes have shown that the polymer is hydrolysed by a complex of at least three enzymes: endoglucanase, cellobiohydrolase and beta-glucosidase. This work involved the cellulolytic enzyme profiles and enzyme induction study of the edible mushroom, *Pleurotus ostreatus* No. 10. Preliminary studies on the application as animal feed enzymes were also conducted. Knowledge of enzyme profiles and induction characteristics of cellulases could lead to the better understanding of cellulose degradation by this mushroom and the possibility of using this fungus as a new source of cellulases for biotechnological applications.

Key words: Cellulases, *Pleurotus ostreatus,* enzyme induction, cellulose degradation, biotechnological applications, animal feed

1 Introduction

The cultivation of edible mushrooms is a prime example of the conversion of low value waste, primary from agricultural practices, to a high value commodity. Mushroom production has increased dramatically over the last decade, especially in Southeast Asian countries and this trend is set to continue. Although many fungi are able to utilize cellulose, studies of cellulolytic system have been very limited in the mushroom fungi.[1, 2] The edible mushroom, *Pleurotus ostreatus* can produce various lignocellulolytic enzymes such as endo-1,4-β-D-glucanase (EC 3.2.1.4; CMCase), exo-1,4-β-D-glucanase (EC 3.2.1.91; Avicelase), β-glucosidase (EC 3.2.1.21), xylanase (EC 3.2.1.8) and laccase (EC 1.14.18.1) to degrade lignocellulosic materials that have numerous applications in industrial processes. This paper describes cellulase production profiles and induction in liquid cultures of *P. ostreatus* No. 10, and the potential for use as feed enzyme. To the best of our knowledge, this is the first report of enzymes derived from this mushroom for use in animal feed applications.

2 Materials and Methods

2.1 Organism and culture conditions

P. ostreatus No. 10 was kindly provided from Mrs. Ahchara Payapanon, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand. The fungus was cultured in Mushroom Complete Medium (MCM) containing (per litre) 20g glucose, 2g yeast extract, 2g peptone, 0.5g $MgSO_4.7H_2O$, 0.46g KH_2PO_4, and 1 g K_2HPO_4. MCM solid medium was supplemented with 20 g agar. For enzyme production, minimal medium containing (per litre) 10g Avicel, 1g K_2PO_4, 0.68g asparagine, 0.5g yeast extract, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 0.1g $FeSO_4.7H_2O$, 0.008g $MnSO_4.4H_2O$, 0.003g $Zn(CH_3COO)_2$, 0.006g $Ca(NO_3)_2.4H_2O$ and 0.003g $CuSO_4.5H_2O$, was used. Mycelial inoculum was transferred from a stock plate to the fresh MCM plates. Then, myce

lium grown over 7 days was harvested and homogenised for 30 sec in 15 ml sterile distilled water using a disperser (model ULTRA-TURRAX® T25 basic, IKA, Germany). Three ml of homogenate was inoculated into 50 ml liquid minimal medium, in 250 ml flasks, with 1% Avicel as sole carbon source and incubated with shaking (200 rpm) at 28°C for 10 days. The culture supernatants were obtained by centrifuging the harvested mycelium at 5,000 rpm for 10 min at 4°C. The solutions were stored at 4°C.

2.2 Enzyme assays

CMCase, Avicelase and xylanase assays were carried out using CMC, Avicel and oat spelts xylan as the substrate, respectively and the reducing sugar produced during incubation was determined following the Somogyi-Nelson method.[3] β-Glucosidase was assayed with p-nitrophenyl-β-D-glucopyranoside as the substrate according to Deshpande et al.[4] Laccase was assayed with syringaldazine according to Leonowicz and Grzywnowicz[5] as the substrate.

2.3 Optimum temperature and pH

The optimum temperature for each enzyme activity was determined by incubating the assay mixture at various temperatures (30, 35, 40, 45, 60, 65 and 70°C). The optimum pH was determined by dissolving the substrate of each enzyme in different buffer solutions (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0).

2.4 Determination of extracellular protein

Extracellular protein in the culture supernatant was determined using the method of Lowry et al.[6] using bovine serum albumin as standard.

2.5 Enzyme profile study

An aliquot (3ml) of mycelial homogenate was inoculated into 250 ml flasks containing 50 ml mushroom minimal medium with 1% Avicel as the sole carbon source. Incubation was carried out for 15 days at 28°C in an orbital shaker at 150 rpm. Culture filtrate was collected everyday and assayed for enzyme activity and protein concentration.

2.6 Induction studies

The fungus was grown for three days in MM medium containing 0.5% glucose and then was transferred to new MM medium containing 0.5% carbon source as inducer. Eleven carbohydrate sources: glucose, xylose, fructose, galactose, sorbose, manose, cellobiose, lactose, carboxymethycellulose, Avicel and xylan, were tested. The enzyme activity of the supernatant was assayed.

2.7 Effect of enzyme on digestibility in animal feed production

The digestibility effect of crude enzyme from *P. ostreatus* No. 10 was tested against soy meal and corn. Enzyme was added to a 250 ml flask containing 100 ml of 10 % (w/v) feed material suspension (soy meal or corn) in citrate phosphate buffer, pH 5.0, to reach a final concentration of 0.05%. The mixture was incubated in orbital shaker at 45°C, 200 rpm. The reaction was stopped and then the mixture was centrifuged to determine the solid content and reducing sugar in supernatant. The digestibility was calculated from the loss of solid content compared with controls.

3 Results and Discussion

3.1 Optimum temperature and pH

As shown in Figure 1, the optimum temperatures and pH values were 50°C, 45°C, 50°C, 45°C and 70°C, and pH 5, 4.5, 5.5, 5 and 6.5 for CMCase, Avicelase, β-glucosidase, xylanase and laccase, respectively

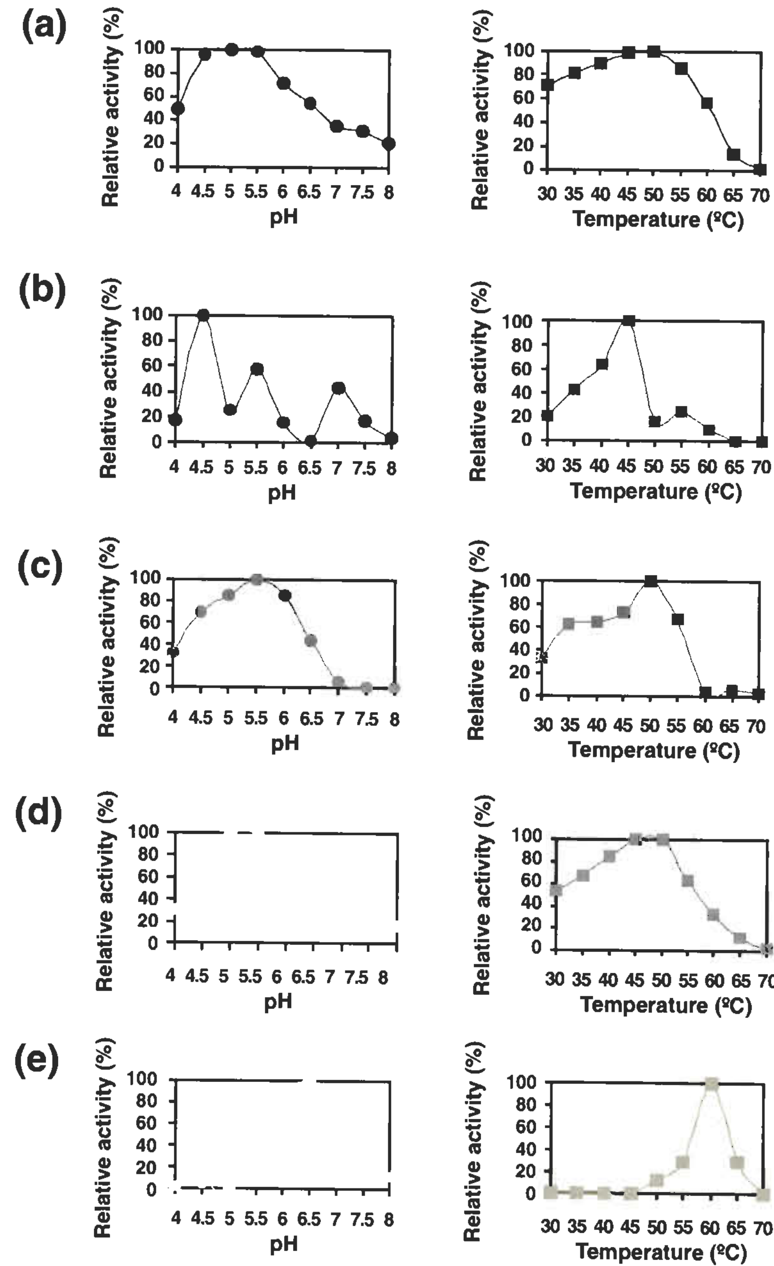

Figure 1 Effect of temperature and pH on activity of CMCase (a) Avicelase (b) β-glucosidase(c) xylanase (d) and laccase (e) from *P. ostreatus* No.10

3.2 Enzyme profiles of *P. ostreatus* No.10 grown in liquid medium

The production of enzymes of the cellulase complex during growth of *P. ostreatus* No.10 in liquid culture was measured on days 5, 4 and 11 with specific activity values of 0.999, 0.034, 0.600 U (mg protein)$^{-1}$ for CMCase, Avicelase and β-glucosidase, respectively. Maximum xylanase production of 1.228 U (mg protein)$^{-1}$ occurred

at 9 days after inoculation whereas the production of laccase reached maximum on day 3 with a specific activity value of 0.393 U (mg protein)$^{-1}$. Enzymatic activities assayed were found to be proportional to protein concentration as shown in Figure 2 and were consistent with those reported by Tan and Wahab.[7]

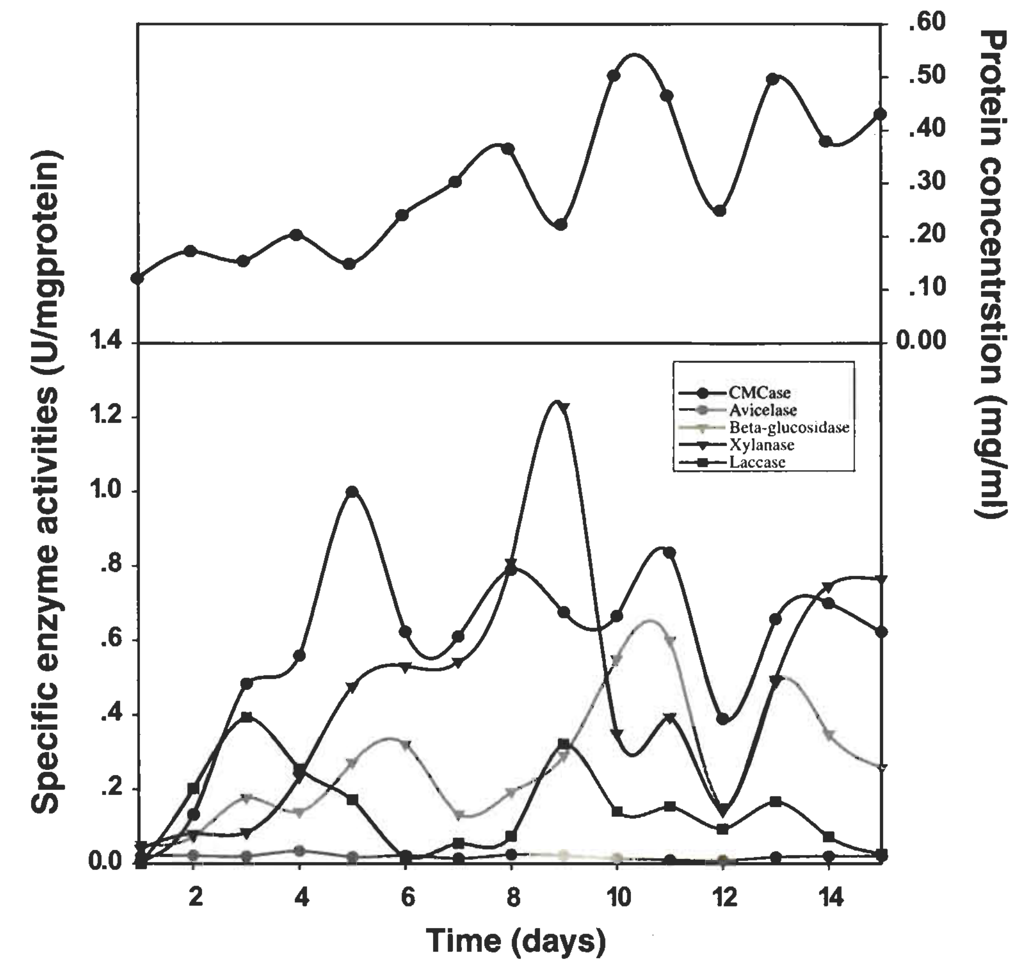

Figure 2. Specific activity of lignocellulolytic enzymes and protein concentration during the growth of *P. ostreatus* No.10

The results also showed that CMCase and xylanase activities were produced at high levels throughout the cultivation, and are in agreement with enzyme production by *Trichoderma reesei* Rut C-30.[8] The lowest activity among the enzymes was Avicelase.These exoglucanases are the rate-limiting step in cellulose degradation. In addition, the enzyme also related to the degradation of lignocellulosic material, the lignin degrading enzyme laccase, was produced prior to cellulase and hemicellulase. It was suggested that the purpose of lignin removal

Table 1 Induction of lignocellulolytic enzymes

Enzyme activity	Most effective inducer
CMCase	Avicel
Avicelase	Lactose
Beta-glucosidase	Avicel
Xylanase	Avicel
Laccase	Xylan

is to expose cellulose and hemicellulose fibres for consumption and further fungal growth.[9] This could lead to better understanding of the process of lignocellulosic degradation in mushroom cultivation and to the development of cellulase, hemicellulase and lignin degrading enzymes production from this fungus for industrial application.

3.3 Induction study

The result was shown in Table 1. They suggested that the high molecular weight carbohydrates such as Avicel and xylan were effective inducers for lignocellulase production in *P. ostreatus* No. 10. The data are consistent with the previous studies reported by Kubicek and Penttila[10] and Lynd et al.[11]

Table 2 Specific activity of *P. ostreatus* No. 10 crude enzyme compared with commercial feed enzymes

Enzyme activity	*P. ostreatus* No.10 (Protein = 0.35 mg/ml)		Feed enzyme A (Protein = 1.07 mg/ml)		Feed enzyme B (Protein = 0.66 mg/ml)	
	EU(unit/ml)	Specific activity	EU(unit/ml)	Specific activity	EU(unit/ml)	Specific activity
CMCase	0.105	0.30	0.169	0.16	0.188	0.28
Avicelase	0.008	2.29×10^{-2}	0.104	9.69×10^{-2}	0.055	8.33×10^{-2}
Xylanase	0.578	1.65	3.697	3.45	0.210	0.32
Pectinase	0.337	0.96	0.085	7.91×10^{-2}	6.319	9.57
β-Glucosidase	0.052	0.15	0.010	9.42×10^{-3}	0.149	0.23
Amylase	0.085	0.24	4.693	4.39	1.817	2.75
Protease	0.092	0.26	0.306	0.29	1.56×10^{-3}	2.36×10^{-3}
β-Glucanase	0.578	1.65	0.423	0.40	0.491	0.74

3.4 Effect of enzyme on digestibility in animal feed production

The specific activity of crude enzyme from *P. ostreatus* No. 10 was assayed and compared to that of commercial feed enzymes (Table 2). The result showed that it has all enzyme activities present in commercial feed enzyme. Although some enzyme activities such as xylanase were lower than in the commercial one, the substitution of such enzyme activities from other sources such as an enzyme cocktail might be the solution. The effect of the mushroom enzyme preparation on soy meal and corn was tested (Table 3). It revealed the possibility of using the preparation in animal feed. These data suggested that it could be a new source for feed enzyme leading to a novel application of mushroom product in the near future.

Table 3 Digestibility effect of *P. ostreatus* No. 10 crude enzyme compared with commercial feed enzymes

Enzyme	Digestibility (%)		Reducing sugar released during digestion(mg/ml)	
	corn	soy meal	corn	soy meal
Crude enzyme of *P. ostreatus*	13.98	3.72	0.008	0.009
Feed enzyme A	8.99	4.27	0.136	0.029
Feed enzyme B	5.16	2.63	0176	0.124

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