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Preparations and Characteristics of Alkaline-active Cellulases from *Coprinaceae*

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ABSTRACT

Coprinus cinereus 2249 producing alkaline-active cellulase was screened from 29 species of *Coprinaceae* and constitutively produced alkaline carboxymethyl cellulase (CMCase) and filter paper cellulase (Fpase). When cultivated at pH 9.0, 25℃ and 5 days, *Copnnus cinereus* 2249 produced higher alkaline activity on 0.5% CMC, 2% wheat bran as carbon source and 0.5% peptone, 0.05% yeast extract as nitrongen source compared with other culture conditions. The level of cellulase production was higher in the presence of wheat bran than in the presence of CMC. The optimum temperature and pH for alkaline-active cellulase activity was 50℃ and 9.0, respectively.

1. Introduction

Nowadays, the demand for recycling of wastepaper has risen dramatically.[1] But, wastepaper contains contaminants such as toners and non-contact polymeric inks from newly developed printing processes.[2,3] Conventional deinking process requires large quantities of chemicals such as surfactant, alkali and high temperature steps, so its methods are both capital and energy-intensive.[4] The use of enzyme in deinking process can minimize both problems.[5,6] Enzymes have been used in pulp and paper industry for over 30 years to facilitate the bleaching, deinking, biopulping, etc.[7-10] However, the great majority of cellulases, so far reported, have optimum pH in the acidic or neutral range, which is no good for deinking because of addition of acid chemicals for keeping optimum enzyme activity conditions.[1,2]

Recently, Some alkalophilic strains of *Bacillus* were isolated.[11-14] But, these enzymes were not effective in deinking. Because they have not completely cellulase components, especially they showed strong activity toward CMC but very little activity toward cellulosic substrates showing high crystallinity. Sreenath *et al.*[15] suggested that the most effective cellulases are those that exhibit activity on filter paper at alkaline or neutral pH and thermal stability is also important for effective processing.[2] *Coprinaceae* species, which in nature grows on horse excrements, can produce alkaline-active cellulases.[16]

The research presented in this paper has focused on preparation of alkaline-active cellulase from *Coprinaceae* and some proper-

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Table 1. Identification of *Coprinaceae*

Scientific name	No.	Collection site	Growth condition in PDA solid-culture
Coprinus atramentarius	2061	Kyonggi	-
〃	861	〃	+
〃	1008-1	Seoul	+
〃	318	Kyonggi	+
Coprinus cinereus	2249	Nepal	+++++
Coprinus comatus	2237	〃	+
〃	2088	Kyonggi	+
〃	536	〃	+
〃	3128	Kyongnam	+
Coprinus disseminatus	2221	Nepal	+++++
Coprinus echiospora	1669	Kangwon	++
〃	2460	〃	+
Coprinus lagopus	2125	Cholnam	++
〃	460	Kyonggi	++
〃	1415	〃	+
Coprinus micaceus	1388	〃	+
〃	2112	Cholnam	-
〃	3295	Cheju	-
〃	2056	Kyonggi	+++++
Coprinus polactiris	2824	Kangwon	+
Coprinus radians	2220	Nepal	+
Coprinus versicolor	871	Kyongnam	++++
Coprinus sp	2927	Taegu	++
〃	2634	〃	++
〃	2695	〃	++
〃	2177	〃	+
〃	2621	〃	+
〃	79	〃	+++++
〃	223	〃	+

Culture condition : Temp. 25℃, Culture periods : 6 days.
Code : + = Positive results, - = Negative results

ties of alkaline-active cellulases.

2. Materials and Methods

2.1 Microorganism

Fungi used showed in Table 1. The fungus was grown on potato dextrose agar at 25℃ for 5 days and then stored at 4℃. Stock cultures were transferred to fresh medium every 3 weeks and incubated under the same condition.

2.2 Media

For production of alkaline-active cellulase, we used eight media (Table 2).

2.3 Screening of optimum fungus for production of alkaline-active cellulase

Table 2. Composition of medium

Medium Ⅰ	Medium Ⅱ	Medium Ⅲ	Medium Ⅳ
Avicel 5g	*Quercus* 5g	*Pinus* 5g	α-cellulose 5g
Peptone 10g	Peptone 10g	Peptone 10g	Peptone 10g
Yeast extract 1g	Yeast extract 1g	Yeast extract 1g	Yeast extract 1g
KH_2PO_4 1g	KH_2PO_4 1g	KH_2PO_4 1	KH_2PO_4 1g
$Na_2HPO_4 \cdot 12H_2O$ 200mg	$Na_2HPO_4 \cdot 12H_2O$ 200mg	$Na_2HPO_4 \cdot 12H_2O$ 200mg	$Na_2HPO_4 \cdot 12H_2O$ 200mg
$MgSO_4 \cdot 7H_2O$ 1g	$MgSO_4 \cdot 7H_2O$ 1g	$MgSO_4 \cdot 7H_2O$ 1g	$MgSO_4 \cdot 7H_2O$ 1g
$CaCl_2 \cdot 2H_2O$ 20mg	$CaCl_2 \cdot 2H_2O$ 20mg	$CaCl_2 \cdot 2H_2O$ 20mg	$CaCl_2 \cdot 2H_2O$ 20mg
$FeSO_4$, $MnSO_4$ 10mg	$FeSO_4$, $MnSO_4$ 10mg	$FeSO_4$, $MnSO_4$ 10mg	$FeSO_4$, $MnSO_4$ 10mg
$ZnSO_4$, $CuSO_4$ 10mg	$ZnSO_4$, $CuSO_4$ 10mg	$ZnSO_4$, $CuSO_4$ 10mg	$ZnSO_4$, $CuSO_4$ 10mg
Urea 20g	Urea 20g	Urea 20g	Urea 20g
Tween-80 1g	Tween-80 1g	Tween-80 1g	Tween-80 1g
Distilled water 1000ml	Distilled water 1000ml	Distilled water 1000ml	Distilled water 1000ml
Medium Ⅴ	**Medium Ⅵ**	**Medium Ⅶ**	**Medium Ⅷ**
Glucose 5g			
Peptone 10g	Dextrose 30g	CMC 10g	Wheat bran 10g
Yeast extract 1g	K_2HPO_4 1g	$(NH_4)_2SO_4$ 0.1g	Peptone 5g
KH_2PO_4 1g	$MgSO_4 \cdot 7H_2O$ 0.5g	KH_2PO_4 0.1g	KH_2PO_4 5g
$Na_2HPO_4 \cdot 12H_2O$ 200mg	KCl 0.5g	$MgSO_4$ 0.1g	Yeast extract 0.5g
$MgSO_4 \cdot 7H_2O$ 1g	$FeSO_4 \cdot 7H_2O$ 10mg	$CaCl_2 \cdot 2H_2O$ 0.1g	$MgSO_4 \cdot 7H_2O$ 0.5g
$CaCl_2 \cdot 2H_2O$ 20mg	Yeast extract 15g	Urea 0.5g	Tween-80 1g
$FeSO_4$ 10mg	Bacto agar 15g	Peptone 1g	Distilled water 1000ml
$MnSO_4$ 10mg	Distilled water 1000ml	Tween-80 1g	
$ZnSO_4$ 10mg		Distilled water 1000ml	
$CuSO_4$ 10mg			
Urea 20g			
Tween-80 1g			
Distilled water 1000ml			

For screening of optimum fungus for production of alkaline-active cellulase, we prescreened five species out of twenty nine species by the rate of growth on potato dextrose agar (pH 5.5, 25℃, 5 days). The prescreened strains were grown on potato dextrose agar plates that adjusted to 5, 7 and 9 by addition of 0.1 N sodium hydroxide and hydrochloric acid for 25℃, 5 days respectively. By the rate of growth on pH 5, 7 and 9, we screened optimum fungus out of five species prescreened.

2.4 Optimum culture temperature

For evaluating effect of culture temperature on mycelium growth, we examined mycelium weights between 25℃ and 41℃ in shake cultures. Shake cultures were carried out in 250 mL Erlenmeyer flasks containing 100 mL of medium Ⅴ (Table 2). The inoculated flasks were shaken continuously at 120 rpm. Mycelium weights were evaluated by dry mycelium weights using glass microfibre filter (Whatman, GF/A 4.7 cm).

2.5 Preparation of crude enzymes

After the fungi had attained confluent growth, spores and mycelia were washed from the surface with 5 mL of sterilized water and used as an inoculum. The cell suspension (2 mL) was added to 100 mL of 2% yeast extract solution that adjusted to 9.0 by addition of 1 N sterilized sodium carbonate in shake cultures. Shake cultures were carried out in 250 mL Erlenmeyer flasks continuously at 120 rpm, 35℃ for 2 days. One-hundred milliliters of various media in a 250 mL Erlenmeyer flask was inoculated with 5 mL of 2% yeast extract fungal inoculum and shaken at 120 rpm, 25℃ for 5 days. Samples from each day were centrifuged at 4000 rpm for 10 min. and the supernatant solution was used as crude enzymes.

2.6 Enzyme assays

For determination of carboxymethylcellulase activity (CMCase), soluble carboxymethyl cellulose (Sigma. Ltd.) was prepared by suspending 1g of CMC in 100 mL of 50 mM glycine-sodium hydroxide buffer (pH 9.0), or tris (hydroxymethyl) aminoethane-hydrochloric acid buffer (pH 7.0), or sodium acetate-acetic acid buffer (pH 5.0) and stirring the suspension until completely dissolved.

CMCase activity was measured in the reaction mixture containing 0.5 mL of CMC in buffer and 0.5 mL of crude enzyme was incubated at 50℃ for 30 min. The reaction was stopped by adding 3 mL DNS color reagent to estimate reducing sugars.[17]

Filter paper culture enzyme activity was measured in buffer at each pH. Filterpaper activity (Fpase) was measured in the reaction mixture containing a piece of filter paper (50 mg, 1×6 cm, Whatman No. 1) in 1.5 mL buffer, and 0.5 mL of crude enzyme was incubated at 50℃ for 60 min.

Except using birch xylan (Sigma. Ltd.) as the substrate, xylanase assays were identical to CMCase assay.

One unit (IU) of each enzyme activity was defined as the amount of protein which produced 1.0 μ mole of reducing sugar, expressed as glucose, per min. Protein was determined by the Lowry Method[18] with bovine serum albumin (Sigma. Ltd.) as a standard.

Buffers used for studying the effect of pH on enzyme activity were 50 mM of sodium acetate-acetic acid buffer (pH 3.6 to 5.6), potassium phosphate-sodium hydroxide buffer (pH 5.8 to 8.0), and tris (hydroxymethyl)aminoethane-hydrochloric acid buffer (pH 8.4 to 9.0), and glycine-sodium hydroxide buffer (9.0 to 10.6). For thermostability studies, the crude enzyme was treated for 30 min. at various temperatures during enzyme activity.

2.7 Partial purification of the enzymes

Crude enzyme passed through a Sephadex G25 medium (1.5×100 cm) and was detected by U.V detector (UV-7000, EYELA, Japan). Enzyme activity at pH 5.0 and 9.0 in each fraction was measured.

3. Results and Discussion

3.1 Screening of optimum fungus

Of the 29 fungal strains in the preliminary screening, five strains were screened. As shown in Table 3, *Coprinus sp.* 79 grew much slower than *Coprinus cinereus* 2249. *Coprinus cinereus* 2249 showed excellent growth at various pH regions, especially pH 9 within 5 days of cultivation. *Coprinus cinereus* 2249 as

Table 3. Comparison of mycelium growth in various pH

Coprinaceae	pH 5	pH 7	pH 9
Coprinus micaceus 2056	++	+	+
Coprinus versicolor 871	+	+	+
Coprinus cinereus 2249	+++	+++	+++++
Coprinus disseminatus 2221	+	+	+
Coprinus sp. 79	++	++	++

Culture condition : Temp. 25℃. Culture time : 5 days
Medium : Potato dextro agar

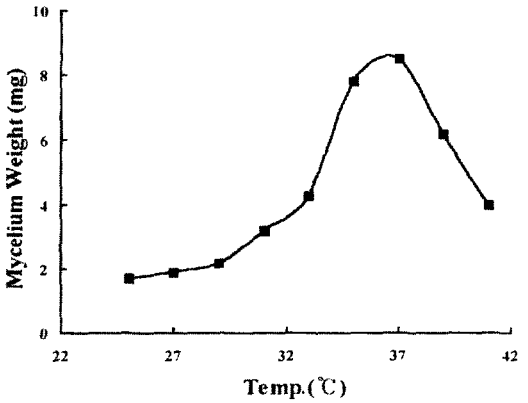

Fig. 1. Temperature and growth with *Coprinus cinereus* 2249 on liquid culture.

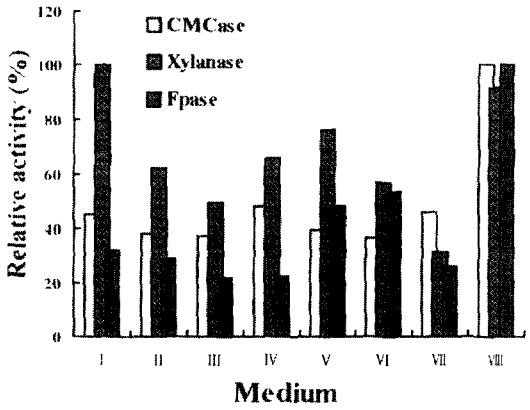

Fig. 2. Enzyme activity on various media.

optimum fungus for alkaline active cellulase production was used for the further experiment.

3.2 Effect of culture temperature on mycelium growth

As shown in Fig. 1, *Coprinus cinereus* 2249 produced higher mycelium weight at 35~37℃ than any other temperature, thus indicating the mesophilic nature of this organism.

3.3 Optimum medium for production of alkaline-active cellulase

To find the most favorable medium for the production of alkaline-active cellulase, we examined various media (Table 2). As shown in Fig. 2, Medium Ⅷ produced the higher CMCase and Fpase activity at pH 9.0 than other media. Through the medium Ⅰ that produce large amounts of xylanase activity, we have found that medium Ⅷ seems to be optimal basal medium for production of alkaline-active cellulases.

3.4 Effects of carbon and nitrogen sources on enzyme production

As shown in Fig. 3, media containing different carbon sources were examined in order to evaluate their effect on enzyme pro-

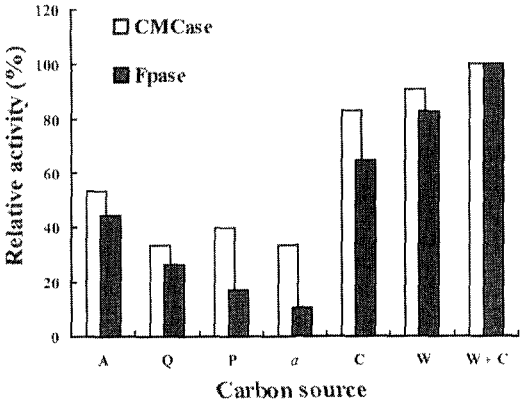

Fig. 3. Effect of different carbon source on the enzyme production.
A: 1% Avicel, Q : 1% Quercus, P : 1% Pinus, α : 1% α-cellulose, C: 1% CMC, W : 1% Wheat bran, W + C : 1% Wheat bran + 0.5% CMC

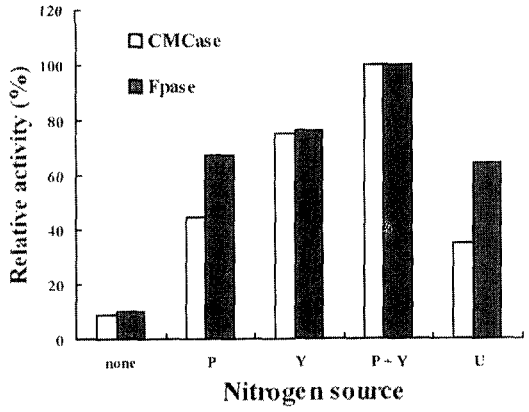

Fig. 5. Effect of nitrogen source on the enzyme production.
P: 0.5% Peptone, Y: 0.05% Yeast extract, U: 0.5% Urea

duction. The carbon sources examined were: avicel(1%), α-cellulose(1%), two kinds of wood meal(*Quercus mongolica* and *Pinus densiflora*, 1%), CMC(1%) and mixture of CMC(0.5%) and wheat bran(1%), wheat bran(1%). Of the carbon sources tested, mixture of CMC and wheat bran gave the highest production of CMCase and Fpase.

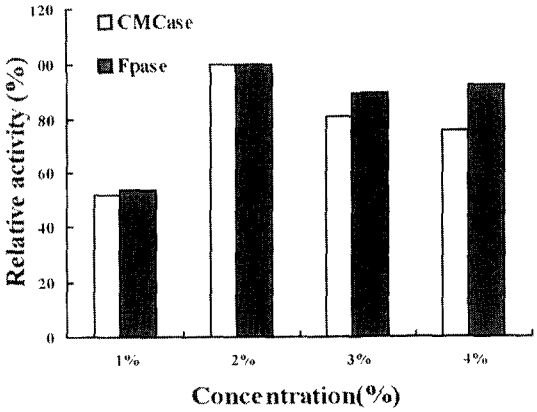

Fig. 4. Effect on concentration of wheat bran on the enzyme production.

Similar results had been reported in the strain of *Bacillus*[12,13] and *Cephalosporium*.[19,20]

We have found that cultivation on wheat bran as carbon source for production of alkaline active cellulases was more effective than CMC. To examine effect of wheat bran, several concentrations of wheat bran for production of alkaline-active enzyme were tested. As shown in Fig. 4, concentration of 2% wheat bran was good for enzyme production.

Higher production of alkaline active cellulase required the presence of complex nitrogens, such as that in 0.5% peptone and 0.05% yeast extract (Fig. 5). Same results had been reported in the strain of *Bacillus*.[11] In contrast to these results improved enzyme activity by addition of sugars, we did not find any improvement in *Coprinus cinereus* 2249.

3.5 Effect of pH, temperature and cultivation time on enzyme production

Enzyme production was improved in shake culture with regard to various pH of cultivation, cultivation time and temperature (Figs. 6, 7 and 8). The effect of initial pH

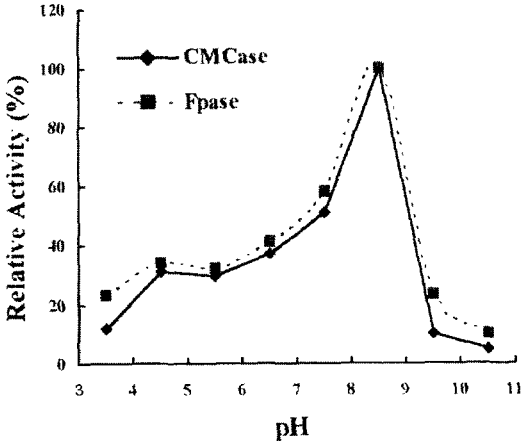

Fig. 6. Effect of initial pH on the enzyme production.

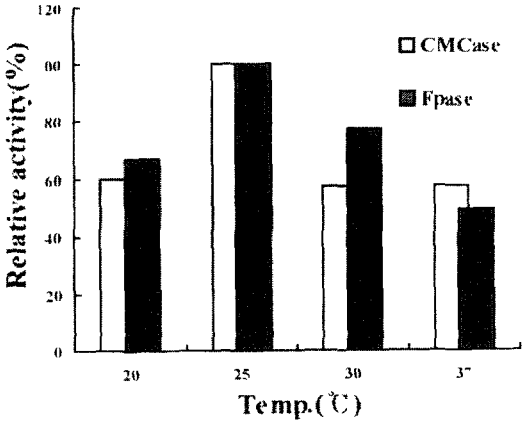

Fig. 7. Effect of temperature on the enzyme production.

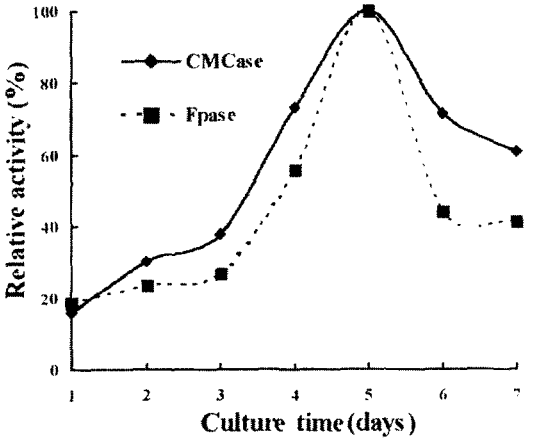

Fig. 8. Effect of culture time on the enzyme production.

of culture broth on the enzyme production was examined. The highest activity of CMCase and Fpase was produced at pH 9.0, thus indicating of alkalophilic nature of *Coprinus cinereus* 2249. But, increasing of initial pH up to 10 dramatically reduced activity.

The optimum temperature and cultivation time for the production of CMCase and Fpase was determined by varying the incubation temperature and time. The highest activity was observed at 25℃ for 5 days.

These results were similar to the strain of *Cephalosporium*[19] and *Bacillus*.[12,13]

3.6 Effect of reaction temperature and pH on crude enzyme

The optimum reaction temperature for the activity toward CMC and Filter paper was determined by varying the incubation temperature. As shown in Fig. 9, CMCase and Fpase activity were active in a wide temperature range, 30℃ to 60℃. The optimum temperature was about 50℃. But, increasing incubation temperature up to 55℃ dramatically reduced CMCase activity. Otherwise, Fpase activity was stable up to 60℃. The effect of pH on the CMCase activity of crude enzyme was examined in buffers of a wide pH range from 4 to 10. As shown in Fig. 10, the enzyme was active over a broad pH range, from 4 to 11, and most active at pH 9.

3.7 Partial purification

As shown in Fig. 11, fraction I showed strong activity at pH 9 and low activity at pH 5. In comparison with fraction I, the

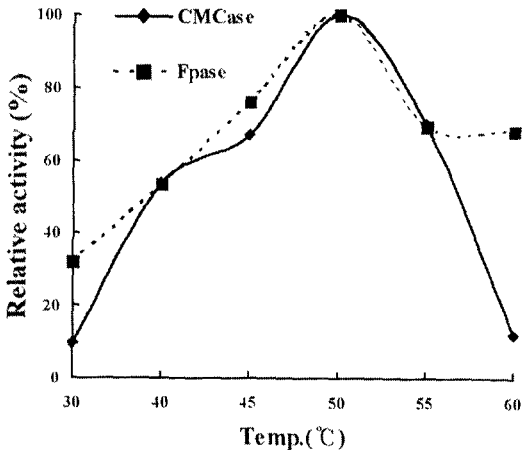

Fig. 9. Effect of temperature on the crude enzyme.

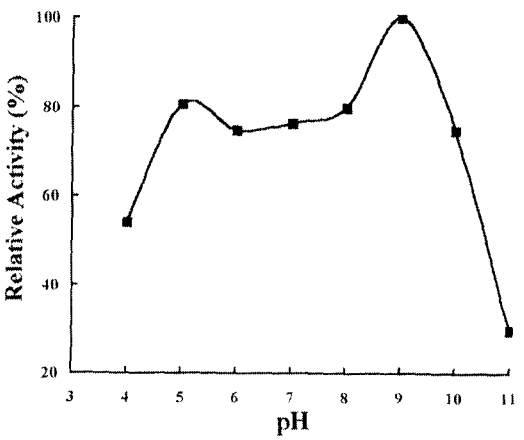

Fig. 10. Effect of pH on the crude enzyme.

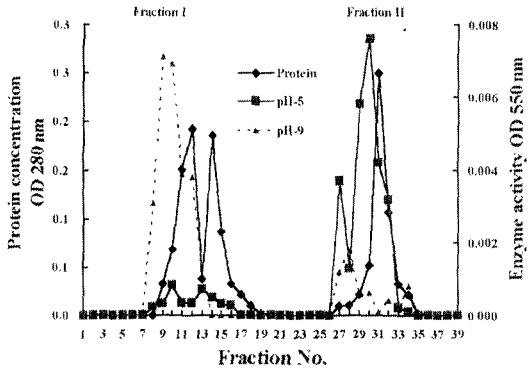

Fig. 11. Sephadex G25 medium gel chromatography of the crude enzyme.

fraction Ⅱ showed higher activity at pH 5. Such results suggested that *Coprinus cinereus* 2249 have two kinds of cellulases, namely acidophilic and alkalophilic cellulase.

4. Conclusions

Among twenty-nine species, we screened *Coprinus cinereus* 2249 for production of alkaline-active cellulase. *Coprinus cinereus* 2249 constitutively produced alkaline carboxymethyl cellulase (CMCase) and filter paper cellulase (Fpase). Enzyme activity was observed at an initial pH 9, thus illustrating the alkalophilic nature of this organism.

We have investigated alkaline activity in various media. Among these media, cultivation on complex carbon source gave a higher cellulase activity than other culture media. When cultivated at pH 9.0, 25℃ and 5 days, *Coprinus cinereus* 2249 produced higher alkaline activity on 0.5% CMC, 2% wheat bran as carbon source and 0.5% peptone, 0.05% yeast extract as nitrogen source compared with other culture conditions. The fungus grew at 35℃ but enzymes were produced at 25℃. Crude enzymes prepared with these conditions showed higher Fpase activity than CMCase activity and produced high activity at pH 9, 50℃.

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