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OPTIMIZATION OF CHITINASE ENZYME FROM *CERCOSPORA* SPECIES

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ABSTRACT : Chitinase producing fungi were isolated from rotten edible mushrooms from local markets of Jabalpur. There were 5 isolates of fungi which grew well at 28°C on Sabarouds dextrose agar and gave clear zone in chitin agar. Isolate A_2 showed maximum diameter of zone hence it was selected for further optimization studies. It was tested for chitinase production on Enzyme production medium (EPM) with shaking at 150 rpm for 12 days. This isolate was tested for optimum condition. The chitinase activity shown by this isolate was 12.02 unit/ml when cultivated on EPM mixed with colloidal chitin and peptone, initial pH 4 and cultivated at 28°C. Based on morphological examination, isolate A_2 was identified as *Cercospora* sp.

Key words : *Cercospora sp., Chitinase activity, Colloidial chitin, Edible mushrooms.*

INTRODUCTION

Chitin came into being in 1823 when Frenchman Oldier boiled the wing of insects with potassium hydroxide only to obtain non-soluble substances which subsequently was named chitin (www.ampleeffect.com/chitin.htm). Chitin, a linear homopolymer of N-acetylglucosamine is widely distributed in nature as a principal structural component in the cell walls of fungi and, in the exoskeleton of crustaceans and arthropods (Gohel *et al.*,2005). Great deal of interest has been generated on chitinases because of their applications. Chitin is the second most abundant renewable natural resource with worldwide annual recovery of 1-100 metric tones (Rattanakit *et al.*,2002).

The first step in chitin digestion is hydrolysis of β-1, 4 linkages between the N-acetyl glucosamine (NAG) molecules mediated by chitinases an extracellular inducible enzymes that are found in a variety of organisms. Recently, chitinases have received increased attention because of their wide range of biotechnological applications, especially as biocontrol agents against fungal phytopathogens, in production of chitooligosaccharides (immune enhancers), N-acetyl glucosamine, protoplast from yeast and fungal species (Dahiya *et al.*,2005) and single-cell protein from chitinous waste (Vyas & Deshpande,1991 and Singh *et al.*,2009). The cell walls of the parasitized *A.bisporus* fruit bodies are thin in comparison to the thicker and more electrondense walls of healthy cells, supporting the view that cell-wall-degrading enzymes may play an important role in the infection process, as previously postulated by Michaels (1973), Trigiano & Fergus (1979) and Kalberer (1984). The cell wall of fungi is a complex structure composed of glucans (a & p), glycans, chitin and lesser quantities of proteins and lipids (Bartnicki-Garcia,1968). Successful cell wall degradation may therefore depend on the activity of various enzymes (Calonje *et al.*,1997). The present work was undertaken to investigate the chitinase enzyme secreted by *Cercospora* sp. isolated from *Agaricus bisporus*. It may be one of the enzymes providing aggressiveness to the isolated mycoparasite and is a potent source of chitinases.

MATERIAL AND METHODS

Sample collection : Samples were collected from rotten mushrooms in sterile polythene bags.

Method for isolation : Isolation was done in Sabarouds dextrose agar media by serial dilution method and incubated at 28°C±1 °C for one week.

Screening of chitinolytic activity : Chitin agar media was used for screening. Colloidal chitin was prepared from commercial chitin by the method of Roberts & Selitrennikoff (1988) & was amended in the chitinase assay medium as a sole carbon source. Fungal cultures were point inoculated over 15 ml of chitin agar media containing bromocresol purple dye as an indicator and incubated at 28°±1°C for 7 days.

Fungal identification : Slide culture of selected fungus was prepared. Lactophenol cotton blue mount was also prepared (Aneja,2003).

Chitinase assay : Colloidal chitin was prepared by the method of Mathivanan (1995). Colloidal chitin broths (100 ml) in 250 ml capacity Erlenmeyer flasks were inoculated with 1.0 ml spore suspensions (adjusted to 1.0 O.D. 600) of the isolates and incubated in rotary incubator at 150 rpm and 37 °C to 40°C for 12 days. The culture broths were centrifuged at 8000 g for 20 min

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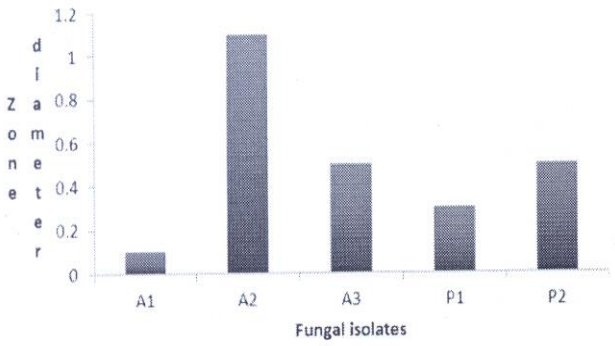
and the cell free supernatant saturated with ammonium sulphate to 60-70% levels and kept at 4°C overnight to extract the enzymes. The precipitates were dissolved in 50 mM phosphate buffer (pH 7.0) and dialysed against distilled water at 4°C overnight through a dialysis membrane with a molecular weight cut off at 12,000 Da. The stocks thus obtained were preserved at 0-4°C in PVC bottles. The assay system of Monreal and Reese for estimating reducing sugars released by enzyme action was adapted for the study. (Monreal & Reese,1969 and Bansode & Bajekal,2006).

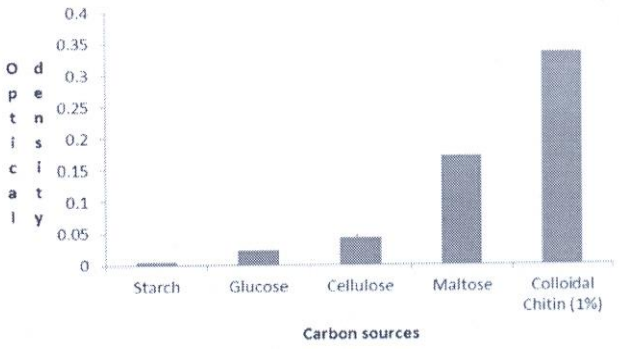
Optimization of chitinase production : An optimum level of carbon and nitrogen source concentration on chitinase production was determined by amending different concentrations of carbon and nitrogen sources in a chitin yeast extract medium. The carbon sources such as starch, glucose, maltose, cellulose and colloidal chitin (1%) and nitrogen sources such as ammonium chloride, potassium nitrate, *L.asparagine*, *L.glutamine* and peptone were supplemented with the CYE medium to study their influence on chitinase production. The impact of pH and temperature on chitinase production was investigated by cultivating isolate in the CYE medium at various pH ranging from 4 to 10 and temperature ranges of 28° to 70°C for 60 hr.

RESULTS AND DISCUSSION

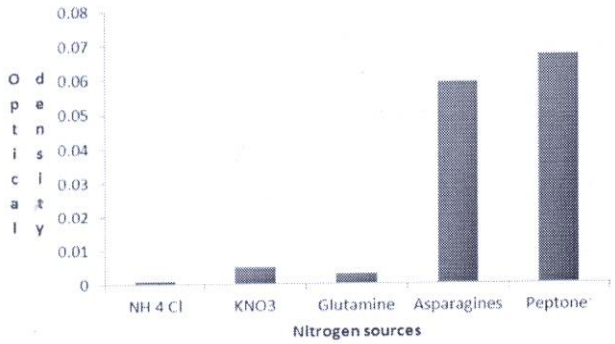
Isolation : In this piece of work five fungal isolates were obtained which were named as A_1, A_2, A_3, P_1, and P_2 (Table.1). Isolate A_2, *Cercospora* sp. (Fig.1) was used in this study for chitinase activity.



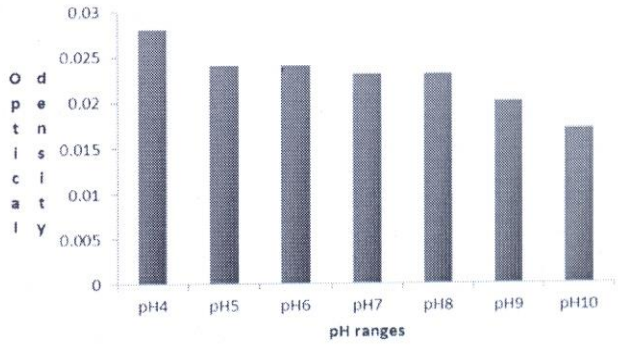
Graph 1 Chitinase production test.



Graph 2 Optimization of carbon source.



Graph 3 Optimization for nitrogen source.



Graph 4 Optimization of pH.

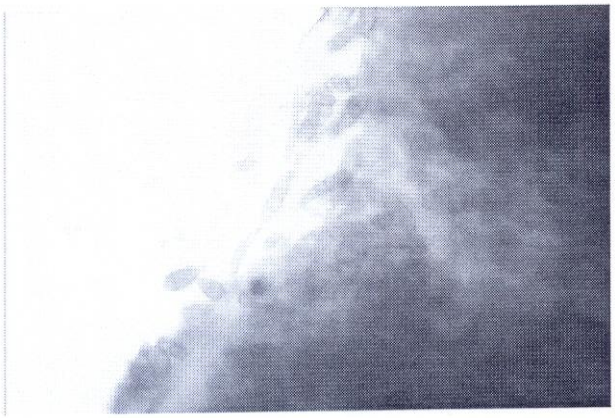


Fig. 1 *Cercospora* sp.

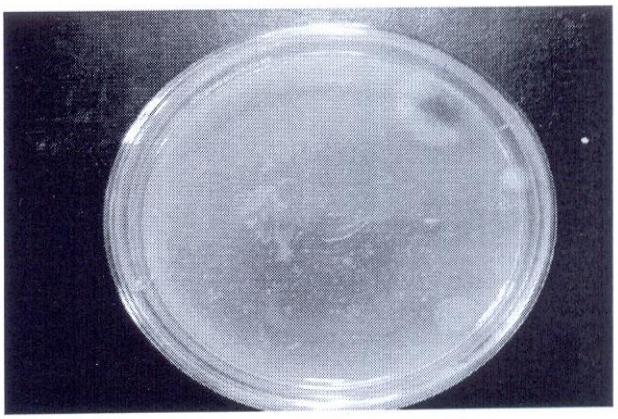


Fig. 2 Chitinase production test.

Screening of chitinolytic activity : Inoculated culture plates containing bromocresol purple dye were observed for the clear zone formation after 7 days of incubation. Purple colored zone of clearance were observed. Colour intensity and diameter of the zone were taken as the criteria to determine the enzyme activity. The isolates were grouped according to the diameter of the zone as : Isolates showing low and highest chitinase activity (Table.2). *Cercospora* sp. showed the highest chitinase activity (Fig.2) (Graph-1).

Table. 1 Fungal isolates.

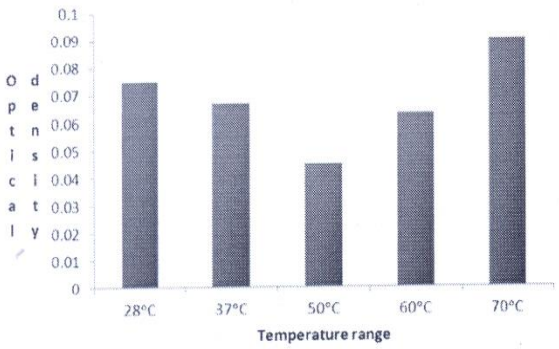
S.	Chitin source	Fungi isolated	Identified fungi
1.	*Agaricus* sp.	A_1	*Aspergillus* sp.
		A_2	*Cercospora* sp.
		A_3	*Aspergillus* sp.
2.	*Pleurotous* sp.	P_1	*Rhizopus* sp.
		P_2	*Rhizopus* sp.

Table. 2 Chitinase production test.

S.	Strain	Result	Diameter of the zone	Group
1.	A_1	+ve	0.1 cm	Low chitinase activity
2.	A_2	+ve	1.1 cm	High chitinase activity
3.	A_3	+ve	0.5 cm	Medium chitinase activity
4.	P_1	+ve	0.3 cm	Low chitinase activity
5.	P_2	+ve	0.5 cm	Medium chitinase activity

Table. 3 Chitinase activity.

S.	Time	O.D
1.	0 min	0.008
2.	1 hr.	0.016



Graph 5 Optimization for temperature.

Table. 4 Optimization for carbon source.

S.	C-source	O.D
1.	Starch	0.004
2.	Glucose	0.022
3.	Cellulose	0.042
4.	Maltose	0.171
5.	Colloidal chitin (1%)	0.335

Table. 5 Optimization for nitrogen source.

S.	N-source	O.D
1.	NH_4CL	0.001
2.	KNO_3	0.005
3.	Glutamine	0.003
4.	Asparagines	0.059
5.	Peptone	0.067

Table. 6 Optimization for pH.

S.	pH	O.D
1.	4.0	0.028
2.	5.0	0.024
3.	6.0	0.024
4.	7.0	0.023
5.	8.0	0.023
6	9.0	0.020
7.	10.0	0.017

Table. 7 Optimization for temperature

S.	Temp. °C	O.D
1.	28	0.075
2.	37	0.067
3.	50	0.045
4.	60	0.063
5.	70	0.09

Chitinase assay : Crude enzyme was produced from the selected strain of *Cercospora* sp. Chitinase assay was done by Monreal and Reese method. O.D of crude enzyme was taken at 540 nm. With increasing time due to enzyme activity chitin breaks into NAG due to which O.D increases after 1 hr of incubation (Table.3). Thus the crude enzyme shows positive chitinase activity.

Optimization of chitinase production : The selected strain of *Cercospora* sp. was cultured on CYE medium amended with different carbon (Table.4) (Graph-2) and nitrogen sources (Table.5) (Graph-3) and at different pH (Table.6) (Graph-4) and temperature (Table.7) (Graph-5) for optimization and to study the influence of these factors on chitinase enzyme production.

Cercospora sp. showed best chitinase activity at pH 4, Temperature 28°C, Peptone as nitrogen source and 1% colloidal chitin as carbon source, when incubated for 7 days. The chitinase activity of *Cercospora* sp. under these optimal conditions was 12.02 unit/ml.

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