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Full Length Research Paper

Biosynthesis of cellulolytic enzymes by *Tricothecium roseum* with citric acid mediated induction

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Tricothecium roseum, **an unexplored fungus for the production of cellulolytic enzymes, was used in this study for carboxymethylcellulase (CMcase) and β-glucosidase production. The culture filtrate of this organism exhibited highest total cellulolytic activity and extracellular protein content on 9th day during the course of its growth on potato dextrose yeast extract (PDYE) broth medium supplemented with 1.0% (w/v) carboxymethylcellulose. Among various soluble carbon and complex nitrogen sources tested in this study, caboxymethylcellulose and peptone supported maximum production of both cellulolytic enzymes. Under all suitable growth conditions, the enzyme biosynthesis was remarkably increased when the inducer citric acid was added to the PDYE media containing carboxymethyl-cellulose. Maximum CMcase production (1.59 U/ml) and β-glucosidase production (1.82 U/ml) were achieved in citric acid amended PDYE media at 37°C. Both the cellulolytic enzyme production was highest at initial pH 6.5 and after 7 days incubation. The study clearly demonstrated that citric acid is a good inducer for extracellular cellulolytic enzyme production by the fungus.**

Key words: *Tricothecium roseum*, cellulolytic enzymes, CMcase, β-glucosidase.

INTRODUCTION

Cellulase production is considered as important process in the economical production of ethanol (Bhat, 2000). Since petroleum products attained high prices, we are in need of finding alternate fuel sources and one of them is bioethanol. The enzymatic digestion cellulosic substrate by fungal cellulases has been proved as economic feasible for the conversion of cellulose into fermentable sugars and fuel ethanol (Mandels and Sternberg, 1976; Doppelbauer et al., 1987). For the complete enzymatic hydrolysis of cellulose, it needs synergistic activity of three cellulolytic enzymes such as cellobiohydralase, carboxymethylcellulase (CMcase) and β-glucosidases (Ryu and Mandels, 1980). Optimization of the culture medium and culture conditions influences the enzyme production. Even though there are many reports on cellulase producing fungi (Shin et al., 2000), only few have high activities for commercial success (Kang et al., 1994; Elad, 2000). In our exploration for cellulolytic fungi, *Tricothecium roseum* was found to be a high producer of cellulolytic enzyme under suitable nutritional and environmental conditions. The present study was attempted to evaluate the influence of citric acid on the induction of cellulase production by the fungus under different cultural conditions, and also to determine the optimum conditions for the enzyme activity.

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MATERIALS AND METHODS

Culture conditions

Tricothecium roseum which was originally isolated from the phyllo-plane of apple trees was obtained from microbial culture collection from Bio-control and Microbial Metabolite Lab, Center for Advanced Studies in Botany, University of Madras, India, was used in this study. The fungus was maintained in Potato-Dextrose Agar (PDA) slants.

Culture conditions for the enzyme production

Sterile 50 ml of three different media (minimal, Czapek-Dox and Potato dextrose yeast extract agar (PDYE)) each amended with 1%

Table 1. Growth (cm) of *Tricothecium roseum* on different cellulosic substrate.

Cellulosic substrate	Incubation time (days)						
	1	2	3	4	5	6	7
CM Cellulose	1.2*	2.4	3.8	5.0	5.6	7.0	7.8
Cellobiose	1.0	2.2	3.4	4.0	5.2	5.6	6.4
Filter paper	0.8	1.4	3.2	3.4	4.2	5.8	6.4

*Values presented are average growth (cm) of two separately conducted experiments.

cellulose as carbon source was distributed in sterile 250 ml Erlenmeyer flask. Meanwhile, the spore suspension was prepared in sterile distilled water from 6-day old cultures of *T. roseum* grown on Potato-Dextrose Agar (PDA) slants. The flasks were inoculated with a density of 2 x 10^6 spores and incubated at 28°C on a rotary shaker (180 rpm). Flasks were withdrawn at 3-day intervals over a period of 2 weeks and filtered through Whatman No.1 filter paper to separate mycelial mat and culture filtrate. Biomass of the culture was dried at 70°C in an oven until constant weight and then measured. Fungal growth was expressed in terms of dry weight of mycelial mat (mg/flask). The content of soluble protein in the culture filtrate was estimated according to method of Lowry et al. (1951) with bovine serum albumin as a standard. Total activity of cellulase complex and or individual component enzyme activities in the culture filtrate were determined as per procedures described below. In view of maximum growth and cellulase activity on the PDYE medium at 9th day interval, subsequent experiments were carried out on this medium supplementation of carbon and nitrogen sources on growth, secretion of extracellular protein and cellulase production by *T. roseum* at only 9th day interval.

Conditions for induction of cellulase using citric acid

Induction of cellulase by citric acid was performed in 100 ml conical flask containing 50 ml PDYE broth and 1.0% CMC supplemented with or without the inducer citric acid (0.3%). Each flask was inoculated with 2 x 10^6 spores and incubated at 28°C for cellulase production. The fungal culture was filtered through Whatman No.1 filter paper and the filtrate was then centrifuged at 8,000 rpm for 15 min. The clear supernatant was recovered and stored at 4°C with few drops of sodium azide to avoid contamination. The optimum cultural conditions, such as incubation period, initial pH and incubation temperature, for production of cellulase in shake-flask culture were determined as described by Shibli et al. (2001).

Enzyme assay

Filter paper activity (FPA) for total cellulase activity in the cultural filtrate was determined according to the method of Mandels et al. (1976). Aliquots of appropriately diluted culture filtrate as enzyme source was added to Whatman No.1 filter paper strip (1 X 6 cm; 50 mg) immersed in one millilitre of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min. carboxymethylcellulase was measured as described previously (Ghosh, 1987) using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.05 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50°C for 1 h and the reducing sugar produced was determined by DNS method. One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min. β-Glucosidase activity was assayed by the method of Herr (1979). β-Glucosidase activity was measured in 1 ml of 5 mM p-nitrophenyl-6-D-glucopyranoside (PNPG) in 0.05 M citrate buffer (pH 5.0) and aliquots of appropriately diluted culture filtrate and incubated at 50°C for 30 min. The reaction was terminated by addition of 4 ml of 0.1 M NaOH-glycine buffer solution and the released p-nitrophenol was read at 410 nm. The activity was expressed in terms of liberation of p-nitrophenol from p-nitrophenyl-6-D-glucopyranoside (PNPG). One unit of the enzyme activity was defined as the amount of enzyme producing 1 µmole of p-nitrophenol per min.

RESULTS AND DISCUSSION

Ability of *T. roseum* to grow in three cellulosic substrates was tested, among which the fungi attained maximum mycelial radial growth of 7.8 cm in 7 days with carboxymethycellulose as a substrate (Table 1). From this result it is very evident that CM cellulose acts as easily utilizable source for the fungi. Kudanga and Mwenje (2005) demonstrated similar results in which the carboxymethycellulose induced the highest production of cellulase by *Aureobasidium pullulans*.

T. roseum was grown on three liquid media; minimal media, Czapeck Dox media and potato dextrose yeast extract media amended with 1.0% CMC at 28°C on a rotary shaker (180 rpm). During cultivation, growth and total cellulolytic activity (filter paper activity) and protein contents in the culture filtrate were monitored at 3-day interval over a period of 2 weeks and are presented in Table 2. During the course of incubation the growth of fungus in terms of dry weight of mycelial mat, increased on all 3 media. Among the 3 media tested, PDYE supported the maximum growth of *T. roseum*. In all growth media, *T. roseum* exhibited the highest cellulolytic activity at 9th day and showed declined cellulolytic activity in successive incubation days. Maximum cellulolytic activity of about 1.87 FPU per milliliter was observed in PDYE medium at 9th day interval. Similar pattern was also observed for the total protein content in this experiment. Hence it is clear that PDYE media is the best among three media for the growth and cellulolytic activity of *T. roseum*.

Cellulolytic activity exhibited by the *T. roseum* is comparable to the activity exhibited by well studied fungi *Trichoderma reesei* which showed activity ranges from 1-2 FPU/ml on different media (Tamada et al., 1989;

Table 2. Growth, total cellulolytic activity (filter paper activity*) and protein secretion by *Tricothecium roseum* on different media.

Incubation time (days)	Minimal media			Czapeck Dox media			PDYE media		
	Dry weight of mycelial mat (mg/flask)	Total cellulolytic activity (FPU/ml)	Total protein content (µg/ml)	Dry weight of mycelial mat (mg/flask)	Total cellulolytic activity (FPU/ml)	Total protein content (µg/ml)	Dry weight of mycelial mat (mg/flask)	Total cellulolytic activity (FPU/ml)	Total protein content (µg/ml)
3	134	0.38	52	182	0.72	64	287	0.78	87
6	161	0.64	127	211	1.17	172	324	1.52	197
9	367	0.92	162	433	1.62	211	586	1.87	234
12	529	0.47	67	560	0.76	98	642	0.94	120

Values are average of results of two separately conducted experiments.
*Filter paperase (F Pase) is expressed in terms of filter paper units (FPU). One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.

Table 3. Effect of supplementation of carbon source on cellulase production by *Tricothecium roseum.*

Carbon source	Dry weight of mycelial mat (mg/flask)	Protein(µg/ml)	Cellulase		
			FPU/ml[a]	CMcase[b] (U/ml)	β-glucosidase[c] (U/ml)
Glucose	763	210	0.23	0.28	0.36
Carboxymethyl cellulose	594	240	1.90	0.63	1.02
cellobiose	572	221	1.72	0.54	0.76

Values are average of two separately conducted experiments.
[a]Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.
[b]One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.
[c]One unit of 6-glucosidase activity is defined as the amount of enzyme liberating 1µmole of p-nitrophenol per min.

Table 4. Effect of supplementation of nitrogen source on cellulase production by *Tricothecium roseum.*

Carbon sources	Dry weight of mycelial mat (mg/flask)	Protein (µg/ml)	Cellulase		
			FPU/ml[a]	CMcase[b] (U/ml)	β-glucosidase[c] (U/ml)
Peptone	611	253	2.32	0.67	0.72
$NaNo_3$	512	201	1.68	0.32	0.48
Yeast extract	567	233	1.74	0.49	0.56

Values are average of two separately conducted experiments.
[a]Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.
[b]One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.
[c]One unit of 6-glucosidase activity is defined as the amount of enzyme liberating 1µmole of p-nitrophenol per min.

Domingues et al., 2000). The activity does not exceed more than 1.5 FPU/ml in the case of *Cellulomonas biazotea* (Rajoka et al., 1998), *Aspergillus niger* and *A. fumigatus* (Immanuel et al., 2007). Influence of different carbon sources in PDYE medium on production of cellulase by *T. roseum* was examined. Among all the carbon sources tested carboxymethylcellulose served as the best for the production of total protein, CMcase activity and β-glucosidase activity as presented in Table 3. Followed by carboxymethylcellulose, cellobiose induced the maximum production. Glucose as a carbon source increased the fungal growth but resulted in minimal production of cellulolytic enzymes and extracellular protein. Rajendran et al. (1994) similarly demonstrated the induction of cellulase production by *Humicola fuscoatra* in presence of cellulosic substrates. Kudanga and Mwenje (2005) also substantiated the same results and it is well in agreement with the present study. Cellulose production by *T. roseum* grown on different nitrogen sources at concentration of 0.03% was compared (Table 4). Among the tested complex nitrogen sources, the effectiveness in supporting cellulase production along

Table 5. Effect of culture temperature, initial pH and incubation period on extracellular CMcase synthesis from *Tricothecium roseum* on inducing medium containing CMC (1.0%) with or without citric acid (0.3%) supplementation.

Incubation condition	Enzyme activity in presence of CMC		Enzyme activity in presence of CMC + citric acid	
Temperature[a]	**CMcase (U/ml)**	**Relative activity (%)**	**CMcase (U/ml)**	**Relative activity (%)**
27°C	0.98	99.0	1.19	72.3
37°C	0.89	83.0	1.59	100.0
45°C	0.80	74.6	1.09	65.2
Intial pH[b]				
4.5	0.85	100.0	1.12	100.0
6.5	0.18	21.5	0.32	30.1
8.5	0.07	7.0	0.13	12.6
Incubation period[c]				
3 days	0.44	49.3	0.61	39.4
5 days	0.57	61.8	0.98	62.3
7 days	0.87	100.0	1.41	100.0
10 days	0.78	90.2	0.89	53.3

[a]Shake flask culture with an initial pH 4.6 for 7 days.
[b]Shake-flask cultures at 37°C for 7 days.
[c]Shake-flask culture with an initial pH 4.6 at 37°C.

Table 6. Effect of culture temperature, initial pH and incubation period on extracellular β-glucosidase synthesis from *Tricothecium roseum* on inducing medium containing CMC (1.0%) with or without antioxidant citric acid (0.3%) supplementation.

Incubation condition	Enzyme activity in presence of CMC		Enzyme activity in presence of CMC + citric acid	
	β-glucosidase (U/ml)	**Relative activity (%)**	**β-glucosidase (U/ml)**	**Relative activity (%)**
Temperature[a]				
27°C	1.02	105.0	1.28	78.3
37°C	0.96	91.0	1.82	99.0
45°C	0.93	87.2	1.15	73.4
Intial pH[b]				
4.5	0.92	100.0	1.12	100.0
6.5	0.23	31.2	0.47	38.5
8.5	0.11	7.57	0.24	24.3
Incubation period[c]				
3 days	0.59	54.4	0.75	43.6
5 days	0.68	72.7	1.34	68.9
7 days	0.96	100.0	1.52	100.0
10 days	0.86	97.7	1.08	62.7

[a]Shake flask culture with an initial pH 4.6 for 7 days.
[b]Shake-flask cultures at 37°C for 7 days.
[c]Shake-flask culture with an initial pH 4.6 at 37°C.

with growth, secretion of extracellular protein and cellulolytic activity by *T. roseum* decreased in the following order: peptone > yeast extract > $NaNO_3$.

It is evident in the present study that complex nitrogen source give good support for the enhanced enzyme production. Ellouz Chaabouni et al. (1994) demonstrated that crude complex substrates used in combination with local cellulose gave high cellulase yields. In contrast, the growth of *T. reesei* on production medium without nitrogen source increased cellulase production (Turker and Mavituna, 1987). The influence of incubation temperature, initial pH and incubation period on CMcase produc-

tion from the fungus when grown in CMC-containing medium supplemented with or without citric acid is given in Table 5. Production of the enzyme was very dependant on the environmental conditions. However, under all conditions, the enzyme production was remarkably enhanced by the addition of citric acid in the medium. CMcase production by the fungus was high in CMC and citric acid containing medium at 37°C, but in CMC containing medium it was high at 27°C. Therefore, the optimum temperature for CMcase production by the fungus might lie between 27 and 37°C. However, the same temperature was optimal for the production of β-glucosidase similar to that of CMcase (Table 6).

Malek et al. (1987) also demonstrated in *Cytophaga* sp. that 37°C is optimum temperature for the maximum cellulase production. Among various pH, maximum production of CMcase and β-glucosidase was observed in the pH 4.5. Media amended with citric acid showed enhanced production in both enzymes at pH 4.5. There are many reports on the requirement of pH of culture medium for extracellular enzyme production by fungi and bacteria, and in most cases the maximum lies between pH 4.5 and 5.5 (Coughlan, 1985). Highest CMcase and β-glucosidase activity was observed after 7 days of incubation, and thereafter the enzyme activity decreased. The reason for this may be because of autolysis of mycelium in prolonged incubation period leading to enzyme instability. In both cases of enzyme production, the media amended with the citric acid showed enhanced production after 7 days of incubation period. The role of a compound to act as inducer of cellulase biosynthesis varies from organism to organism. Sophorose is a strong inducer of cellulase in *Trichoderma* species (Loewenberg and Chapman, 1977).

In this study, the citric acid was a good inducer for CMcase and β-glucosidase biosynthesis by *T. roseum*. For many other organisms, cellobiose and cellulose are potential inducers of cellulase (Mandels and Reese, 1960; Lin and Wilson, 1987). The present study clearly shows that the inducer, citric acid has the ability to induce cellulolytic enzyme production by *T. roseum*. Therefore, it could be incorporated in production medium for increased production of cellulase by *T. roseum*. This unexplored fungus merits further attention as a potential source of extracellular cellulolytic enzymes.

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