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Biochemistry of postharvest spoilage of sweet potato (*Ipomoea batatas* L.). 2. Comparison of cellulolytic enzyme production in cultures and fungi-infected sweet potato tubers

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ABSTRACT

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The study was conducted to determine the production *in vitro* and *in vivo* of cellulases by *Botrydiplodia theobromae* and *Rhizopus oryzae*. Isolates of these organisms were obtained from the postharvest decay of sweetpotato tubers.

Results revealed that *B. theobromae* and *R. oryzae* which were isolated from postharvest spoilage of sweetpotato tubers produced endo-β-1,4-glucanase and exo-V-1,4-glucanase in culture and in fungi-infected tissues of sweetpotato tubers. The optimum temperature and pH for cellulase synthesis and activity were 30°C and pH 6.5, respectively.

Keywords: cellulase, fungi, spoilage, sweet potato

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INTRODUCTION

Sweetpotato (*Ipomoea batatas* L.) which is one of the important tuber crops in Asia (Villareal, 1982), is susceptible to a number of postharvest diseases (Ray and Balagopalan, 1997). *Botryodiplodia theobromae* and *Rhizopus oryzae* Went & Prins. are two important and most frequently occurring phytopathogenic fungi associated with internal moldiness of sweetpotato tubers in India (Ray and Misra, 1995; Ray and Punithalingam, 1995; Ray *et al.*, 1997).

Many phytopathogens produce extracellular cellulase which degrade cellulose components of the host cells (Fessehatzion and Olutiola, 1987; Nathawal *et al.*, 1991). Some of these microorganisms produce a distinct C_1-cellulase (cellulase 1,4-β-cellobio hydrolase; C_1:EC 3.2.1.91) which helps hydrolize insoluble and complex forms of cellulose to soluble and simple forms, mainly Cx-cellulase (cellulase β-1-4-glucan glucano hydrolase; Cx: EC 3.2.1.4) and β-glucosidase (cellobiase) while others produce C_1-cellulase as well (Bagga and Sandhu, 1987; famurewa and Olutiola, 1991). On the other hand, certain fungi including some members of phycomycetes produce only cellobiase (Mandels and Steinberg, 1976; Reese and Levinson, 1952). The study was undertaken to demonstrate production *in vitro* and *in vivo* of cellulases by *B. theobromae* and *R. oryzae*. Effects of temperature and pH on cellulase activity were also investigated.

MATERIALS AND METHODS

Fungal isolates

The isolates of *B. theobromae* and *R. oryzae* used in this study were isolated from the postharvest decay of sweetpotato tubers (Ray and Misra, 1995). Spore suspensions of both the fungi were prepared separately from 7 day old cultures grown at room temperature (28 ± 2^0C) on potato dextrose agar (PDA). Spores were harvested in sterile distilled water and diluted to a concentration of 5.5×10^6 spores/ml. The same concentration was used in all the experiments.

Sweetpotato tubers

Freshly harvested sweetpotato tubers (var. Pusa Safed) were collected from the experimental farm of the Regional Center of Central Tuber Crops Research Institute, Bhubaneswar during 1998-1999 rabi (January-February) season. The tubers were used within 3-4 days after harvest.

Enzyme synthesis

The utilization of insoluble (native) form of cellulose was investigated using the folded, oven-dried and pre-weighed filter papers (Whatman No. 1, 11 cm.). Individual filter paper circles after folding were placed in 250 ml Erlenmeyer flasks containing 100 ml liquid medium (KH_2PO_4, 1.0; $NaNO_3$, 2.0 g; $MgSO_4.7$, 0.5 g; KCl, 0.5 g; $FeSO_4.7\ H_2O$, 0.01 g; yeast extract, 10 g; distilled water, 1 L) and pH was adjusted to 6.0. The flasks were sterilized by autoclaving at 121°C for 15 min. Two ml of the spore suspensions (5.5 x 10^6 spores/ml) of the fungi *B. theobromae* and *R. oryzae* were inoculated into the flasks and the flasks were incubated for 20 days at room temperature (28 ± 2^0C). At 4-day interval, the pH of the medium was determined and the filter papers were then removed (neither washed nor the mycelium removed) and dried at 80°C for 24 h. A comparison of the dry weight of the filter paper at the end of incubation period was made with its dry weight at the start to determine the weight loss. Uninoculated filter papers served as controls. Three replicates were made in each case and the mean value of the three determinations was taken.

Utilization of soluble cellulose by the organisms was determined by growing the fungi in the above basal liquid medium except that instead of filter paper, carboxyl methyl cellulose (CMC) (low viscosity) at 10% level was added. CMC was deleted in some of the experiments to determine whether cellulolytic enzymes were produced in its absence. Erlenmeyer flasks (250 ml) containing 100 ml basal medium with CMC (10%) were inoculated with 2 ml of spore suspension (5.5 x 10^6 spores/ml) of either *B. theobromae* or *R. oryzae*, and the flasks in triplicate replications were incubated for 10 days at room temperature (28 ± 2^0C). It took nearly 3-4 days for spores to grow into mycelial mass when CMC was used as carbon source, and after 4 days of inoculation, at regular interval the content of growth flasks were pooled and filtered. The mycelial mat (cell mass after filtration) corresponding to each

harvest was dried in oven at 8°C at constant mass. The culture filtrate (CF) was used as the enzyme source.

Extraction of enzymes from infected sweetpotato tubers

Healthy and freshly harvested tubers were taken and surface sterilized with 5% sodium hypochlorite solution. These tubers were inoculated separately with mycelial discs of either *B. theobromae* or *R. oryzae* following the method described by Ray and Punithalingam (1966). The tubers were kept for 4 to 10 days at room temperature (28± 2°C) for rot development. Lesion areas and surrounding tissues were removed with a sterile scalpel weighed into 10 g samples and extracted by grinding with distilled water in a Waring Blendor in the ratio 1:3. The ground tissues were strained through three layers of cheese cloth to remove the pulp. The liquid fraction (not extract, RE) was cleared by centrifugation at 10,000 x g in a refrigerated centrifuge and was adjusted to the volume. The RE served as the enzyme source.

Enzyme assay

Carboxyl methyl cellulase (CMCase) (E.C.3.3.1.4) activity was determined by quantifying the reducing sugar released by 3,5-dinitrosalicylic acid (DNSA) reagent method using glucose as standard (Mahadevan and Sridhar, 1998). The assay medium was 0.55% CMC in 0.55 M acetate buffer (pH 5.2), and 4 ml of this was incubated with 1 ml of sodium acetate buffer and 2 ml of enzyme source (CF or RE) and incubated by combining 1 ml of the assay mixture with 3 ml of DNSA reagents (1 g DNSA, 200 mg crystalline phenol, 20 ml 2 M NaOH and 20 g potassium sodium tartarate in 100 ml distilled water). The mixture was boiled for 5 min in a water bath and cooled under running tap water. The absorbance was measured at 575 nm in a Bio-Aquarious UV-VIS Spectrophotometer and the amount of reducing sugars was calculated using a standard curve prepared from aqueous solution of D-Glucose of various concentrations (10-100 ug/ml). Enzyme (CMCASE) activity was expressed as mg glucose released/24 h/ml filtrate.

Effects of temperature on growth, cellulase synthesis and acitivity

Erlenmeyer flasks (250 ml) containing 100 ml basal medium for cellulase synthesis as earlier described were inoculated with two ml of spore suspensions of either *B. theobromae* or *R. oryzae* and incubated at 10^0C interval from 10 to 40^{0}C for 10 days. Triplicate flasks were removed from each group temperature group, the contents were filtered and cellulase activity of the culture filtrate (CF) was determined as described above.

Effects of pH on cellulase activity

The effect of growth medium pH was studied by incubating the growth flasks (in triplicates) containing 100 basal medium with 10% CMC of different pH (3.0-9.0).

RESULTS AND DISCUSSION

B. theobromae and *R. oryzae* associated with soft rot of stored sweetpotato produced extra cellular cellulase both in culture and in fungi-infected tissues (Table 1). The cellulase activity of rotted tubers infected with the fungi and of culture filtrate (CF) were 1.4 and 2.05 units for *B. theobromae*, and 2.73 and 2.32 units for *R. oryzae*, respectively, after 12 days incubation period. Earlier reports also showed that *R. oryzae* associated with soft-rot of potato tubers produced extra cellular cellulase both in culture and *Rhizopus*-infected tissues (Amadioha, 1993). Similarly, Adisa and Fajola (1983) reported cellulolytic enzyme production by *B. theobromae* during fruit rot of of *Citrus sinensis*. Further, the increase in enzyme activity were concomitant with the increase in cell mass of the fungi up to 8 days of the incubation period and thereafter, it declined. *R. oryzae* produced comparatively more cellulase than *B. theobromae* in CF. There was a decrease in cell mass after 8 days of growth probably due to autolysis (Lahoz *et al.*, 1976) or due to nutrient limitation (Scot, 1976).

After 12 days of incubation at room temperature (28 $\pm$ 2^{0}C), *B. theobromae* and *R. oryzae* significantly degraded the filter papers resulting in

Mycelial growth (cell mass) and cellulase activity (mg glucose/24 h/ml) of *B. theobromae* and *R. oryzae* in liquid cellulose medium during 12 days incubation period

	B. theobromae		*R. oryzae*	
	CF	RE	CF	RE
	0.6 ± 0.02* (176 ± 12)**	0.0 ± 00	1.3 ± 0.2 (175 ± 23)	1.0 ± 0.2
	2.68 ± 0.27 (828 ± 56)	0.8 ± 0.03	2.98 ± 0.34 (855 ± 53)	1.7 ± 0.2
	2.05 ± 0.33 (788 ± 24)	1.4 ± 0.21	2.32 ± 0.41 (630 ± 27)	2.73 ± 0.03

Standard error
Cell mass in mg in parentheses
Culture filtrate
Raw extract

Degradation of filter paper by cellulolytic enzymes produced by *B. theobromae* and *R. oryzae* at 28 ± 2°C and final pH of the liquid medium

	Incubation (days)	Wt. of filter paper (mg) Initial	Final	Wt. loss (%)	pH of the medium
	04	360 ± 1.2	354 ± 2.5	1.4 ± 0.8	6.8 ± 0.20
	08	360 ± 1.4	339 ± 2.8	5.8 ± 1.3	7.0 ± 0.50
	12	360 ± 1.3	208 ± 4.3	42.1 ± 2.6	7.1 ± 0.40
	16	362 ± 1.3	92.6 ± 2.9	74.4 ± 2.5	7.2 ± 0.24
	20	362 ± 1.0	78.3 ± 1.7	78.3 ± 1.7	7.0 ± 0.15
	04	362 ± 1.4	345 ± 2.9	4.6 ± 0.8	6.0 ± 1.8
	08	360 ± 1.3	320 ± 2.7	11.1 ± 1.3	7.2 ± 0.21
	12	361 ± 1.6	188.0 ± 4.8	47.9 ± 3.7	7.1 ± 0.25
	16	361 ± 1.3	88.3 ± 4.7	75.5 ± 2.5	7.0 ± 0.31
	20	362 ± 1.0	88.0 ± 4.3	75.5 ± 3.0	7.0 ± 0.12

Standard error

Table 3. Effect of temperature on mycelial growth (cell mass. mg) and cellulase activity (mg glucose/24 h/ ml) of *B. theobromae* and *R. oryzae* in liquid cellulase medium after 10 days incubation period

Temperature (°C)	*B. theobromae*		*R. oryzae*	
	Cell mass	Cellulase	Cell mass	Cellulase
10	8 ± 1	0.25 ± 0.07	54 ± 10	0.18 ± 0.08
20	350 ± 24	0.75 ± 0.12	398 ± 21	0.55 ± 0.12
30	850 ± 53	2.85 ± 0.07	845 ± 34	2.43 ± 0.24
40	612 ± 24	0.93 ± 0.18	748 ± 28	0.55 ± 0.18

± Standard error

Table 4. Effect of carboxy methyl cellulose (CMC) on growth (cell mass, mg) and cellulase activity (mg glucose / 24 h/ ml) of *B. theobromae* and *R. oryzae* in liquid culture medium after 12 days incubation period

Fungi	Culture Medium	Cell mass	Cellulase
B. theobromae	with CMC	566 ± 32	2.35 ± 0.11
	without CMC	28.8 ± 2.3	0
R. oryzae	with CMC	610 ± 18.4	2.61 ± 0.14
	without CMC	15.3 ± 1.6	0

± Standard error

Table 5. Effect of pH on growth (cell mass, mg) and cellulase activity (mg glucose / 24 h/ ml) of *B. theobromae* and *R. oryzae* in liquid cellulase medium after 10 days incubation period

pH	*B. theobromae*		*R. oryzae*	
	Cell mass	Cellulase	Cell mass	Cellulase
3.0	198 ± 12	0.45 ± 0.11	270 ± 21	0.63 ± 0.08
4.0	395 ± 11	0.88 ± 0.11	298 ± 30	1.00 ± 0.10
5.0	648 ± 22	2.25 ± 0.21	530 ± 27	2.28 ± 0.16
6.0	790 ± 31	2.55 ± 0.18	740 ± 28	2.80 ± 0.22
7.0	795 ± 28	2.88 ± 0.22	760 ± 14	2.80 ± 0.21
8.0	45 ± 8	0.38 ± 0.11	295 ± 22	0.31 ± 0.07
9.0	0	0	0	0

± Standard error

loss of dry weight (Table 2). There was a net weight loss of 78.3% for *B. theobromae* and 75.5% for *R. oryzae* at the end of 20 days incubation period. The final pH of the culture medium remained almost neutral (6.8-7.2). This result showed that both the fungi could produce C_1 cellulase in culture. The effects of temperature on the growth and production of the cellulases by the fungi in liquid media showed that activity of cellulases and mycelial growth were highest when the organisms were grown at 30°C (Table 3). The earlier results have shown that growth of *B. theobromae* was optimum at a temperature of 25-30°C with the peak at 28°C (Ray and Punithalingam, 1996) and that of *R. oryzae* was at 30°C (Ray *et al.*, 1997). Similarly, previous works have demonstrated that the postharvest grow best between 25-30°C (Adisa and Fajola, 1983; Amadioha, 1993; Somner, 1982).

The rot causing organisms produced neglible amount of mycelium when grown in a medium without CMC and the filtrates from these cultures were unable to hydrolyze CMC to reducing sugars (Table 4). The organisms showed significant growth in medium containing CMC and the culture filtrates were able to hydrolyze CMC to reducing sugars.

The cellulase synthesis and growth of the fungi were affected by pH of the medium (Table 5). While there was a general increase in growth and cellulase synthesis between pH 6 and 7, the growth and cellulase activity declined thereafter. This observation corroborated with earlier results (Amadioha, 1993; Ray and Punithalingam, 1996).

Both *B. theobromae* and *R. oryzae* produced cellulases in culture and infected sweetpotato tubers suggesting that the rot causing organisms were cellulolytic fungi. The action of the cellulase produced by these organisms might be conceived to act during pathogenesis in two ways: first, it was apparently involved in rot production and second, the hydrolytic products of cellulase activity might have provided the fungi with carbohydrates for its continued growth and survival in the host tissues. Under these conditions, the host tissues would probably be depleted of sugars.

The utilization of cellulases by fungi depends on their ability to produce two types of enzymes : β-1,4-glucano hydrolase or endo-β 1,4-glucanase (C_x) (Mandels and Steinberg, 1976). The present study shows that both *B. theobromae* and *R. oryzae* were capable of degrading native cellulases (filter

paper) as well as derived soluble cellulose (CMC). The development as the microorganisms have to break the skin of the tuber which is more or less insoluble in nature (Wood, 1960). This proves that both the fungi had ability to produce both C_1 and C_x enzymes. Moreover, when CMC was not added in the growth medium, there was virtually little growth and no cellulase activity.

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