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Note

Production of extracellular amylase and hemicellulase from four fungal pathogens

TSEAA SHAMBE
Department of Chemistry, University of Jos, P.M.B. 2084, Jos, Plateau State (Nigeria)
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Fungal plant pathogens are known to produce extracellular enzymes that cleave virtually every glycosidic linkage in plant cell-wall polysaccharides[1]. The carbohydrate in the environment of the pathogen regulates the growth of microorganisms and the enzymes produced[2,3]. The growth of fungus in submerged culture solution is often accompanied by pH changes with time[4,5]; the changes are dependent on sources of carbon if the initial culture pH is kept constant[4].

Alternaria (SA1222), *Colletrotrichum* (Coll), *Drechslera* (SA1208), and *Fusarium moniliforme* (SA1190) are fungal pathogens that attack agricultural crops, which are either staple crops or crops of economic importance. These crops, *e.g.*, rice (*Oryzo sativa*) and sorghum (*Sorghum bicolor*), are grown throughout West Africa. Sorghum and millet (*Pennisetum typhoides*), grown throughout the arid Savannah zones of West Africa, produce a low yield of amylase when malted, and consequently a low yield of fermentable sugars. It is necessary, therefore, to produce amylases and hemicellulases from microorganisms for the purpose of hydrolyzing hemicellulose and starch.

Poor growth was observed for all the fungal samples cultured on agar plates, irrespective of the sources of carbon. After 24 h, all the fungal pathogens grew profusely on hemicellulose B, starch, or sucrose as source of carbon, except SA 1222, which grew poorly on hemicellulose B, but profusely on sucrose or starch. The maximum growth was observed between 120 and 144 h. Growth response of fungal pathogens with source of carbon has earlier been reported[2,3].

Maximum enzyme activity (Tables I and II) was observed after 96 h for all the fungal pathogens, irrespective of the source of carbon used in the culture media; similar results were obtained previously by Dekker and Richards[6], and Shambe[5]. The growth of SA 1222 and SA 1208 was observed throughout the solution, rather than only at the surface in the upper layer of the solution. In the case of Coll and SA 1190, the growth was only at the surface in the upper layer, which suggests a requirement for aeration. Degradation by SA 1190, in culture solution, of its carbon

TABLE I

ACTIVITY[a] OF HEMICELLULASE PRODUCED BY SA 1222, COLL, SA 1190, AND SA 1208 UNDER DIFFERENT CONDITIONS OF GROWTH

Growth conditions	*Carbon source*[b]	*Fungus*				*Time (h)*
		SA 1222	*COLL*	*SA 1190*	*SA 1208*	
Shaking	H	0.37	0.08	0.71	0.13	48
	S	0.08	0.25			48
Stationary	H	0.67	0.41	0.75	0.19	48
	S	0.13	0.37			48
Shaking	H	0.67	0.41	0.75	0.08	96
	S	0.25	0.25	0.39	0.21	96
Stationary	H	0.67	0.41	0.97	0.21	96
	S	0.13	0.37	0.85	0.25	96
Shaking	H	0.65	0.41	0.63	0.13	166
	S	0.22	0.24			166
Stationary	H	0.64	0.41	0.75	0.13	166
	S	0.13	0.35			166

[a]Mol. reducing sugar · min^{-1} · mL^{-1}. [b]Abbreviations: H, hemicellulose; S, starch.

TABLE II

ACTIVITY[a] OF AMYLASE PRODUCED BY SA 1222, COLL, SA 1190, AND SA 1208 UNDER DIFFERENT CONDITIONS OF GROWTH

Growth conditions	*Carbon source*[b]	*Fungus*				*Time (h)*
		SA 1222	*Coll*	*SA 1190*	*SA 1208*	
Shaking	H	0.11	0.25			48
	S	0.07	0.25	0.28	0.36	48
Stationary	H	0.13	0.07			48
	S	0.13	0.41	0.25	0.15	48
Shaking	H	0.33	0.07	2.40	0.21	96
	S	0.00	0.33	0.29	0.19	96
Stationary	H	0.45	0.07	1.28	0.21	96
	S	0.00	0.27	0.40	0.17	96
Shaking	H	0.31	0.06			166
	S	0.04	0.22	0.04	0.00	166
Stationary	H	0.40	0.07			166
	S	0.04	0.13	0.24	0.29	166

[a,b]See footnotes to Table I.

source resulted in precipitation within 48 h. A similar observation had been made by Dekker and Richards[7] on the action of purified hemicellulase II (HC-II) on hemicellose B. The enzymic activities depended on the source of carbon and condition of growth. The slight decrease after maximum activity (96 h) may be due to the

formation of enzymic inhibitors or of other enzymes that may hydrolyze amylase and hemicellulase or to partial, irreversible denaturation of the enzymes due to change of pH. The change in pH (Table III) for SA 1222, Coll, and SA 1190 grown on starch, after 96 h, is most probably due to production of basic or acidic products, since these culture solutions showed profuse growth up to 120 h. The differences in pH for some species of fungus, grown on different sources of carbon, confirm earlier observation that the source of carbon determines the nature and composition of extracellular fluid[5,6]. The degradation products (Table IV) for hemicellulose B are different from those observed by Dekker and Richards[6], but in agreement with those observed earlier by Shambe[5]. The hydrolysis products of starch by SA 1190 amylase were mostly glucose with a trace of maltose.

EXPERIMENTAL

Materials. — Hemicellulose B was extracted from undelignified rice-straw with 10% sodium hydroxide[8]. Authentic samples of xylobiose (Xyl_2), xylotriose (Xyl_3), xylotetraose (Xyl_4), arabinoxylobiose ($AraXyl_2$), arabinoxylotriose ($AraXyl_3$), and arabinoxylotetraose ($AraXyl_4$) were prepared from degradation of hemicellulose B by hemicellulases produced by *Cephalosporium sacchari*[5]. L-Arabinose, D-xylose, D-glucose, D-galactose, and maltose were analytical grades and obtained from British Drug House (BDH). All other chemicals were analytical grade and used without further purification.

Culture of fungi. — The four fungal pathogens, *Alternaria*, *Colletrotrichum*, *Drechslera*, and *Fusarium moniliforme* were maintained pure on potato dextrose and were cultured on agar plate, at 28°, under illumination according to the procedure of Hultin and Nordström[9], by use of 0.5% (w/v) rice-straw hemicellulose B or 1% (W/V) starch or sucrose.

Production of extracellular amylase or hemicellulase. — The four fungal samples were grown in submerged liquid-culture on media containing 1% (w/v) analytical commercial starch solution or 0.5% (w/v) rice-straw hemicellulose B. After inoculation, the culture media (25 mL in a 50-mL flask) were inoculated at 28°, with or without shaking (20 strokes/min). Samples were withdrawn at time intervals, and centrifuged at 18 000*g*, for 0.5 h at 10° to remove mycelia and spores before they were used in enzyme assays. The results are shown in Tables I and II.

Assays for amylase and hemicellulase activity. — The enzymic digests containing 0.5% (w/v) of analytical commercial starch or rice-straw hemicellulose in 0.05M sodium acetate buffer (pH 5.5, 5.0 mL), and the fungal extracellular fluid (0.10 mL), were incubated for 0.5 h at 37°. The reducing sugars were estimated as glucose or xylose for amylase and hemicellulase activity, respectively, with the method of Nelson[10]. Enzyme activity was expressed as μmol of reducing sugar produced by the digest/min/mL of extracellular fluid.

Variation of pH of culture solution with time. — The culture solutions (50-mL each in a 100-mL conical flask) were inoculated at 28° without shaking. Aliquots

TABLE III

VARIATION OF pH OF SUBMERGED CULTURE SOLUTIONS WITH TIME AND CARBON SOURCE AT 28°

Carbon source	*Fungus*				*Time (h)*
	SA 1222	*COLL*	*SA 1190*	*SA 1208*	
Hemicellulose	5.60	5.60	5.60	5.60	00
Starch	5.60	5.60	5.60	5.60	00
Hemicellulose	5.90	5.80	5.70	5.60	24
Starch	5.80	5.80	5.60	5.80	24
Hemicellulose	6.10	6.6	6.30	6.10	48
Starch	6.10	6.50	6.00	6.20	48
Hemicellulose	5.90	6.80	5.00	7.10	72
Starch	6.00	6.40	5.40	5.70	72
Hemicellulose	5.90	7.50	4.60	7.30	96
Starch	6.60	6.30	4.70	5.00	96
Hemicellulose	6.10	7.20	4.30	7.40	120
Starch	7.50	5.70	3.80	5.20	120

TABLE IV

DEGRADATION OF HEMICELLULOSE AND STARCH BY EXTRACELLULAR AMYLASE AND HEMICELLULOSE FROM FOUR FUNGAL PATHOGENS

Substrate	*Fungus*	*Products of hydrolysis*		*Time (h)*
		Major	*Minor*	
Hemicellulose	SA 1222	$AraXyl_2$; $AraXyl_3$	Ara; Xyl_2; Xyl_3	20
Starch	SA 1222	Glc	Maltose	20
Hemicellulose	SA 1222	$AraXyl_2$	Xyl_2	72
Starch	SA 1222	Glc	Maltose	72
Hemicellulose	Coll			20
Starch	Coll	Glucose	Maltose	20
Hemicellulose	Coll	Ara; $AraXyl_2$; Xyl_2	Xyl	72
Starch	Coll	Glc	Maltose	72
Hemicellulose	SA 1190	$AraXyl_2$; Xyl_2; $AraXyl_3$	Xyl_4	20
Starch	SA 1190	Glc	Maltose	20
Hemicellulose	SA 1190	Xyl; Xyl_2; Xyl_4	$AraXyl_2$; Ara	72
Starch	SA 1190	Glc	Maltose	72
Hemicellulose	SA 1208	Xyl_2; Xyl_4	Ara; Xyl	20
Starch	SA 1208	Glc	Maltose	20
Hemicellulose	SA 1208	Xyl_3; Xyl_4	$AraXyl_3$; Ara; Xyl	72
Starch	SA 1208	Glc	Maltose	72

were taken at time intervals, centrifuged at 18 000*g*, and the pH measured at 25°. The results are shown in Table III.

Analysis of enzymic digests. — The products obtained from the enzymic hydrolysis of starch or hemicellulose B and the standard sugars were chromatographed on Whatman No. 1 paper in 6:6:5:4 (v/v) 1-butanol–ethyl acetate–pyridine–

water and 10:4:3 (v/v) ethyl acetate–pyridine–water for neutral sugars, and the spots made visible with alkaline silver nitrate[11] or the *p*-anisidine hydrochloride[12] reagents on duplicate papers. The results are shown in Table IV.

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