DECOMPOSITION OF CELLULOSE BY THE FUNGUS CURVULARIA LUNATA WAKKER—*Studies on enzyme activity*

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ABSTRACT

Active cellulolytic enzymes have been found in the metabolic liquor as well as in the mycelial mat of the fungus *Curvularia lunata* Wakker. The properties of the enzymes from the metabolic liquor have been investigated in detail and their purification has been attempted.

Introduction

Fungal deterioration of military stores made of cotton textiles in the tropical and sub-tropical regions of the world is a problem of great economic importance which has received considerable attention in many countries. A systematic study of decomposition of cellulose by the causative fungi is of great value in laying down suitable measures of control of attack. Some work has been recorded on the cellulose-destroying enzymes elaborated by the fungi *Aspergillus oryzae*,[1] *Fusarium solani*,[2] *Penicillium pinophilum*,[3] *Collybia velutipes*,[4] *Verticillium alboatrum*[5] and *Myrothecium verrucaria*[6]. It was thought of considerable interest to investigate some fungi recorded in India and shown as causing high destruction of cellulosic material. *Curvularia lunata* Wakker is one such fungus and the present paper deals with the factors which influence the activity of the cellulolytic enzymes elaborated by this fungus. Subsequent papers in this series will deal with the nutritional requirements of this fungus and the inhibitors for the enzyme systems associated with the degradation of cellulose.

Experimental procedure

Culture medium—Preliminary investigations* showed that the fungus grew on Omeliansky's medium [7] described below to which 2·0% cuprammonium cellulose had been added and whose *pH* had been adjusted to 6·2—6·4.

Ammonium sulphate $[(NH_4)_2\ SO_4]$	1·0 gm
Dipotassium phosphate (K_2HPO_4)	1·0 gm
Magnesium sulphate $(MgSO_4,\ 7H_2O)$	0·5 gm
Calcium carbonate $(CaCO_3)$	2·0 gm
Sodium chloride $(NaCl)$	Trace
Distilled water	1000 ml.

*Unpublished work, Defence Research Laboratory (Stores), Kanpur, India.

Inoculum—The fungus was grown for ten days on Potato-Dextrose-Agar slants containing filter paper strips. The spores collected at this period of growth were suspended in sterilized water and the suspension filtered through a sterile muslin cloth. The filtrate was diluted with sterilized water to a density of about 50,000 spores per ml. Two ml. of the spore suspension were added to 40 ml. of the sterilized culture medium which was then incubated at 28°±2°C.

Separation of enzymes—For the extracellular enzyme, the metabolic liquor was filtered through clean glass wool. The filtrate was centrifuged (at 1500 r.p.m.) for 15 minutes and the clear liquor was used for assessment of the extracellular enzymatic activity. For the determination of activity of intracellular enzyme, the mycelial mat was partly dried by pressing it between folds of filter paper. It was then crushed in an agate pestle and mortar and subsequently dried at 7°C in a vacuum descicator to constant weight. It was found that dialysis of metabolic liquor or the crushed mycelial mat did not lead to enzyme of greater activity. On the other hand, some reduction in the enzyme activity was observed during 3-4 days of dialysis.

Assessment of enzymatic activity—A known volume of the clear metabolic liquor containing the extracellular enzyme or a known weight of the dried mycelial mat having the intracellular enzyme was mixed with cuprammonium cellulose substrate, buffered by citric acid—sodium monohydrogen phosphate and incubated for a definite period at the required temperature. A thin layer of toluene was provided to exclude contamination. A control was run exactly under identical conditions but after the enzyme had been destroyed by autoclaving at 20 lbs./sq. inch for 30 minutes. After incubation, the reducing sugars formed were estimated by the modified copper iodometric method of Shaffer and Somogyi[8]. The method recommended by Jensen and Hagedorn[9] was also occasionally employed.

Results

Effect of period of growth on enzyme activity—40 ml. of the culture medium were taken in 500 ml. Erlenmeyer flasks and the fungus was grown at 28°±2°C. The cellulolytic activities of the metabolic liquor as well as the crushed mycelial mat were estimated at various periods of growth of the fungus. The mat after separation and drying as described earlier was weighed. The reducing sugars in the metabolic liquor were also estimated. The results are shown in Table I. Intracellular activity is high at early stages of growth while the extracellular activity is maximum at about 15—20 days.

Optimum pH for enzyme activity—The enzymatic activity of the metabolic liquor was assessed at different levels of *pH* ranging from 3·6 to 7·5 using citrate buffer. The period of incubation was 72 hours at 38°C. The results are recorded in Table II. Maximum activity was observed at *pH* 4·6—5·0.

Optimum temperature for the enzyme activity—The enzyme-substrate mixture was incubated at different temperatures ranging from 25° to 90°C for 50, 72, and 96 hours. The *pH* was maintained at 5·0 using citrate buffer. There were two replicates for each period of incubation. The results are summarised in Table III. The optimum temperature for maximum cellulase

activity is 37°C for 72 and 96 hours of incubation while it is 40°C for 50 hours of incubation.

Heat stability of the enzyme—The metabolic liquor was stored at different temperatures for one hour. In one set, these ranged from 20° to 90°C and in the second from 0° to 60°C. After storage, the metabolic liquor was incubated with substrate and buffer (pH 5·0) at 37° C for 96 hours and the enzymatic activity estimated. The results are given in Table IV. It will be observed that the activity of the enzyme is destroyed above 55°C.

Optimum period of incubation of enzyme with substrate.—The enzyme-substrate mixture (pH 5·0) was incubated at 37°C for different periods ranging from 0 to 96 hours. The enzyme activity was assessed by determining the reducing sugars produced at different periods of incubation. The experiment was not concerned with the study of the kinetics of the reaction but confined itself only to the investigation of the optimum period of incubation for evaluation of enzyme activity of the metabolic liquor. A number of test tubes containing the enzyme—substrate-buffer mixture was incubated, two of these were removed at different intervals for assessment of the enzymatic activity. The results are given in Table V. Maximum hydrolysis took place during 74—84 hours. Incubation for a longer period did not bring about any appreciable increase in cellulose hydrolysis.

Cellulase activity in relation to substrate concentration—One ml. of the metabolic liquor was incubated at 37°C and at pH 5·0 for 96 hours with different concentrations of cuprammonium cellulose suspension. The reducing sugars present at the end of incubation were estimated. The results are shown in Table VI. With the increase in concentration of the substrate the quantity hydrolysed also increases upto the stage when the substrate concentration is 0·15%. With higher concentrations, increase in the reducing sugars produced is comparatively less.

Cellulase activity in relation to enzyme concentration—Different volumes of the metabolic liquor were added to substrate-buffer mixture and the resulting mixture diluted to a constant volume by the addition of distilled sterile water. Each mixture was incubated at 37°C and pH 5·0 for 96 hours. The reducing sugars produced at the end of this period were determined. The results are given in Table VII. An increase in quantity of the metabolic liquor is attended by an increase in cellulose hydrolysis in earlier stages. Thereafter, the hydrolysis is not affected by the quantity of the metabolic liquor.

Effect of storage of metabolic liquor on cellulase activity—The metabolic liquor was stored at 7°C and 30°C and the cellulolytic activity was estimated after different periods of storage ranging from 0 to 15 days. The results are given in Table VIII. It will be observed that a gradual fall in the activity of the enzyme takes place as a result of storage.

Enzymatic hydrolysis of lower oligosaccharides—The metabolic liquor as well as the mycelial mat were incubated with different lower oligosaccharides, cellobiose, cellotriose and cellotetraose. The incubation was done at 37°C and pH 5·0 for 96 hours. The results are summarised in Table IX. It appears that the fungus does not elaborate uniformly the same type of enzyme at different stages of growth.

Purification of the enzyme from the metabolic liquor—By precipitation—The soluble salts and sugars were removed from the metabolic liquor by dialysis in cellophane bags against running distilled water for 2—3 days. Acetone, ethyl alcohol and chloroform were separately added to 150 ml lots of the dialysed liquor and the precipitates obtained were filtered and weighed. The cellulolytic activity of the precipitates was also investigated. Results are given in Table X. It appears that considerable part of the cellulolytic activity is lost during precipitation by the organic solvents investigated.

By centrifuging—The metabolic liquor was centrifuged at 30,000 r.p.m. in Sharple's Super Centrifuge. The enzyme did not separate.

By concentration—The metabolic liquor was concentrated by distillation under reduced pressure (60—80 mm) at 40°—50°C. In a second set, the metabolic liquor was supercooled to −5°C and the water was frozen by the addition of a small crystal of ice. The non-solidified portion was removed from the solid ice by decantation. The enzymatic activity of both the solid and supernatant liquid was assessed. The solid part was devoid of any cellulolytic activity. The results are given in Table XI. A reduction in the cellulolytic activity results by concentrating the metabolic liquor by distillation under reduced pressure. The enzyme can be concentrated to some extent by freezing.

By adsorption on alumina column—Alumina (B.D.H. for chromatographic adsorption) was made into a slurry with distilled water. The slurry was poured into a tube to make a column about 15 cms. long. The alumina column was thoroughly washed with distilled water. The metabolic liquor was passed through this column at the rate of about 40 ml/hour. The column was then removed intact from the glass tube and cut into four successive zones from the top (*a*) 2·5 cm (*b*) 5 cm (*c*) 5 cm and (*d*) 2·5 cm long. Each of these zones was separately eluted with citrate buffer (pH 5·0) and the enzymatic activity of the elute determined. Table XII gives the results obtained by using the metabolic liquor at different periods of growth of the fungus. Observations with concentrated (by freezing method) and unconcentrated metabolic liquor are given in Table XIII. It will be observed that the enzymatic activity is lost during adsorption on the alumina column.

Discussion

Growth of the fungus and enzyme activity of the metabolic liquor—It is evident that an active extracellular cellulolytic enzyme is elaborated by the fungus *C. lunata*. It appears that in the initial stages of growth of the fungus when the spores are not able to produce an active extracellular enzyme, the cells decompose cellulose with the help of an active intracellular enzyme. The activity of the cells slowly decreases with the growth of the fungus while the production of extracellular enzyme slowly increases (Table I). Maximum elaboration of the extracellular enzyme occurs in 15—20 days. After this period the fungus still continues to grow, no doubt, by utilising reducing sugars produced by it. The enzyme activity of the metabolic liquor during this period is low.

Optimum pH. temperature and incubation period—The enzyme has low activity at pH lower than 4·2 and higher than 6·0. It is rapidly deactivated below pH 3·8 or above 6·5. Even slight alkalinity (pH 7·5) seems to denature the protein moiety and deactivate the enzyme. The value of optimum pH (4·6 to 5·0) agrees with the results obtained by Thomas[10] on *Stachybotrys atra* and Narayanamurti and Verma[11] on *Polystictus sanguineus*. Temperature seems to play a dual role on the enzymatic reaction namely the acceleration of the chemical reaction and the denaturation of the protein moiety resulting in the inactivation of the enzyme. For short incubation periods the acceleration of the chemical reaction is predominant, while for longer periods (Table III), it is the denaturation of protein moiety. Optimum temperature for 96 hours incubation is about 37°C. Similar results were obtained by Saunders *et al*[6] (40°C) and Greathouse[12] (37°—40°C) on *M. verrucaria*; Reese [13], however, has reported an optimum temperature of 55°C in the case of *Aspergillus fumigatus*. Optimum activity at 50°C has been recorded [14] for *M. verrucaria*, *A. luchuensis*, *A. fumigatus* and *Actinomyces* sp.

The activity of the enzyme is destroyed at about 60°C. Saunders *et al*[6] reported that the enzyme from *M. verrucaria* underwent rapid inactivation above 50°C. The optimum incubation period determined now for the enzyme elaborated by the fungus *C. lunata* is about 96 hours. This period of incubation has also been used by other workers [11, 12, 13] in their studies.

Substrate concentration and enzyme concentration—An increase in the concentration of substrate results in a corresponding increase in the quantity of reducing sugars produced upto 0·15% concentration of the substrate after which this relationship fails. These results are similar to those of Kuhn [15] on sugar hydrolysis by invertase. The results shown in Tables VI & VII support the hypothesis postulated by Michaelis and Menten [16] that the initial rate of hydrolysis of substrate is proportional to the concentration of an intermediate substrate-enzyme complex.

Storage of metabolic liquor—The metabolic liquor loses its cellulolytic activity during storage much more at 30°C than at 7°C. Saunders *et al*[6] have reported that the enzyme from *M. verrucaria* is stable for many months when stored at 5°C.

Activity on lower oligosaccharides—Intracellular as well as extracellular enzymes derived from the fungus *C. lunata* (Table XI) are capable of hydrolysing cellotriose and cellotetraose. Grassmann *et al.* [17] have reported the isolation of cellobiase factor from cellulase from *A. oryzae*; this factor was found to hydrolyse hydrocellulose, cellotriose and cellobiose. Certain metabolic filtrates of cellulose destroying micro-organisms growing on cellulose have been recorded [20] as being capable of digesting cellulose derivatives, partially substituted carboxymethyl cellulose but not cellobiose.

Purification of the metabolic liquor—The maximum amount of precipitate obtained by using organic solvents is with ethyl alcohol. The precipitate, however, showed very low enzymatic activity. Ploetz [18] investiagted the metabolic liquor from *Merulius lacrymans*. He separated the cellulase from

lichenase by fractional precipitation with ethanol-ether mixture. Work reported in this paper on the enzyme from *C. lunata* has indicated that concentration by freezing is partially successful. Adsorption of the enzyme from *C. lunata* on alumina column results in loss of enzymatic activity, although Trager [19] and Grassmann *et al.* [17] have reported the isolation of active cellulase and cellobiase on alumina columns.

Acknowledgements

The authors are thankful to Dr. V. Ranganathan for initiating and guiding the work. Thanks are also due to Shri S. K. Ranganathan, Shri L.R. Sud and Dr. P. K. Vijayaraghavan for their interest in the investigations and to Dr. P. N. Agarwal for guidance in preparing this paper.

References

1. Karreth, A., *German Patent* **654**, 490, (1937).
2. Karreth, A., *German Patent* **745**, 081, (1944).
3. Clark, E.D., and Scales, F.M., *J. Biol. Chem.*, **24**, XXXI (1916).
4. Norkrans, B., *Physiol Plantarum.*, **10**, 454, (1957).
5. Talboys, P.W., *J. Text. Inst.* (Abstr.), **50**, A117, (1959).
6. Saunders, P. R., Siu, R. G. H., and Genest, R., *J. Biol. Chem.*, **174**, 697, (1948).
7. Omelinasky, W., *Centr. Bakt.*, II Abt. **8**, 226, (1902).
8. Shaffer, P.A., & Somogyi, M., *J. Biol. Chem.*, **100**, 695, (1933).
9. Hagedorn, H.C. & Jensen, B. N., *Biochem. Z.*, **131**, 46, (1923).
10. Thomas, R., *Australian J. Biol. Sci.*, **9**, 159, (1956).
11. Narayanamurti, D., & Verma, G. M., *Holz als Roh und Werkstoff*, **11**, 7 (1953).
12. Greathouse, G. A., *Textile Research Journal*, **20**, 227, (1950).
13. Reese, E. T., *Thesis State* College Pennsylvania (1946).
14. Anonymous, *Technical Progress Report. Office of the Quartermaster General, U. S. A.*, (Dec. 1949).
15. Kuhn, R., *Z. Physiol. Chem.*, **125**, 28, (1923).
16. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 333, (1913).
17. Grassmann, W., Zechmeister, L., Toth, L. & Stadler, R., *Ann. Chem.*, **503**, 167, (1933).
18. Ploetz, T., *Z. Physiol. Chem.*, **261**, 183, (1939).
19. Trager, W., *Biochem. J.*, **26**, 1762, (1932).
20. Siu, R. G. H., *Microbial decomposition of cellulose*, Reinhold Publishing Corporation, New York-18, U. S. A. (1951), p-288.

TABLE I

Effect of period of growth on enzyme activity and quantity of mycelium produced

Age of culture	Cellulase activity (% hydrolysis of cellulose)		Weight of mycelium	Total reducing sugars in metabolic liquor as glucose
	Extracellular	Intracellular		
days			*mgm*	*mgm*
6	8·0	Not done	**	1·93
8	8·0	30·0	154	3·30
10	13·3	19·3	194	7·72
15	16·0	4·6	222	9·51
20	25·3	*Nil*	226	14·62
27	13·4	*Nil*	234	12·21
37	*Nil*	*Nil*	220	8·93

**The mycelium could not be separated due to sparse growth.

TABLE II

Effect of pH on cellulolytic activity of the enzyme

pH	Cellulase activity (% hydrolysis of cellulose)
3·6	*Nil*
3·8	5·0
4·0	7·7
4·2	11·0
4·4	15·0
4·6	29·4
4·8	29·4
5·0	31·0
5·2	24·7
5·4	22·3
5·6	22·3
6·0	11·0
6·5	2·3
7·0	*Nil*
7·5	*Nil*

TABLE III

Effect of temperature on cellulolytic activity at pH 5·0

Temperature of incubation	Cellulase activity (% hydrolysis of cellulose) Average of two runs		
	50 hrs	72 hrs	96 hrs
°C			
25	2·5	1·8	5·0
32	4·8	7·8	9·8
37	7·3	10·2	13·8
40	9·0	8·5	8·8
50	1·8	2·0	1·8
55	*Nil*	*Nil*	*Nil*
60	*Nil*	*Nil*	*Nil*
70	*Nil*	*Nil*	*Nil*
90	*Nil*	*Nil*	*Nil*

TABLE IV

Heat stability of cellulolytic enzyme

Set I		Set II	
Temperature at which maintained for one hour	Cellulase activity (% hydrolysis of cellulose)	Temperature at which maintained for one hour	Cellulase activity (% hydrolysis of cellulose)
°C		°C	
20	33·2	0	10·0
34	33·3	10	12·0
50	29·8	18	12·3
70	*Nil*	31	12·3
80	*Nil*	37	12·3
90	*Nil*	55	5·0
..	..	60	*Nil*

TABLE V

Effect of period of incubation on enzymatic activity
(Temp. 37°C, pH 5·0)

Period of incubation	Cellulase activity (% hydrolysis of cellulose)	
	Set I	Set II
hrs		
0	*Nil*	*Nil*
4	*Nil*	..
8	*Nil*	*Nil*
22	3·5	..
24	....	3·5
28	4·5	4·5
32	..	5·0
46	6·5	..
52	..	7·5
54	8·0	..
60	..	9.0
70	9·5	..
74	10·0	..
84	..	10·5
96	9·5	10·0

TABLE VI

Effect of substrate concentration on cellulase activity

Substrate concentration	Cellulase activity (expressed as reducing sugars)	
	Set I	Set II
%	*mgm*	*mgm*
0·02	0·016	*Nil*
0·05	0·022	0·009
0·10	0·052	0·014
0·12	0·065	0·027
0·15	0·070	0·034
0·20	0·073	0·036

TABLE VII

Effect of enzyme concentration on cellulase activity

Quantity of metabolic liquor	Volume of distilled water added for dilution	Cellulase activity (% hydrolysis of cellulose)
ml	*ml*	
1·0	6·5	4·5
1·5	6·0	7·0
2·5	5·0	8·5
4·0	3·5	9·0
6·0	1·5	8·5
7·5	0	9·0

TABLE VIII

Effect of storage on cellulase activity

Period of storage	Cellulase activity (% hydrolysis of cellulose)	
	Stored at 7°C	Stored at 30°C
in days		
0	17·8	17·8
4	17·8	12·5
8	14·6	6·5
10	14·2	5·3
11	16·0	6·0
12	12·0	3·5
15	8·5	*Nil*

TABLE IX

Effect of cellulose activity of some oligosaccharides

Nature of enzyme	Enzyme activity (% hydrolysis of substrate)		
	Cellobiose	Cellotriose	Cellotetraose
Intracellular after 15 days growth ..	15·0	*Nil*	9·0
Intracellular after 20 days growth ..	*Nil*	12·0	*Nil*
Extracellular after 20 days growth I ..	*Nil*	*Nil*	18·0
Extracellular after 20 days growth II ..	*Nil*	6·5	2·3

TABLE X

Precipitation of the enzyme by organic solvents

Set No.	Nature of enzyme	Amount of the enzyme	Quantity of enzyme taken for assessment	Cellulase activity (% hydrolysis of cellulose)
	Control (Dialyzed metabolic liquor)	150 ml	5 ml.	32·0
I	Precipitated by acetone	47·7 mgm	20·0 mgm.	6·0
	Precipitated by ethyl alcohol ..	85·1 mgm.	20·0 mgm.	18·5
	Control (Dialyzed metabolic liquor)	150 ml.	5 ml.	36·5
	Precipitated by acetone ..	49·0 mgm.	20·0 mgm.	6·8
II	Precipitated by ethyl alcohol ..	94·7 mgm.	20·0 mgm.	14·3
	Precipitated by chloroform ..	56·5 mgm.	20·0 mgm.	*Nil*

TABLE XI

Concentration of cellulase

Method adopted	Set No.	Extent of concentration	Cellulase activity (% hydrolysis of cellulose)	
			Before concentration	After concentration
Distillation under reduced pressure at 40°—50°C	I	1/10 vol.	57·5	7·5
	II	,,	20·0	*Nil*
By freezing (at —5°C)	I	2/5th vol.	40·0	62·1
	II	2/5th vol.	33·5	52·8
		1/4th vol.	33·5	55·0

TABLE XII

Adsorption of metabolic liquor on alumina at different periods of growth of the fungus

Age of culture (days)	Cellulase activity (% hydrolysis of cellulose)					
	Metabolic liquor (prior to adsorption)	column portion				
		a	*b*	*c*	*d*	Chromatographed liquor
7	11·0	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*
14	12·0	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*
21	32·0	6·5	*Nil*	*Nil*	*Nil*	*Nil*
21	32·0	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*
21	34·0	2·0	*Nil*	*Nil*	*Nil*	*Nil*

TABLE XIII

Adsorption of metabolic liquor (concentrated and unconcentrated) after 21 day's growth of the fungus

No. of Set	Extent of concentration by freezing	Cellulase activity (% hydrolysis of cellulose)					
		Prior to adsorption	column portion				
			a	*b*	*c*	*d*	Chromatographed liquor
I	Control (unconcentrated).	40·0	2·0	*Nil*	*Nil*	*Nil*	*Nil*
	Concentrated to 2/5th of its volume.	62·5	8·0	*Nil*	*Nil*	*Nil*	*Nil*
II	Control (unconcentrated)	33·5	1·5	*Nil*	*Nil*	*Nil*	*Nil*
	Concentrated to 2/5th of its volume.	52·8	7·5	*Nil*	*Nil*	*Nil*	*Nil*
	1/4th of its volume.	55·0	7·5	*Nil*	*Nil*	*Nil*	*Nil*