Zag. J. Pharm. Sci., Vol. 3, No. 2, August 1994

BIOCHEMICAL STUDIES AND PARTIAL PURIFICATION OF CELLULASE ENZYME BIOSYNTHESIZED BY TRICHODERMA VIRIDE AND FUSARIUM OXYSPORIUM

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ABSTRACT

Precipitation of carboxymethyl cellulase enzyme biosynthesized by *Trichoderma viride* and *Fusarium oxysporium* was affected using organic solvents such as ethyl alcohol, acetone and butanol . Best results were obtained when 66% ethyl alcohol was used as a solvent. The precipitate was applied on a column chromatography with sephadex G-150 for fractionation and purification and the most active fractions were then collected. The optimal biochemical conditions under which the partially purified carboxy methyl cellulase (CMCase) exhibited its maximal activities were incubation temperature 50°C for 45 minutes at pH 5.0 in presence of 1% carboxy methyl cellulose. Zinc sulphate and calcium chloride were used as stimulators for the enzyme.

INTRODUCTION

The biosynthesis of cellulase enzyme was studied by many investigators[1-4]. Previously, precipitation of cellulase enzymes by ammonium sulphate and ethyl alcohol were reported[5]. In addition, the fractionation and purification of these enzymes by column chromatography containing sephadex G-150 and G-200 gel filtration were also described[6].

The optimum pH and temperatures reported for cellulase enzyme biosynthesized by Neocallimastrix frontalis were 6 and 5°C, respectively[7]. Moreover, reports about the characterization of extracellular cellulase enzyme produced by A. niger were endoglucanase with 48% of it's original activity and the exoglucanase contained 42% according to the following formula[4]:

Cellulose —endo-exo glucanase→ cellobiase —Cellobiose→ glucose

Two isolated fungal strains Trichoderma viride and Fusarium oxysporium were proved to be active cellulolytic organisms [8]. The precipita- tion. purification and characterization of the biosynthesized enzyme were studied [8]. The optimal conditions under which maximal activities of the partially purified enzyme were also described [8].

EXPERIMENTAL

Organisms used : Trichoderma viride and Fusarium oxysporium are local isolates from Egyptian soil, Sharkia Governorate. Taxonomic identification was confirmed as reported [9-11].

Growth medium : Modified Czapek's cellulose medium was used for the growth and cellulolytic activities of the two organisms used with the following composition (g/1): cellulose 10; $NaNO_3$ 3.0; K_2HPO_4 1.0; KCl 0.5; $MgSO_4 . 7 H_2O$ 0.5; $FeSO_4 . 5 H_2O$ 0.01 and distilled water 1000 ml. Portions (49 ml) of the

medium was autoclaved in 250 ml Erlenmeyer flasks. The culture flasks were inoculated with 1 ml spore suspension and incubated at 30°C for 15 days[8].

Enzyme assay : The cellulolytic activity was determined as described before[12,13], as follows: one ml of the enzyme preparation was incubated with 9 ml of 1% carboxymethyl cellulose in 5 mM citrate buffer (pH 5.0) for 30 minutes at 40°C. At the end of the reaction time, the reducing sugar liberated was determined[14].

One unit of cellulase enzyme is defined as the amount of enzyme that liberates 1 mg of glucose from CMC per one ml of the total solution.

The conversion rate (%) was calculated using the following equation :

$$\text{Percentage of conversion} = \frac{\text{Total glucose formed}}{\text{weight of substrate}} \times 10$$

Precipitation and partial purification of cellulase enzyme :

At the end of incubation period, the fungal mats and the suspended solids were separated by centrifugation (at 2000 rpm for 20 minutes). The clear supernatent was used as a source for the crude enzyme. The precipitation of the enzyme was carried out at 15°C overnight using several solvents, precipitate was then purified by passing through a column filled with sephadex G-150 (2.5 x 39cm) followed by another filled with sephadex G-200 (2 x 20 cm) equilibrated with citrate buffer pH 5. Two ml from each fractions were collected at flow rate of 20 ml/h. Fractions with high specific activity were collected and subjected to further biochemical studies[15].

Biochemical studies on partially purified CMCase enzyme :

The effect of the pH values, temperature, substrate, concentration of enzyme, micro-elements and time on the partially purified enzyme activity were determined according to previously described methods [16].

RESULTS AND DISCUSSION

The experimental organisms were cultivated in the liquid culture for 15 days at 27°C. At the end of the incubation period, the cultures were centrifuged at 2000 rpm for 20 minutes. To the clear supernatant 44, 66 and 83% of ethanol, acetone or butanol were added in order to determine the most effective precipitate of the cellulolytic enzyme. Table (1) indicates that 66% ethanol is the most suitable for precipitating the highest amount of the enzyme. This may be due to its high miscibility with water. Ethanol did not affect the enzyme activity [15,17].

The precipitated enzyme was dissolved in citrate buffer (pH 5) and applied on column chromatography packed with sephadex G-150. The fractionation pattern are illustrated in Fig. (1). The results indicated that the most active fractions were those numbered 30 through 43 (26 ml) for Trichoderma viride and those numbered 32 through 42 (22 ml) for Fusarium oxysporium. The two peaks containing maximum cellulolytic activities are shown in Fig. 1, similar to those previously published [18,19].

The results in Table (2) proved that carboxymethyl cellulose (CMC) is the most suitable for enzyme activity compared to the other tested substrates[7]. This indicated that the enzyme secreated by the two organisms is mainly CMCase. The ability of the enzyme to hydrolyse other polysaccharides may be due to the partially purified enzyme. Also the principal CMCase enzyme is contaminated with other enzymes from the tested organisms. The inhibitory effect of both cellobiose and glucose can be discussed on the base that these substances are end products of the cellulases activities. The presence of which might act as repressors of the enzymes formed[8].

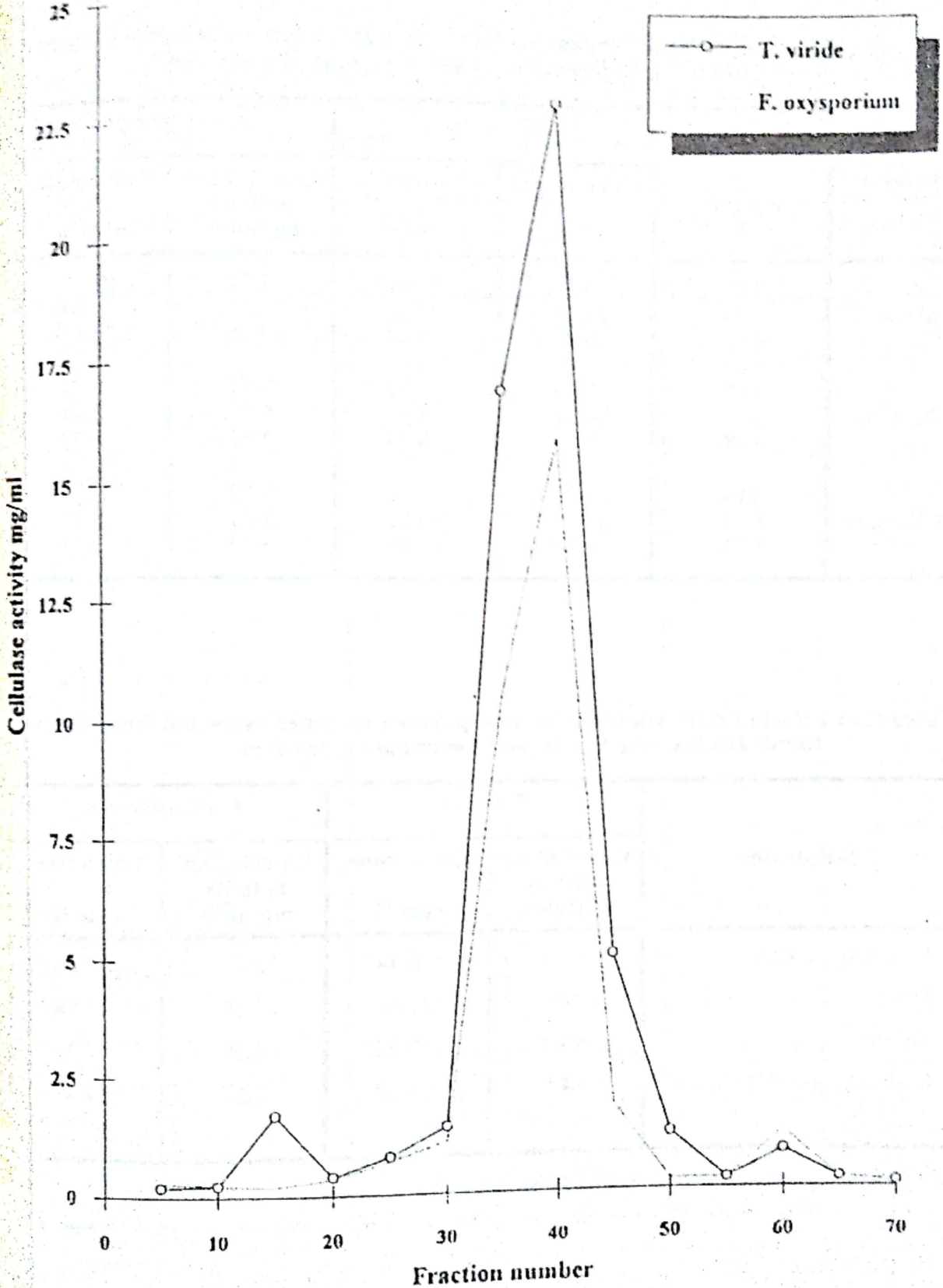

Fig. (1) : Fraction numbers of cellulase activity precipitated at 66% ethyl alcohol of *T. viride* and *F. Oxysporium*.

Table (1) : Effect of different organic solvents concentration on cellulase enzymes produced by *Trichoderma viride* and *Fusarium oxysporium*.

Solvent	Concentration of solvent	*T. viride*		*F. oxysporium*	
		Cx cellulase activity mg/ml/h.	Conversion rate %	Cx cellulase activity mg/ml/h.	Conversion rate %
Ethyl alcohol	44%	10.05	11.16	1.34	1.49
	66%	24.12	27.20	17.15	19.04
	83%	6.70	7.44	10.05	11.16
Acetone	44%	3.35	3.72	0.67	0.74
	66%	7.71	8.56	6.70	7.44
	83%	7.37	8.18	3.35	3.72
n-Butanol	44%	3.35	3.72	0.65	0.72
	66%	11.73	13.02	7.71	8.56
	83%	6.70	7.44	3.35	3.72

Table (2) : Effect of different substrates on purified enzymes extracted from the cultured *Trichoderma v iride* and *Fusarium oxysporium*.

Substrates	*T. viride*		*F. oxysporium*	
	Cx cellulase activity mg/ml/h.	Conversion rate %	Cx cellulase activity mg/ml/h.	Conversion rate %
Cellulose powder	5.80	6.44	2.40	2.66
Starch	12.40	13.67	9.80	10.88
Cellobiose	8.40	9.32	6.50	7.22
Carboxymethyl cellulose (CMC)	24.5	27.20	22.0	24.42

Cx : cellulase activity is expressed as mg glucose/ml/h.

Table (3): Effect of different pH values on partially purified Cx cellulase biosynthesized by *Trichoderma viride* and *Fusarium oxysporium*

pH values	*T. viride*		*F. oxysporium*	
	Cx cellulase activity mg/ml/h.	Conversion rate %	Cx cellulase activity mg/ml/h.	Conversion rate %
1- Citrate buffer				
3.0	12.5	13.88	13.0	14.43
3.5	15.5	17.21	15.0	16.66
4.0	19.0	21.10	17.5	19.43
4.5	21.5	23.88	20.0	22.20
5.0	24.5	27.20	22.0	24.24
5.5	20.5	22.67	19.3	21.42
2- Phosphate buffer				
6.0	19.0	21.10	18.3	20.31
6.5	17.5	19.43	17.5	19.43
7.0	15.0	16.66	15.0	16.66
7.5	12.5	13.88	13.0	14.43
3- Ammonium chloride-HCl buffer				
8.0	12.0	13.32	12.5	13.88
8.5	11.0	12.21	10.5	11.66
9.0	9.8	10.88	8.5	9.44
9.5	7.5	8.33	7.5	8.33
10.0	2.5	2.78	5.0	5.55

Table (4) : Effect of different incubation temperatures on partially purified Cx cellulase activity formed by *Trichoderma viride* and *Fusarium oxysporium* using carboxy methyl cellulose as substrate.

Incubation temperature	*T. viride*		*F. oxysporium*	
	Cx cellulase activity mg/ml/h.	Conversion rate %	Cx cellulase activity mg/ml/h.	Conversion rate %
5 °C	5.60	6.22	5.00	5.56
10°C	8.60	9.56	7.50	7.78
15°C	15.0	16.66	13.40	14.88
20°C	18.2	20.22	16.20	18.00
25°C	20.4	22.66	17.00	18.88
30°C	22.8	25.34	18.40	20.44
35°C	23.60	26.22	21.00	23.34
40°C	26.00	28.88	22.20	24.66
45°C	26.80	29.76	23.40	26.00
50°C	27.80	30.88	23.60	26.22
55°C	26.20	29.10	23.00	25.56
60°C	23.00	25.56	19.60	21.76

Table (5) : Effect of different incubation periods on partially purified Cx cellulase enzyme extracted from *Trichoderma viride* and *Fusarium oxysporium.*

Incubation periods (minutes)	*T. viride*		*F. oxysporium*	
	Cx cellulase activity mg/ml/h.	Conversion rate %	Cx cellulase activity mg/ml/h.	Conversion rate %
5	5.40	6.00	3.10	3.44
10	12.20	13.56	10.50	11.67
15	19.80	22.00	15.90	17.67
20	22.10	24.56	17.80	19.84
25	24.50	27.22	19.90	22.11
30	26.20	29.10	22.20	24.66
35	27.00	30.00	22.50	25.00
40	27.30	30.33	22.60	25.11
45	27.40	30.44	22.60	25.11
50	27.20	30.22	22.40	24.89

Cx: cellulase activity is expressed as mg glucose/ml/h.

Table (6) : Effect of different concentrations of the partially purified Cx cellulase biosynthesized by *Trichoderma viride* and *Fusarium oxysporium*

Enzyme concentration (ml)	*T. viride*		*F. oxysporium*	
	Cx cellulase activity mg/ml/h.	Conversion rate %	Cx cellulase activity mg/ml/h.	Conversion rate %
0.1	8.50	9.44	7.10	7.89
0.2	12.30	13.67	10.10	11.22
0.3	17.80	19.84	14.10	15.67
0.4	21.90	24.33	18.90	21.00
0.5	26.20	29.10	22.10	24.55
0.6	27.90	30.10	23.00	25.55
0.7	28.90	31.78	23.10	25.67
0.8	28.90	32.11	23.20	25.78
0.9	28.90	32.11	23.20	25.78
1.0	28.90	32.11	23.20	25.78

Table (7) : Effect of different metallic ions on the partially purified Cx cellulase extracted from *Trichoderma viride* and *Fusarium oxysporium*

Metallic ions	*T. viride*		*F. oxysporium*	
	Cx cellulase activity mg/ml/h.	Conversion rate %	Cx cellulase activity mg/ml/h.	Conversion rate %
Control	26.10	29.00	22.10	24.56
Ferrous sulphate	24.30	27.00	18.90	21.00
Copper sulphate	15.40	17.11	8.50	9.44
Cobalt carbonate	3.10	3.44	2.50	2.78
EDTA	26.50	29.44	22.30	24.78
Zinc sulphate	27.30	30.33	21.90	24.33
Calcium chloride	27.10	30.11	22.50	25.00
Calcium sulphate	26.80	29.78	22.40	24.89
Manganous chloride	17.40	19.33	19.80	22.00
Cobalt chloride	2.50	2.78	0.00	0.00
Manganous sulphate	21.60	24.00	20.10	22.33
Barium chloride	20.5	22.78	17.80	19.84

Cx cellulase activity is expressed as mg glucose/ml/h.

The effect of pH on cellulase activity in Table (3) showed that weak acid citrate buffer (pH 5.0) was the most suitable for maximal values of CMCase activity and conversion rate of T. viride and E. oxysporium. Significant effect on the enzyme activity of both fungal strains was noticed when the pH value of the reaction mixture was shifted from the alkaline side or to the acidity.

The result in Table (4) showed that the optimum incubation temperature for maximal CMCase activity was 50°C for both tested organisms, lower temperatures reduced enzyme activity. Denaturation of the enzyme at high temperature was observed. This may explain the severity of high temperature on the enzyme formed. The effect of temperature is known to depend on the amino acid component and the nature of the enzyme.

Table (5) indicated that the optimal incubation period for maximum CMCase activity was 45 minutes. Longer time decreased the enzyme activity due to the accumulation of the end products which supressed the enzyme activity [18,19].

As known the enzyme reaction depends mainly on the enzyme concentration. Table (6) showed that the optimal concentrations of the enzyme secreted by T. viride is 0.7 ml and 0.8 for E. oxysporium. The enzyme concentrations is versatile since these concentrations are dependent on enzyme specificity[7].

Investigating the effect of some metallic ions on the CMCase was studied. It is evident from Table (7) that Zn sulphate acts as stimulator since it increased the rate of CMC hydrolysis by 4.6% for T. viride enzyme. $CaCl_2$ was a good stimulator for the enzyme synthesized by E. oxysporium. The other tested elements were either less stimulatory or were inhibitory to the enzyme activity[20,22].

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دراسة بيوكيميائية وتنقية جزئية لإنزيم السيليوليز المنتج بواسطة تراكودرما فيريدى وفيوزاريوم اكسوسبوريوم

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** قسم النبات - كلية العلوم - جامعة طنطا*

لقد تم ترسيب انزيم السيليوليز المنتج بواسطة تراكودرما فيريدى والفيوزاريوم اكسوسبوريوم باستخدام المذيبات العضوية مثل الكحول الإيثيلى والأسيتون والبيوتانول بتركيزات ٤٤٪ ، ٦٦٪ ، ٨٣٪. وقد وجد أن الكحول الإيثيلى بتركيز ٦٦٪ هو افضل المذيبات العضوية لترسيب الانزيم. وقد اذيب الراسب فى المنظم استرات (pH 5) ووضع فى العمود الكروماتوجرافى مملوء بالسيفادكس لتنقية الأنزيم ووجد أن أنسب العوامل البيوكيميائية التى تؤثر فى نشاط الأنزيم النقى هى ١٪ كربوكسى ميثيل سيليولوز بعد ٤٥ دقيقة من التحقيق فى ٤٠م عند منظمة سترات (pH 5) لكل من الكائنين تحت الدراسة وقد وجد أن كبريتات الزنك وكلوريد الكالسيوم لهم تأثير منشط لنشاط الأنزيم النقى بمعدل نشاط ٤ر٦ ، ١ر٨٪ لكل من تراكودرما فيريدى والفيوزاريوم اكسوسبوريوم بالتتالى.