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PRODUCTION OF CELLULOLYTIC ENZYMES BY *Trichoderma viride* GROWN ON SUGAR BEET INDUSTRIAL WASTES

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ABSTRACT

Four fungal strains were grown using sugar beet industrial by-products. These wastes were sugar beet pulp, sugar beet leaves, sugar beet molasses as low cost carbon source. The optimization for the enzymatic production was examined such as growth temperature, initial pH of fungal growth and the effect of nitrogen source. Some properties of cellulases obtained by *Trichoderma viride* were also studied such as temperature, pH, incubation time and thermal stability of the enzymatic activity. Cellulases activities were found at the maximum point after the 6th day of shake flask cultivation for the three tested enzymes, FP-ase, CMC-ase and Cb-ase by *Aspergillus niger*. In case of *Aspergillus fumigatus* data showed the same behaviour except CMC-ase activity which produced after 9th day of fermentation where a mixture of leaves and molasses was used as carbon source. For *Trichoderma viride*, sugar beet pulp gave the maximum enzymatic production of both CMC-ase and Cb-ase after the 6th day of fermentation, while FP-ase was reduced after that time. Using beet leaves for growing *Trichoderma harzianum*, both FP-ase and Cb-ase were in their optimal production while CMC-ase reached its peak when the fungus grown on sugar beet leaves with molases in 1:1 ratio. The growth temperature of 30°C and pH 5 were the optimum for the selected fungal strain, *Trichoderma viride* for the three tested cellulases. The cultivation medium containing peptone and $(NH_4)_2SO_4$ as N source showed to be the best for the production of the three tested cellulases. For the cellulolytic activities, the optimum temperature and pH were 60°C and 5.5, respectively. For the incubation time, 18hr, 1 hr and 45 min, proved to be the best for FP-ase, CMC-ase and Case, respectively. The thermal stability of the cellulases was remained to 70°C.

Keywords: Cellulases, Fermentation, Environmental pollution, Sugar beet by-product, *Trichoderma viride*.

INTRODUCTION

Cellulose present in the rapidly renewable lignocellulosics is considered the most important reservoir of carbon for the production of glucose, a fuel and chemical feedstock. The enzymatic saccharification of cellulose to glucose has received much attention during the last two decades. The breakdown of native cellulose to soluble sugars by microorganisms is a process that involves the action of multi-system. The cellulases systems of the aerobic fungi consists of : 1- C_1 [Endo, β-D-glucanase (E.C. 3.2.1.4)], 2- C1 [Exo, β-D-glucanase (3.2.1.91)], 3- Endo, β-D-glucanase (E.C. 3.2.1.74)] and 4- β-glucosidase (E.C. 3.2.1.21), Bhat and Bhat (1997). Sugar beet wastes appear to be favourable substrate, as it is abundantly and cheaply available and have a cellulose content. The wastes accumulate in agroindustrial yards, causing environmental pollution, fire hazards and

disposal problems and have no significant industrial or commercial uses. Considering these facts, the study was conducted to optimize the fermentation conditions for the biosynthesis of cellulases by the examined fungus, *T. viride*, selected on the basis of a screening study carried out during the work. Also, some factors affecting. *T. viride* cellulases activities were done.

MATERIALS AND METHODS

Sugar beet wastes:

The wastes were obtained from Delta Beet Sugar Company, Belquas, Dakahlia Governorate, Egypt. The pulp and leaves were dried in the oven at 60°C to constant weight and ground into fine powder in a laboratory mill. The powder was sieved and kept in a desicator until used.

Organisms:

Four fungal strains were used in this study. Two strains of the genus *Aspergillus* namely *A. niger* and *A. fumigatus*, and two strains of the genus *Trichoderma* namely *T. viride* and *T. harzianum*. These strains were kindly taken from Plant Pathology Dept., Agric. Res. Center, Giza, Egypt.

The fungal strains were propagated and maintained on Potato Dextrose Agar slants (Demain and Solomon, 1986) at 4°C and subcultured at monthly intervals.

Inoculum preparation:

Inocula were prepared by transferring 12 days old conidia grown on PDA slopes by 5 ml sterile distilled water. A portion of one ml of this spore suspension, which contains about $10^5 - 10^7$ spores / ml was used for the inoculation of each 250 ml Erlenmeyer flask contain 50 ml of the production medium of Chen and Wayman medium (1991).

Cellulases assay:

Filter-paper-cellulase activity (FP-ase):

FP-ase activity was determined by measuring the reducing sugars released from Whatman filter paper No. 1 (Muniswaran and Charyulu, 1994). One unit of FP-ase was defined as the amount of enzyme that liberated 1 mg glucose/60 min under the optimal conditions.

Carboxymethyl cellulases activity (CMC-ase):

CMC-ase activity was determined by measuring the reducing sugars released from 0.5%CMC (Muniswaran and Charyulu, 1994). One unit of CMC-ase was defined as the amount of enzyme that liberated 1 mg glucose/30 min under the optimal conditions.

Cellobiase activity (Cb-ase):

Cb-ase activity was determined by measuring the reducing sugars released from cellobiose (Muniswaran and Charyulu, 1994). One unit of Cb-ase was defined as the amount of enzyme that liberated 1 mg glucose/15 min under the optimal conditions.

Culture conditions and optimization studies:

A series of 250 ml Erlenmeyer flasks, each containing 50 ml of Chen and Wayman medium (1991).the medium is consisted of (g\L) K_2HPO_4,2.0; $(NH_4)SO_4$,2.0; $MgSO_4.7H_2O$,0.3; $CaCl_2$,0.3 and peptone 1.0. The wastes of sugar beet by-products (pulp,1%; leaves,1%; pulp,0.5% + leaves,0.5%; pulp,0.5%: + molasses,0.5%; or leaves,0.5%; + molasses,0.5%) were added as a sole carbon source and the medium was sterilized at 121°C for 15 min and inoculated with 1 ml of spore suspension. Cultivation was performed on a rotary shaker (180 rev/min). After incubation period the cultures were harvested by filtration through glass wool filter and then centrifuged. The clear supernatant was used for enzyme assay. Some factors affecting cellulases production such as carbon source, time course, incubation temperature, initial pH and nitrogen source elemination were studied.

Factors affecting cellulolytic activity:

Optimal temperature:

It was determined using substrates buffered at temperatures ranging from 30-70°C under standard conditions.

Optimal pH:

The optimal pH for each enzyme was determined using 0.05 M citrate buffer (pH 4.0-6.6), 0.05 M sodium phosphate buffer (pH 7.0-8.0) under standard conditions.

Optimal time of enzymes activities:

It was done by incubating the reaction mixture of each enzyme for different incubation times under standard conditions.

Thermal stability:

It was investigated by measuring the residual activities after incubating the culture filtrate at 50 and 60°C for 15, 30 and 45 min.

Reducing sugars determination:

Reducing sugars were colorimetrically assayed by the method of Somogyi (1952).

RESULTS AND DISCUSSION

Factors affecting cellulases production:

Carbon source and time course:

They were examined by growing the four tested organisms in Chen and Wayman medium (1991), supplemented with one of the sugar beet wastes (1%) as a carbon source, at 30°C for 3, 6, 9, 12 and 15 days. Data in Tables 1 and 2 showed the effect of time course and carbon source on cellulases production by the tested fungi. Tabulated data indicated that the production of cellulases by the tested fungi increased with time increased up to 6 days of fermentation and started to decrease. Results are in agreement with the previous data of Muniswaran and Charyulu (1994) who found that the maximum FP-ase and CM-Case production from *T. viride* was obtained after 7 days of incubation. Sharma *et al.* (1991) obtained cellulases after the 5th day of fermentation, while Youssif (1996) obtained cellulases at the 7th day of fermentation. Maximum enzyme production by *A. niger* was reported

at the 7th day of incubation (Abd-El-Naby (1988). Kang *et al.* (1995) found that the maximum yield of β-glucosidase produced by *A. niger* KKS mutant grown on rice straw was obtained after 7 days of fermentation. Selim (2001) found that the CM Case and FPase of *T. viride* grown on banana peel were maximized in their productivity on the sixth day of fermentation.

Table 1: Effect of sugar beet wastes and time course on cellulases production by fungi grown at 30°C (reaction mixture was done at 40°C for 1 h for FP-ase, 30 min for CM-Case, and 15 min for Cbase using citrate phosphate buffer at pH4.8).

Time (days)	Enzyme activity U/ml	Waste used									
		P	L	P+L	P+M	L+M	P	L	P+L	P+M	L+M
		Aspergillus niger					*Aspergillus fumigatus*				
3	FP-ase	16.7	26.4	20.2	12.5	23.8	9.2	12.6	15.2	11.8	26.2
	CMC-ase	29.9	20.2	24.9	29.0	58.1	22.6	21.6	25.7	17.2	17.0
	Cb-ase	19.7	18.6	23.8	21.0	13.4	12.5	12.4	11.4	11.0	14.9
6	FP-ase	28.3	31.8	31.9	26.5	29.5	23.3	27.4	49.3	25.8	45.0
	CMC-ase	38.5	74.3	47.9	47.2	82.4	51.5	58.5	68.1	47.6	40.4
	Cb-ase	34.7	43.9	37.8	37.3	30.0	21.8	20.1	19.3	23.9	21.9
9	FP-ase	18.6	24.2	20.8	23.4	24.2	19.3	21.8	29.2	18.7	41.7
	CMC-ase	27.1	41.2	27.0	35.3	56.1	32.5	48.9	50.0	38.4	54.1
	Cb-ase	24.4	27.6	29.0	31.2	22.2	13.3	18.5	15.5	20.7	13.5
12	FP-ase	9.9	19.2	16.0	13.9	22.5	17.5	10.2	24.7	12.2	27.2
	CMC-ase	18.0	32.9	13.4	26.4	48.5	26.0	33.5	35.1	28.6	32.7
	Cb-ase	15.0	18.1	23.9	21.3	18.8	9.1	15.8	10.5	12.6	9.9
15	FP-ase	9.0	12.2	9.1	6.5	18.8	8.4	6.6	17.9	8.6	18.2
	CMC-ase	16.0	21.7	8.1	15.1	28.9	14.4	22.1	23.2	14.4	17.3
	Cb-ase	11.8	9.9	16.0	16.3	13.0	7.2	9.8	7.1	6.3	5.6

L: Leaves,1%; P: Pulp, 1%; M: Molasses, 1%; P+M: Pulp, 0.5%;+ Molasses 0.5%
L+P: Leaves,0.5%;+ Pulp, 0.5%; L+M: Leaves,0.5%+ Molasses 0.5%

On the other hand, data in Tables 1 & 2 showed that the enzyme production is influenced by the type of waste and organism. The best carbon source of cellulases production by *A. niger* was 1% leaves while it was 0.5% pulp + 0.5% leaves with *A. fumigatus* for FP-ase and CMCase and 0.5% pulp + 0.5% molasses for cellobiase production. On the other hand, 0.5% pulp + 0.5% leaves was the most favorable carbon source for FPase, CMC ase and cellobiase production by *T. viride* while 1% leaves was the best carbon source for FPase and cellobiase production by *T. harzianum* and 0.5% pulp + 0.5% leaves of CMCase production. Results are in agreement with the previous suggestions (Mandels, et al., 1974; Toyam, 1976 and Knp and Leeg, 1986) that when *T. reesei* is grown on different cellulosic materials as its sole carbon source, large differences in the cellulases and xylanase were observed.

Table 2: Effect of sugar beet wastes and time course on cellulases production by fungi grown at 30°C (reaction mixture was done at 40°C for 1 h for FP-ase, 30 min for CM-Case, and 15 min for Cb-ase using citrate phosphate buffer at pH4.8).

Time (days)	Enzyme U/ml	Waste used									
		P	L	P+L	P+M	L+M	P	L	P+L	P+M	L+M
		Trichoderma viride					*Trichoderma harzianum*				
3	FP	14.5	22.6	22.8	25.2	24.1	15.9	29.6	15.0	13.7	13.7
	CMC	47.4	43.3	95.1	51.5	56.7	22.0	26.5	69.5	55.3	39.2
	Cb	12.2	20.2	40.1	11.5	17.3	13.0	12.2	12.9	6.0	11.0
6	FP	51.0	49.5	61.8	58.0	56.0	28.9	38.3	32.5	24.0	23.1
	CMC	78.3	100	125.3	71.6	98.6	43.3	67.0	70.5	35.5	74.5
	Cb	39.6	33.2	73.2	33.4	28.2	36.0	46.3	42.0	19.6	21.2
9	FP	41.2	40.5	43.5	33.3	41.2	22.5	25.3	20.5	18.9	19.6
	CMC	76.8	87.0	112.9	62.3	62.9	33.8	66.0	59.8	26.3	68.8
	Cb	23.3	28.4	50.6	22.5	14.3	20.4	25.7	25.8	17.8	18.0
12	FP	29.4	26.8	40.3	14.9	28.9	12.5	18.8	17.4	14.9	16.7
	CMC	46.9	71.6	83.0	42.2	46.9	16.5	43.0	37.0	13.9	52.5
	Cb	18.9	18.3	31.2	12.2	12.3	15.0	14.5	11.9	13.3	14.3
15	FP	19.3	18.7	29.5	13.0	15.9	9.7	14.8	9.3	11.3	9.3
	CMC	31.0	47.9	54.4	27.4	27.3	26.5	29.5	33.5	10.5	31.4
	Cb	12.9	11.9	25.0	11.1	11.3	7.7	10.1	8.0	10.5	9.2

Growth temperature:

It was studied by growing *T. viride*, the most active organism, on Chen and Wayman medium (1991) using 0.5% pulp + 0.5% leaves as carbon source. Data in Table (3) show that the production of the three enzymes gradually increased with increasing of temperature up to 30°C, then decreased at 33 and 36°C. Mukhopadhyay and Malik (1980) reported that the optimum temperature for cellulases production by *T. reesei* was 28-30°C. Many investigators reported optimum temperature for cellulolytic enzymes production between 27-30°C (Webb *et al.*, 1986; Doppelbauer *et al.*, 1987 and Youssif, 1996). Selim (2001) found that the optimum temperature for enzyme production by *T. viride* was 30°C.

Table 3: Effect of incubation temperature on the production of cellulases by *T. viride* after 6 days of incubation using 0.5% pulp + 0.5% leaves as carbon source.

Temp. (°C)	FP-ase		CMC-ase		Cb-ase	
	U/ml	%	U/ml	%	U/ml	%
24	25.71	40.33	50.45	39.20	23.90	31.70
27	40.21	63.07	97.70	75.94	52.20	69.14
30	63.75	100.0	128.65	100.0	75.50	100.0
33	40.85	64.08	106.55	82.8	63.90	84.60
36	29.55	46.35	95.70	74.39	46.20	61.19

Initial pH:

Results listed in Table (4) showed that the production of the three cellulases enzymes increased with the increase of the value of pH starting from pH 3 up to pH 5, then the production decreased from pH 6 up to pH 9. Cochet (1991) reported that the optimum culture pH for CMC-ase and FP-ase production by *T. reesei* was 4.0. Mashitah and Roziah (1996) found that the greatest production of cellulases by *Myceliophthora thermophila* grown on oil palm residues was obtained when the pH of the cultivation medium was 5.5-5.8. Selim (2001) found that the optimum pH for CMCase and FPase production by *T. viride* was 5.0.

Table 4: Effect of pH values on the production of cellulases by *T. viride* after 6 days of incubation at 30°C (reaction mixture was done at 40°C for 1 h FP-ase – 30 min. CMC-ase – 15 min CMC-ase using phosphate citrate buffer at 4.8).

PH	FP-ase		CMC-ase		Cb-ase	
	U/ml	%	U/ml	%	U/ml	%
3	20.34	31.66	40.90	31.68	26.70	35.79
4	35.00	54.47	88.65	68.35	43.15	57.34
5	64.25	100.0	129.70	100.0	74.60	100.0
6	57.65	89.73	99.10	76.41	57.80	77.48
7	35.93	55.92	81.65	62.95	33.70	45.17
8	25.63	39.89	75.50	58.21	21.20	28.42
9	20.23	31.49	55.90	43.09	13.60	18.23

N-source elemination:

The effect of N-elemination on the production of cellulases by *T. viride* was tested at three levels of pH values 5, 6 and 7. As shown in Table (5) the culture medium M_1 showed to be the best at pH 5 and pH 6 for FP-ase and Cb-ase production. Data showed that both ammonium sulphate and peptone are required to support cellulolytic enzymes production by *T. viride*. Deprivation of ammonium sulphate and/or peptone resulted in suppression of enzyme production. These results are confirmed with those obtained by Srivastava *et al.* (1987) and Abd El-Naby (1988). They reported the suitability of ammonium sulphate as the inorganic N-source and peptone as organic N-source for the production of active cellulase and cellobiase by *Aspergillus* sp. Enari and Markennan (1977) reported that good cellulase production can be obtained with peptone as the organic N. Source. Muniswaran and Charyulu (1994) stated that in the presence of peptone, a more effective N-source, *T. viride* produced high amounts of cellulases. Finally Selim (2001) obtained similar results on *T. viride* cellulase production. From the above mentioned data, it could be seen that suitability of N-source for cellulases production from the tested fungus is depend on the pH value of the cultural medium and M1 medium was the optimum at different pH's.

Some properties of cellulases:

Table 5: Effect of N-source elimination on the production of cellulases by *T. viride* after 6 days of incubation at 30C (reaction mixture was done at 40°C for 1 h FPase, 30 min for CMCase and 15 min for cellobiase using phosphate citrate buffer at 4.8).

Initial pH	Medium used	FP-ase		CMC-ase		Cb-ase	
		U/ml	%	U/ml	%	U/ml	%
5	M_1	63.75	100.0	125.25	100.0	76.52	100.0
	M_2	51.50	80.78	105.60	84.31	50.90	66.39
	M_3	27.78	43.58	62.79	50.09	30.60	39.99
	M_4	20.06	31.45	39.40	31..46	18.80	24.57
6	M_1	58.38	100.0	98.30	100.0	54.30	100.0
	M_2	47.65	81.62	88.30	89.83	50.10	92.27
	M_3	27.30	46.76	54.10	55.04	23.90	44.01
	M_4	18.25	31.26	36.60	37.23	14.90	27.44
7	M_1	36.25	100.0	82.00	100.0	32.80	100.0
	M_2	27.30	75.31	70.90	85.46	24.50	74.70
	M_3	20.83	57.46	55.30	67.44	15.90	48.48
	M_4	17.23	47.53	20.35	24.82	11.90	36.28

M1; complete medium.
M2; complete – $(NH_4)_2SO_4$.
M3; Complete – peptone
M4; Complete – (peptone + $(NH_4)_2SO_4$).

Culture filtrate of *T. viride* grown on Chen and Wayman medium (1991) supplemented with leaves + pulp (1%) as a carbon source, at 30°C, pH 5.0 for 6 days was used as enzymes source. Some factors affecting enzymes activity were studied.

Effect of temperature on cellulases activity:

The effect of temperatures is presented in Table (6). It obvious from data that the optimum temperature for CMCase, FPase and Cbase was 60°C. Kanamoto *et al.* (1979) reported that the optimum temperature for two CMC hydrolyzed enzymes (F-1 and F-v) of *A. aculeatus* were 45 and 55°C, respectively. Khalaf-Allah *et al.* (1993) stated that the optimum temperature for cellulase activity is 50°C. Also Kang *et al.* (1995) found that the optimum temperature for CMC-ase activity of *A. niger* mutant was 60-70°C.

Table 6: Effect of temperature of reaction mixture on cellulases activities (reaction mixture was done at pH 4.8 for 15, 30 and 60 min for cellobiase, CMCase and FPase, respectively).

Temperature of reaction mixture	CMCase U/ml	FPase U/ml	Cellobiase U/ml
30	70.19	49.51	58.07
40	125.25	63.80	76.52
50	130.79	65.71	78.18
60	140.17	70.01	84.05
70	70.28	25.09	40.03

Optimum pH:

The optimum pH for each enzyme was determined using 0.05 M citrate buffer (pH 4.0-6.6) and 0.05 M phosphate buffer (pH 7.0-8.0) under standard conditions. As shown in Table (7) the pH level of 5.0 and 5.5 were more favourable for FPase activity while the level of 5.5 and 6.0 were more favourable for CMCase activity. On the other hand, the pH level of 4.5 was the best for Cb-ase activity. Kanamoto *et al.* (1979) found that the optimum pH of two carboxymethyl cellulose hydrolyzing enzymes (F-1 and F-v) of *A. aculeatus* were 4.0-4.5 and 5.0, respectively, and Kang *et al.* (1995) found that the optimum pH of β-glucosidase produced by *A. niger* was 4.3. Hauka *et al.* (2000) reported that CMC-ase, FP-ase and Cb-ase of *A. niger* showed their pH optima at 5.5, 6.5 and 5.5, respectively.

Table 7: Effect of pH on cellulases activities (reaction mixture was done at 60°C for 15, 30 and 60 min for cellobiase, CMCase and FPase, respectively).

PH	CMCase U/ml	FPase U/ml	Cellobiase U/ml
3.0	98.82	63.22	71.55
3.6	136.55	68.78	100.74
4.0	113.74	60.21	100.74
4.6	121.64	68.80	101.95
5.0	143.1	71.50	84.85
5.6	148.41	71.50	77.04
6.0	148.11	70.00	72.58
6.5	147.56	69.36	63.76
7.0	134.33	67.22	61.91
7.5	127.01	69.60	53.09
8.0	109.14	59.01	48.79

The buffers used were citrate-phosphate buffer (pH 3.0-5.6) and sodium phosphate buffer (pH 6.0-8.0).

Optimum time of reaction:

To examine the optimum time of reaction for the cellulolytic enzymes the activities were measured at different incubation time 1, 2, 6, 12, 16, 18, 20 and 24 hr for FP-ase and 15, 30, 45, 60, 75, 90, 105 and 120 min for CMC-ase and Cb-ase. Data in Tables (8,9) showed that 18 and 20 hr proved to be the optimum incubation time for FPase while the time of 60 min showed to be the optimum for CMC-ase and Cbase. Similar results were obtained on cellulases of *A. niger* by Hauka *et al.* (2000).

Table 8: Effect of incubation time on cellulases activities (reaction mixture was done at 60°C, pH 5.5 for CMC-ase and 4.5 for Cb-ase).

Incubation time (min)	CMCase U/ml	Cb-ase U/ml
15	137.81	101.95
30	148.41	149.13
45	150.91	180.07
60	178.13	210.11
75	178.11	210.11
90	178.99	210.09
105	178.12	208.11
120	178.14	209.30

Table 9: Effect of incubation time on FPases activities (reaction mixture was done at 60°C, pH 5.5).

Incubation time / h	1	2	6	12	16	18	20	24
U/ml	71.05	107.68	109.82	136.50	138.73	159.92	159.92	159.92

Thermal stability of cellulases:

It was investigated by measuring the residual activities of the enzymes after incubating the culture filtrate at 50 and 60°C for 15, 30 and 45 min. Data in Table (10) showed that even at 60°C there is no harmful effect on the enzymes activities. It could be concluded that the three enzymes are thermostable and are suitable to use in some industrial processes using high temperatures. Similar results were obtained by Abd El-Naby (1988) on cellulases of *A. niger* and *T. viride* and Hauka *et al.* (2000) on cellulases of *A. niger.*

Table 10: Effect of thermal stability on cellulases activities (crude enzymes was done at 50 and 60°C for 15, 30 and 45 min, then residual activity was measured. Reaction mixture was done at optimum conditions for enzyme activities.

Temp. (°C)	Time (min.)	CMCase U/ml	Cellobiase U/ml	FPase U/ml
50	15	178.13	210.11	159.92
	30	178.13	210.11	159.92
	45	178.13	210.11	159.92
60	15	178.13	210.11	159.11
	30	169.14	209.19	159.11
	45	167.29	207.01	159.11

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إنتاج الإنزيمات المحللة للسليولوز من فطر ترايكودرما فيردي النامي على المخلفات الصناعية لبنجر السكر

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استخدم في هذا البحث أربعة سلالات فطرية محلية هي أسبرجلس نيجر، أسبرجلس فيوميجاتس، ترايكودرما فيردى، ترايكودرما هارزيانم ونميت على البيئة المعدنية، وكانت مصادر الكربون هي لب البنجر، ورق البنجر، مولاس البنجر بمعدل ١% وخليط منهم (لب ٠,٥% + ورق ٠,٥% ، مولاس ٠,٥% + لب ٠,٥% ، ورق ٠,٥% + مولاس ٠,٥%) وخلصت النتائج إلي أن فطر ترايكودرما فيردي كان أنشط الفطريات إنتاجا للإنزيمات الثلاث المحللة للسليلوز وهي Fp-ase، Cb-ase و CMC-ase ووصل أقصى درجة من الإنتاج بعد ستة أيام من التحضين وكان مصدر الكربون (١%) خليط من لب البنجر وورق البنجر بنسبة ١:١. بالنسبة لفطر أسبرجلس نيجر فقد وصلت الإنزيمات الثلاث إلى أعلى إنتاجية في اليوم السادس من التحضين وقد سلك نفس السلوك فطر أسبرجلس فيوميجاتس لإنزيمى Fp-ase ، Cb-ase أما إنزيم CMC-ase فقد وصلت أعلى إنتاجيه له بعد ٩ أيام وكان مصدر الكربون خليط من المولاس وورق البنجر. فطر الترايكودرما هارزيانم أعطى أعلى إنتاجية من إنزيمي Cb-ase ، CMC-ase عندما كان مصدر الكربون هو ١% من المولاس وورق البنجر بنسبة ١:١. وعند دراسة الظروف المثلى لإنتاج الإنزيمات من أنشط الفطريات المستخدمة في الدراسة وهو فطر تريكودرما فيردى. وقد وجد أن أنسب ظروف للإنتاج هى ٣٠°م، درجة pH هي ٥ بعد ستة أيام. عند دراسة تأثير حذف مصادر النيتروجين من البيئة الأساسية إتضح أهمية وجود كل من الببتون وكبريتات الأمونيوم كمصادر للنيترجين فى البيئة وأدى حذف أي منهما أو كليهما من البيئة إلى نقص في إنتاج الإنزيمات تحت الدراسة.كذلك وجد أن أنسب درجة حرارة للإنزيمات الثلاث هي ٦٠ °م وأنسب درجة pH هى ٥,٥. وقد كان أنسب وقت للتحضين هو ١٨ ساعة بالنسبة لإنزيم Fp-ase، ٦٠ دقيقة بالنسبة لإنزيم CMC-ase، ٤٥ دقيقة بالنسبة لإنزيم Cb-ase. ولقد وجد أن الإنزيمات الثلاث تحت الدراسة كان لديها ثبات حرارى حتى ٧٠ °م.

وتهدف هذه الدراسة إلي تعظيم تدوير المخلفات الزراعية الصناعية باستخدام الكائنات الحية الدقيقة وذلك لحماية البيئة من التلوث بهذه المخلفات إذا تركت دون إستخدام.