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PRODUCTION OF CELLULASE ENZYMES IN A MIXED CULTURE OF *Trichoderma reesei* AND *Apergillus niger*

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ABSTRACT

Trichoderma reesei showed higher production of CMCase (3.65 times),FPase (3.68 times),xylanase (1.63 times), and hydrolytic power (5.4 times) than *Aspergillus niger*. However, β-glucosidase production was only about 0.135 of that of *A. niger*. Mixed cultivation of the two strains *Trichoderma reesei* and *Aspergillus niger* with modification of medium composition increased all cellulase activities. It was 8.1 times for CMCase, 5.9 times for FPase, 1.2 times for β-glucosidase, 3.6 times for xylanase as compared with *Aspergillus niger*. The hydrolytic power (%) of the cellulase complex produced by the mixed culture was 15.5 times in comparison with *Aspergillus niger*. A comparative study was made on cellulase activities produced by the mixed culture and that reported by other cellulase producers. The cellulase enzymes production by mixed culture is comparable to that of cellulases potent producers

Keywords: cellulase production, Mixed culture, *Trichoderma ressei, Aspergillus niger.*

INTRODUCTION

In Egypt, considerable amounts of lignocellulosic materials are discarded in the form of straw as it is of a very low nutritional value for ruminant livestock. More than 3.5 million tones of rice straw are annually generated in Egypt. Part of rice straw is being used properly on pulp and paper industry. However, most of rice straw used for fuel in Egyptian villages causing health problems and environment pollution. Utilization of rice straw as fuel is no longer acceptable means of use, therefore, there is a great attention in Egypt to recycle the rice straw as agricultural waste.

Natural cellulosic materials can provide energy in the form of glucose through their enzymatic hydrolysis by cellulolytic enzymes of microbial origin (Thomas *et al.*, 1995, Bisaria *et al.*, 1997 and Bhat, 2000). In most cellulolytic microorganisms the complete enzymatic hydrolysis of native cellulose to glucose is catalyzed by a multiple cellulase system (Himmel *et al.*, 1996, Teeri, 1997, Baker *et al.*, 1998, Palma *et al.*, 2000, and Lynd *et al.*, 2002). This cellulase system must contain the following enzymes:

1) **CMCase,** Endo-1, 4-β-glucanase (1,4-β-D-glucan glucanhydrolase, (EC 3.2.1.4)
2) **FPase**, Exo-1, 4-β-glucanase (1, 4-β-D-glucan cellobiohydrolase, EC 3.2.1.91)
3) **β-glucosidase (Cellobiase),** (β-D-glucosidase glucohydrolase, EC 3.2.1.21)

It was shown that the cellulose hydrolysis by these three types of enzymes follows a mechanism with two consecutive reactions (Béguin & Aubert, 1994 and Sheehan & Himmel, 1999). The endo- and exoglucanase are responsible for the first reaction and β-glucosidase for the second one, according to the following scheme:

There are two important and difficult points in this process; the first is how to produce the active enzyme, and the second is how to accelerate the hydrolysis rate which is the limiting step in the production of glucose. An extenensive search is being made to select suitable organisms that possess a high celluylolytic activity (Béguin, 1990 and Esterbauer *et al.*, 1991). Generally, the cellulase systems of the most hypercellulase microorganisms are deficient in β-glucosidase. Although β-glucosidase dose not directly participate in cellulose hydrolysis, it plays an important role in saccharification of cellulose. During the hydrolysis of cellulose with this type of cellulase systems, cellobiose accumulates and inhibits the action of cellobiohyrolase. From the hydrolysis results reported by various researchers, we can conclude that a cellulase system having a ratio of FPase: β-glucosidase activities close to 1.0 is necessary to obtain the highest rate of hydrolysis and highest glucose content in the produced hydrolyzate (Chahal *et al.*, 1996).

Microorganisms of the genera *Trichoderma* and *Aspergillus* are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use. Microorganisms of the genus *Trichoderma* produce relatively large quantities of endo-?-glucanase and exo-β-glucanase, but only low levels of ?-glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo-?-glucanase and ?-glucosidase with low levels of exo-?-glucanase production (Teeri,1997 and Juhasz *et al.*, 2003) *Trichoderma reesei* showed significantly higher yields of C_1 and Cx cellulases but lower yield of ?-glucosidase, but it was interesting to note that ?-glucosidase production by *Aspergillus niger* was about 6-7 times that of *Trichoderma reesei*.

The development in the production of cellulase enzymes depended on the study of pure or monospecies cultures despite the knowledge that cellulytic microorganisms in natural environments exist as complex or multispecies communities. Also, the modern fermentation industry similarly has been dominated by the pure culture approach, again despite the fact that traditional food, and waste treatment processes are developed by using of mixed cultures. For these reasons, in the recent years the properties of mixed cultures for cellulases production have attracted increasing attention (Bisaria *et al.*, 1990, Duenas *et al.*, 1995, Gutierrez &Tengerdy, 1997, Gutierrez & Tengerdy, 1998, Gutierrez *et al.*, 1999, and Massadeh *at al.*, 2001). The type of two-microorganisms interaction can be synergetic, therefore the mixed culture of the two strains *Trichoderma reesei* and *Aspergillus niger*

can provide a means for obtaining a higher yield of a complete cellulase system and may be superior to the one obtained by pure culture of each strain.

The current study deals with the efficient production of cellulases, xylanase, and ?-glucosidase in a mixed submerged cultivation of *Trichoderma reesei* and *Aspergillus niger*. Experiments were carried out to optimize the nutrition requirement for maximum production of the cellulase enzymes in the mixed shake co-cultivation of the two strains.

MATERIALS AND METHODES

Microorganisms. *Trichoderma reesei* NRRL-3653 was obtained from ARS, culture Collection, USDA (Peoria, IL), and *Aspergillus niger* DSM-823 from Deutsch Sammelung von Mikroorganisms, G.ttingen, Germany. These strains were individually maintained on potato dextrose agar (PDA) at 4°C and subcultured at two months intervals.

Rice Straw (RS). Air-dried rice straw was obtained from Moshtohor-Kalioubia Governorate. It was milled to particles that passed through a 40 mesh screen.

RS-Holocellulose preparation. The ground rice straw (RS) was delignified to obtain the RS-Holocellulose according to Sidhu & Sandhu (1980) as follows: Rice straw (50 g) was suspended in water (1600 ml) and treated with sodium chlorite (15 g) and conc. acetic acid (5 ml) for 4h at 70-80°C. The treated rice straw was then thoroughly washed repeatedly with water and dried

Culture medium. The basal medium of Mandels & Weber (1969) was used in this study. Its composition as follows (g/l): 1.4 $(NH_4)SO_4$, 0.3 urea, yeast extract o.1, Tween 80 1.0, peptone 0.25, 2 KH_2PO_4, 0.3 $MgSO_4.7H_2O$, 0.3 $CaCl_2$, 0.005 $FeSO_4.7H_2O$, 0.0016 $MnSO_4.H_2O$, 0.0014 $ZnSO_4.7H_2O$, 0.002 $CoCl_2.6H_2O$, and hollocellulose 1% (w/v). The medium was adjusted to pH 5.0 before sterilization.

Inoculum. The inoculum's for each strain was prepared in 250-ml Erlenmeyer flasks containing 50-ml basal medium supplemented with 20 g/l glucose. After sterilization, the flasks were inoculated with a loop of 5 days old culture, and then incubated on rotary shaker (150 rpm) for 24 h at 30 °C. At the end of the incubation period, the broth containing the fungal biomass was used to inoculate the fermentation medium.

Fermentation. The batch culturing was conducted by submerged method on rotary shaker (150 rpm). Portions of 20 ml of nutrient medium were introduced into 100-ml Erlenmeyer flasks containing holocellulose (0.2g, 1%) as carbon source. Sterilization of the medium was carried out at 121°C for 20 min. Inoculation was performed with one ml of pure culture and one ml from each strain in mixed culture . After fermentation, the cells were removed by centrifugation (8000xg, 10 min) or by filtration. The clear solution thus obtained was used as the enzymes source.

Enzyme activities assay.

CMCase. One ml of the appropriately diluted enzyme sample was mixed with 1%(w/v) CMC dissolved in acetate buffer (0.1 M ,pH 4.5) and incubated for

10 min at 50°C in a water bath. Reducing sugars released were estimated by the dinitrosalicyclic acid (DNS) reagent method according to Miller (1959) using glucose as a standard.

FPase. Whatman (No.1) filter paper was used as substrate. One ml of acetate buffer(0.1 M ,pH 4.5) was mixed with 50 mg of 0.3mm diameter filter paper disks and one ml of the diluted enzyme was added. The reaction mixture was incubated for 60 min at 50°C. Reducing sugars in the supernatant were estimated as given above.

Xylanase. One ml of the enzyme suitably diluted was mixed with 1 ml of (1%w/v) oat spelt xylan suspended in acetate buffer (0.1 M, pH 4.5). After incubation for 10 min at 50°C, the reducing sugars were then measured as given above using xylose as a standard.

β-Glucosidase. The activity was determined according to Ghose, (1987) by incubating one ml of diluted enzyme with one ml of 1% salicin in acetate buffer (0.1 M, pH 4.5) at 50° for 30 min, the released glucose measured as given above.

Enzymes unit. In all the above assays one international unit (IU) of enzyme activity was defined as of enzyme releasing 1 µmole of reducing sugar (glucose or xylose) from the substrate per minute.

Hydrolytic power estimation. Holocellulose (50 mg) was mixed with one ml acetate buffer (0.1M, pH 4.5) and one ml of the culture filtrate. After incubation at 50°C for 24h in water bath, the released reducing sugars (mg) were determined as given above. The degree of hydrolysis (%) was calculated from the following equation recorded by Szczodrak *et al.* (1984).

$$\text{Hydrolytic power (\%)} = \frac{162}{180} \times \frac{\text{Reducing sugars (mg)}}{\text{Holocellulose (mg)}} \times 100$$

RESULTS AND DISCUSSION

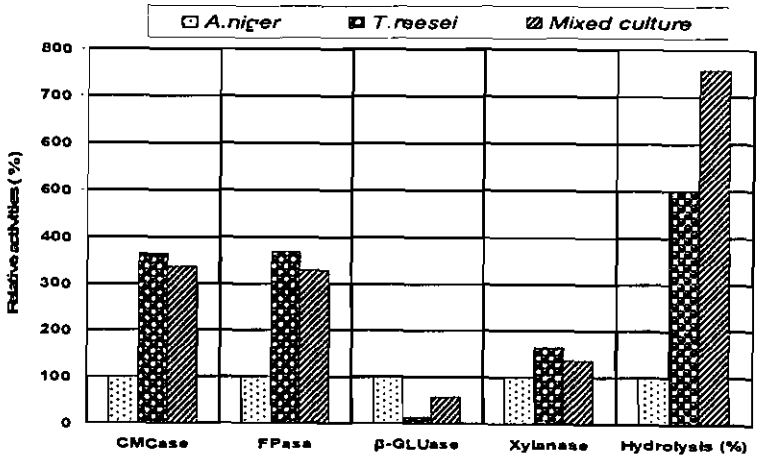
1. Production of cellulase enzymes by pure and mixed culture cultivation.

The pure cultures of the two strains *Trichoderma* reesei and *Aspergillus niger* were cultivated in batch fermentation mode under shaking conditions (150 rpm) using Mandels-medium supplemented with 1% hollocellulose as carbon source for 4 days at 30°C. The assay of enzyme activities was performed in the culture filtrates and the results obtained from these experimental runs are summarized in Table (1) and Fig (1). The strain *Trichoderma reesei* produced more than 3.5 times the amount of CMCase and FPase, and about 1.6 times the amount of xylanase when compared to these produced by *Aspergillus niger* under the same conditions. The hydrolytic power (%) of delignified rice straw (Holocellulose) by cellulase enzymes system from *Trichoderma reesei* was higher 500% than that obtained by *Aspergillus niger*. The results show that ?-glucosidase production by *Trichoderma reesei* was significantly lower (13.5%) than by *Aspergillus niger*. For economic hydrolysis of cellulosic materials all the cellulase enzyme components and β-glucosidase are required at high

strength. To overcome this problem and take advantage of the higher yield of CMCase, FPase, and xylanase by *Trichoderma reesei* and of ?-glucosidase by *Aspergillus niger*, the mixed culture of these two strains was carried out under the same conditions. As recorded in Table (1) and Fig (1), the higher yield of CMCase, FPase, and xylanase was almost retained as by the pure culture of *Trichoderma reesei* but ?-glucosidase production increased to about 56% of the value obtained by pure culture of *Aspergillus niger*. The hydrolytic power (%) of delignified rice straw (holocellulose) by the mixed culture was increased to reach 756%.

Table (1): Production of cellulases, β-glucosidase and xylanase by mixed culture of *Trichoderma reesei* and *Aspergillus niger*.

	Enzyme Activities(IU / ml)				FPase: β-GLUase ratio	Hydrolytic Power%
	CMCase	FPase	β-GLUase	Xylanase		
A. niger	3.8	0.133	0.52	49.3	0.256	3.4
Relative	100%	100%	100%	100%		100%
T. reesei	13.9	0.490	0.07	80.8	5.8	18.5
Relative	365%	368%	13.5%	163%		544%
Mixed culture(+)	12.8	0.405	0.29	67.3	1.39	25.7
Relative	336%	305%	56%	136%		756%



The enzyme activities of *Aspergillus niger* were taken as 100%.

Fig. (1). Relative activities (%) of different enzymes by pure and mixed culture of *Trichoderma reesei* and *Aspergillus niger*.

2. Optimization of some medium components for mixed culture.

For optimization of nutrientional requirements of the mixed culture of *Trichoderma reesei* and *Aspergillus niger* to obtain the maximum production of cellulase enzymes, the components of Mandels medium were tested in the presence of 1% hollocellulose as carbon source (Duff *et al.*, 1987). The experimental results indicate that only $(NH_4)_2SO_4$, urea, and KH_2PO_4 were found to be important. The requirement of all other components for production of cellulase enzymes remained unchanged in Mandels medium.

The (NH_4) SO_4 has been shown to be the preferred nitrogen source for cellulase production by *Trichoderma reesei*. The effect of the presence of different $(NH_4)_2SO_4$ concentrations (0.13-0.17%) in Mandel-medium for cellulase enzymes production with the mixed culture was investigated. It was found that 0.15% $(NH_4)_2SO_4$ was the optimum requirement for cellulases and β-glucosidase production by mixed culture cultivation as shown in Table (2). In many microorganisms, (NH_4) SO_4 has been shown to be the best nitrogen source for cellulases production by *A. fumigatus* (Trivedi & Rao, 1979),*Trichoderma sp.* (Mandels & Sternberg,1976),and *Streptomyces sp.* (Ishaque & Kluepfel,1980).

Mandels medium containing 0.15% $(NH_4)_2SO_4$ was supplemented with different concentrations of urea (0.01-0.07%). At urea concentration 0.05%, the maximum production of all enzyme activities was obtained. Some investigators recoded that urea seems to be unfavorable nutrient for both cellulase production and growth (Kassim, 1983). In studying the salts requirement, the results indicated that KH_2PO_4 was found to be essential with optimum concentration of 0.5 % (Table 2). The above results are in agreement with those reported by Trivedi & Desai (1984).

Table (2): Effect of some medium components on the production of cellulases, β-glucosidase, and xylanase by mixed culture of *Trichoderma reesei* and *Aspergillus niger*.

Concentrations		Cellulases (IU / ml)				Hydrolytic Power %
		CMCase	Fpase	β-GLUase	xylanase	
		$(NH_4)_2SO_4$				
	0.13	8.5	0.350	0.20	56.5	18.6
Control (+)	0.14	12.8	0.405	0.29	67.3	25.7
++	0.15	14.3	0.425	0.38	78.4	30.4
	0.16	11.5	0.387	0.31	60.3	28.5
	0.17	9.2	0.360	0.18	56.0	24.3
		Urea				
	0.01	6.2	0.240	0.17	50.0	20.3
	0.02	7,66	0.270	0.25	63.7	23.9
++	0.03	14.3	0.425	0.38	78.4	30.4
	0.04	10.8	0.430	0.38	91.5	32.1
+++	0.05	16.5	0.536	045	105.3	34.6
	0.06	10.3	0.410	0.30	73.6	30.7
	0.07	7.5	0.250	0.22	65.2	28.6
		KH_2PO_4				
	0.10	10.7	0.068	0.085	96.8	32.7
+++	0.20	16.5	0.536	0.450	103.6	34.6
	0.30	11.7	0.468	0.387	108.0	36.2
	0.40	12.6	0.483	0.410	112.0	37.5
++++	0.50	18.7	0.574	0.490	118.6	39.5
	0.60	12.9	0.450	0.440	110.4	34.2
	0.70	12.5	0.432	0.416	102.3	31.9

Control (+): Mixed culture of *Trichoderma reesei* and *Aspergillus niger* in Mandels medium.

3. Effect of carbon source (hollocellulose) concentrations on the mixed culture.

Since cellulose is a superior carbon source for cellulase enzymes production by most cellulytic microorganisms, it was of interest to investigate its optimum concentration. The use of available lignocellulosic wastes as carbon source in the growth medium would reduce the cost of cellulase enzymes production. Therefore, delignified rice straw (hollocellulose) was found to be the most suitable cellulosic material for induction and biosynthesis of cellulase enzymes than other lignocellulosic wastes when used as a carbon source. Poor induction of cellulase is observed in much other lignocellulosic material owing to their structural complexity. The fermentation was carried out with mixed culture in modified Mandels medium containing 0.15% $(NH_4)_2SO_4$, 0.05% urea, and 0.5% KH_2PO_4. The data listed in Table (3) indicated that the delignified rice straw (hollocellulose) was good inducer for cellulases. The maximum free enzymes activities in culture filtrate was produced in presence of 1.75% of hollocellulose. Increase in the concentration of hollocellulose above 1.75% resulted in decrease of cellulase enzymes concentrations in the culture supernatant, possibly due to specific binding of enzymes to the substrate. Adsorption of cellulases on cellulosic substrate has been studied by few workers (Castanon and Wilke, 1981; Goel & Ramachandran, 1983). It is of interest to note that the delignification caused an easy penetration of fungus mycelium and enzymes to substrate to yield more soluble products that induce more biosynthesis of the enzymes (Rao *et al.*, 1983 and Acebal *et al.* 1986).

Table (3): Effect of holocellulose concentrations on the production of cellulase enzymes by mixed culture of *Trichoderma reesei* and *Aspergillus niger*.

		Cellulases (IU / ml)				Hydrolytic
		CMCase	FPase	β-GLUase	Xylanase	Power %
Hollocellulose	(g/l)					
	0.50	9.5	0.327	0.285	57.3	18.4
	0.75	12.6	0.401	0.349	95.6	27.8
Control (++++)	1.00	18.7	0.574	0.490	118.6	39.5
	1.25	19.5	0.592	0.527	132.7	40.7
	1.50	21.6	0.624	0.554	142.7	42.5
+++++	1.75	24.3	0.645	0.581	162.5	46.3
	2.00	19.5	0.602	0.536	128.3	39.8

Control (++++): Mixed culture in Mandels medium after modification.

4. Time course of cellulase enzymes production by mixed culture.

The assay of enzyme activities was performed routinely in the culture filtrate of the mixed culture grown on modified Mandels medium supplemented with 1.75% of hollocellulose. The time course of the induction of cellulase enzymes, pH, reducing sugars, and hydrolytic power (%) were followed for 10 days and the experimental data were recorded and illustrated in Fig.(2) .

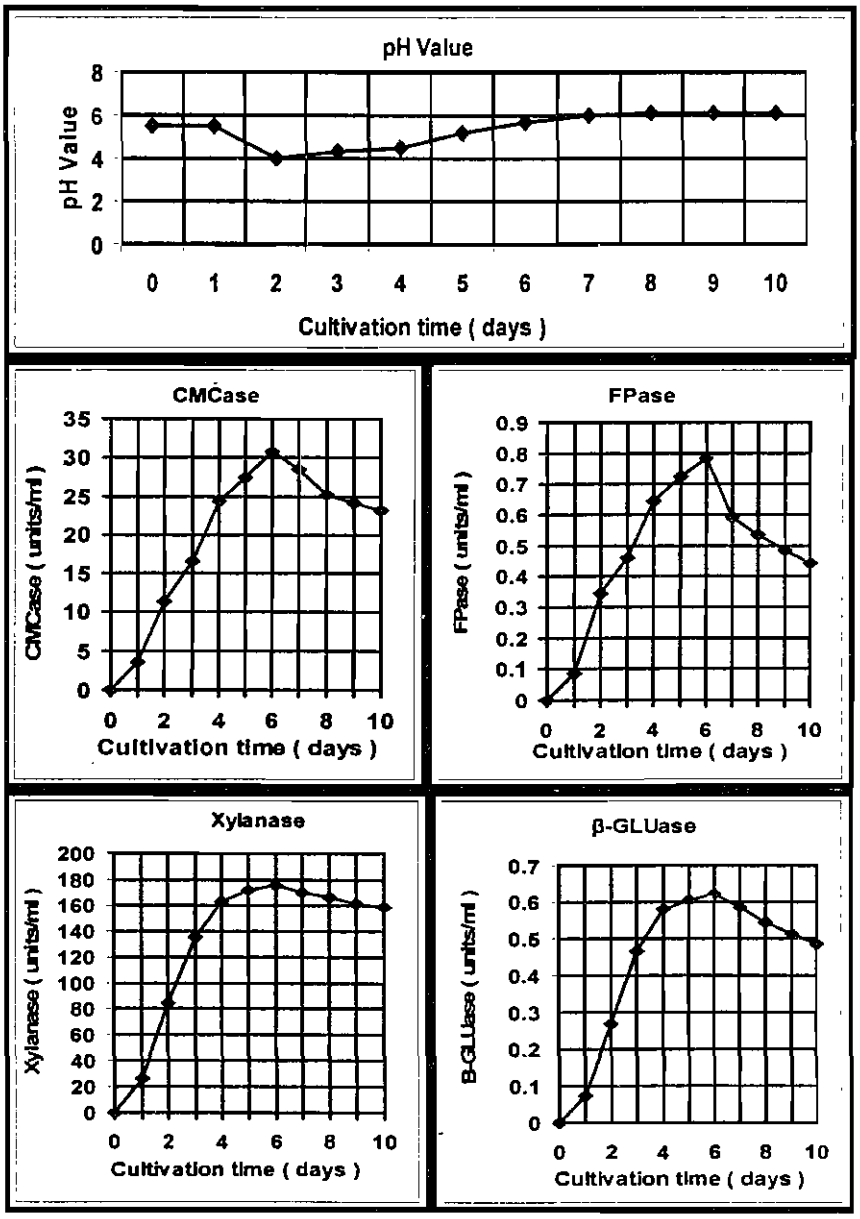


Fig. (2): Production of cellulase enzymes by mixed culture of *Trichoderm reesei* and *Aspergillus niger* on modified Mandels medium .

During cultivation, the pH of culture filtrate fell gradually from 5.5 to 4.4 up to the 4th day and increased to 6.1 up to the 8th day, and then remained almost constant. This pH profile may not inactivate the β-glucosidase enzyme. A sharp fall to pH 3 during cellulase production by *T.viride* has been documented to cause inactivation of β-glucosidase (Mandels *et al.*, 1975). The reducing sugars in culture filtrate were increased up to 5th day to reach a value of 0.835 mg/ml and then began to decrease (Fig. 2). These concentrations were not sufficient to repress the production of CMCase but higher concentrations resulted in pronounced repression. Since glucose is an end product of cellulose hydrolysis it inhibits CMCase. It may be suggested that CMCase produced in the mixed culture obeys the phenomenon of catabolite repression. The similar nature of catabolite repression was observed in various fungi (Eriksson & Hamp, 1978). The maximum free cellulase activities were obtained after 6 days of fermentation, possibly due to the production of reducing sugars in the culture medium, which resulted in the end-product inhibition of the enzymes.

Table (4): Production of cellulase enzymes by mixed culture of *Trichoderma reesei* and *Aspergillus niger* and by other potent cellulase producers.

Organism(s)	Mandels medium				Modified Mandels medium			
	CMCase	FPase	β-GLUase	Xylanase	CMCase	FPase	β-GLUase	Xylanase
Mixed culture	12.8	0.405	0.29	67.3	30.7	0.783	0.621	175.8
Relative (%)	100	100	100	100	240	193	214	261
T. reesei NRRL-11236	11.3	0.536	0.093	122.4	16.7	0.571	0.152	136.5
Relative (%)	88	132	32	182	130	141	52	203
T. viride NRRL-11336	3.1	0.123	0.043	26.5	5.9	0.264	0.085	32.8
Relative (%)	24	30	15	39	46	65	29	49
T. harzalnum NRRL-13019	7.8	0.267	0.037	47.1	6.5	0.265	0.129	38.5
Relative (%)	61	66	13	70	51	65	44	57

• Enzyme activities of mixed culture in Mandels medium were taken as 100%.
• Enzyme activities were (IU / ml).

5. Comparison of the mixed culture of Trichoderma reesei and Aspergillus niger with other cellulases potent producer.

The results obtained in the present study, show the effectiveness of the medium modification as inducers of cellulases synthesis. The two organisms grow well in modified Mandels medium and produce a thick mycelia mat. They were also capable of utilizing the hollocellulose to high extent. About 140%, 93%, 114%,and 161% higher production of CMCase, FPase, β-glucosidase, and xylanase,respectively, was obtained in comparison with the unmodified Mandels medium. From Table (4), it is clear that the enzymes yield are comparable to other cellulase potent producer, it is also worth noting that β-glucosidase production is about 7.4 times and 4.8 times that of *T.viride* and *T. harzianum*, respectively.

The high production of β-glucsidase is of significance in maximizing the saccharification of cellulosic wastes. Also, the FPase : β-glucosidase ratio for the mixed culture in modified Mandels medium was almost equal to one (~ 1.26) which is necessary for high rate of cellulose hydrolysis with high glucose content in its hydrolyzate. In comparison, the ratio of FPase: β-glucosidase was 0.256 and 5.8 in pure culture of *Aspergillus niger* and *Trichoderma reesei*, respectively, and 1.62 in mixed culture with Mandels medium.Amongst the most potent cellulase producers reported are *Trichoderma reesei* Rut-30 (Tangnu *et al.*, 1981), a mutant of *Thermomonospora curvata* (Fennington *et al.* 1982), and *Cellulomonas uda* (Nakamura & Kitamura, 1982 which have been reported to produce CMCase activities of over 100, 20, and 32 units/ml of the culture supernatant, respectively. Further, the mixed culture produced CMCase activity equal to *Cellulomonas uda* (~30 units/ml), which at this level was reported to be a potent strain for production of this activity.

The data presented in the present work suggest that mixed cultivation of *Aspergillus niger* and *Trichoderma reesei* is a potential source for cellulase enzymes production as evidenced by its high hydrolytic power, if exploited further.

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