Optimization of cellulase production by submerged fermentation of rice straw by Trichoderma harzianum Rut-C 8230

G Kocher, K Kalra, G Banta

Citation

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Abstract

The cellulase production by Trichoderma harzianum MTCC 8230 was studied using rice straw as substrate that supported better cellulase production than carboxymethyl cellulose (CMC). The optimum conditions for cellulase production were $(NH_4)_2SO_4$, 0.5 g L^{-1} as nitrogen source, pH (5.0), temperature (28°C) and incoulum size (10^8 spores ml^{-1}). Tween 80 (0.1%) improved the overall cellulase production as under these optimized conditions, C_1, Cx and C_B activities of 0.127, 0.15 and 1.65 U ml^{-1} respectively were produced. The enzyme was concentrated 2.65 folds with a yield of 73.8% using ammonium suplhate precipitation followed by dialysis.

INTRODUCTION

The research on application of lignocellulosics for bioethanol production is catching up very fast to meet its increasing demand for the production. Paddy or rice straw is one such lignocellulosic material that is produced abundantly as a byproduct of rice crop with an annual worldwide production of 800 MT (Wati et al, 2007). So much so that rice straw is burnt in fields which leads to severe environmental constraints. As rice straw contains bound sugars in the form of cellulose and hemicellulose meshed with lignin, it needs to be promoted as a fermentation raw material for bioethanol production. The utilization of rice straw for the purpose passes through 3 major steps- physicochemical pretreatment, cellulase saccharification and glucose fermentation to ethanol with the first 2 steps constituting major inputs (Ward, 2002; Juhasz et al, 2004).

Cellulase is an inducable enzyme complex involving synergistic action of endoglucanase (Cx), exoglucanase (C_1) and Cellobiase (C_B). It is produced by a number of bacteria and fungi though species of Trichoderma and Aspergillus are most reported (Zaldivar et al, 2001). However, the high cost of cellulase production (due to use of pure chemicals in production) coupled with low enzyme activities limits its industrial use. Therefore, efforts are needed to economize cellulase production by media optimization and use of supplements/ additives. In literature, many agricultural substrates like straw, bran, bagasse etc. have been reported (Griffin et al, 1974, Deumas et al, 1995, Pei-jun et al, 2004). In addition, research is being carried out on isolation of potential cellulase producing microorganisms from diverse habitats (Ray et al, 2007).

The present study reports optimization of cellulase production by an isolate from soil, identified as Trichoderma harzianum MTCC 8230 from MTCC laboratory, Institute of Microbial Technology, Chandigarh, India, while using acid pretreated rice straw as substrate.

MATERIALS AND METHODS

Inoculum preparation and Enzyme production: Spores of Trichoderma harzianum MTCC 8230 maintained on CMC agar slants were suspended in CMC broth containing g L^{-1} of {Carboxymethyl cellulose (CMC) -1.1, Yeast extract 0.1, $(NH_4)_2SO_4$-0.5, KH_2PO_4-10.0, $MgSO_4.7H_2O$-0.1, NaCl-0.2, pH-5.0} and incubated on orbital shaker at 28±2°C for 3 days and used to inoculate enzyme production media for submerged fermentation. For enzyme production, erlenmeyer flasks containing 100 ml of basal synthetic medium containing g L^{-1} of {$(NH_4)_2SO_4$, 0.5; KH_2PO_4, 10; K_2HPO_4, 5; $MgSO_4$, 0.1; NaCl, 0.2; Yeast Extract, 0.1 with 1 g pretreated rice straw (Pretreatment carried out with 1.0% H_2SO_4 followed by authoclaving at 15 psi for 90 min) or 1 g CMC} were inoculated with 10^6 spores ml^{-1} prepared inoculum were incubated at 28±2°C on orbital shaker (150

rpm) for 10 days. The mycelium free extract was used as crude cellulase preparation and used for estimating enzyme activities.

Optimization of cellulase production: The cellulase production by T.harzianum was optimized for incubation time (up to 10 days), temperature (20-37°C with an interval of 5°C), pH (4.0-8.0 with an interval of 0.5), inoculum size (10^6 -10^8 spores ml^{-1}), nitrogen sources {$(NH_4)_2SO_4$, peptone, soybean meal, rice bran and $NaNO_3$) and supplementation of Tween 80 (0.0.5 – 0.3%, v/v)}. For studying the effect of N sources, 0.5 g 100ml^{-1} of different nitrogen sources were sterilized separately and added aseptically before fermentation. Samples were drawn aspetically at different periods of time during fermentation and spun at 8000 rpm, 10 min for removing mycelium. The supernatant was used as crude enzyme filtrate for estimation of filter paper activity (C_1) and carboxymethylcellulase activity (Cx) by the method of Mandel et al (1976), while cellobiase (C_B) activity was assayed by the method of Srivastva et al (1987). One enzyme unit (IU) was defined as the amount of cellulase which is capable of producing one micromole (µM) of reducing sugars in one minute. Protein was estimated by the traditional Folin phenol reagent method.

Partial purification of cellulase: The crude cellulase (1150 ml) was partially purified by saturated ammonium sulphate precipitation (0-80%). The precipitates were dissolved in 50 ml of Tris buffer (pH 6.0) followed by its dialysis against the same buffer at 4°C.

RESULTS AND DISCUSSION

The profile of cellulase production at different time intervals revealed that rice straw induced C_1, C_x and C_B activities of 0.09, 0.12 and 1.12 IU ml^{-1} respectively by T.harzianum 8230 at 8 days of incubation (Table 1). These activities were higher as compared to Trichoderma reesei QM 9414 taken as control as well as cellulase producing medium supplemented with CMC as carbon source. Therefore, further experiments were conducted to optimize the cellulase production by T.harzianum by using pretreated rice straw as sole carbon source in the cellulase production medium. Use of different inorganic and organic nitrogen sources revealed $(NH_4)_2SO_4$ as the best nitrogen source (Table 2). Both the Cx and C_1 activities were higher than other nitrogen sources, C_B activity was lowest with $(NH_4)_2SO_4$ among the five nitrogen sources tested. However, $(NH_4)_2SO_4$ with higher C_1 and Cx activities was chosen to further optimize the cellulase production for temperature, pH, inoculum size and the supplementation of Tween 80. Gashel (1992) also reported higher cellulase production with KNO_3 than NH_4Cl and urea. Pei-Jun et al (2004) on the other hand, reported combination of $(NH_4)_2SO_4$ and wheat bran for optimum cellulase production by T.koningii.

Figure 1

Table 1: Effect of incubation period on cellulase production (IUml) in MTCC 8230.

Isolate	Cellulase production (IUml^{-1} of enzyme filtrate) at Days														
	2			4			6			8			10		
	C_1	Cx	C_B	C_1	Cx	C_B	C_1	Cx	C_B	C_1	Cx	C_B	C_1	Cx	C_B
T.harzianum[1]	0.02	0.04	0.10	0.04	0.10	0.71	0.05	0.12	0.96	0.09	0.12	1.12	0.08	0.03	1.10
T.harzianum	0.05	0.09	0.17	0.07	0.10	0.37	0.08	0.12	0.40	0.08	0.12	0.43	0.08	0.10	0.39
T.reesei QM9414	0.02	0.01	0.10	0.04	0.10	0.20	0.06	0.15	0.29	0.05	0.15	0.34	0.05	0.15	0.27

- *T.harzianum*- cellulase production using carboxymethyl cellulase as carbon source
- *T.harzianum*[1]- cellulase production using rice straw as carbon source
- C_1 - Filter Paper Activity, Cx - Carboxymethyl Cellulase activity, C_B - Cellobiase activity,

The incubation conditions were Inoculum Concentration- 10^6 spores ml^{-1} , pH - 6.5,Temperature-28±2 ° C

Figure 2

Table 2: Effect of supplementation of different nitrogen sources on cellulase production by

Nitrogen source	Maximum cellulase production (IUml^{-1} of enzyme filtrate)		
	C_1	Cx	C_B
$(NH_4)_2SO_4$	0.093 (8)	0.12 (6)	1.12 (8)
$NaNO_3$	0.03 (8)	0.06 (2)	1.27 (8)
Peptone	0.05 (2)	0.04 (8)	1.47 (6)
Soymeal	0.03 (8)	0.06 (10)	1.34 (8)
Rice bran	0.04 (8)	0.06 (8)	1.25 (6)

- Figures in parenthesis indicate the time at which maximum activity was achieved.
- All fermentation conditions were as in Table 1.

Among the 4 pH levels tested, a pH of 5.0 was optimum after 8 days of incubation though activities at pH of 6.0 were comparable with those of pH 5.0 (Table 3). pH, temperature, aeration, growth period and additives have been reported to be important parameters in optimizing cellulase production (Immanuel et al, 2006). Among these, pH is of major interest (Juhasz et al, 2004) as they reported a high C_B production in buffered medium while C_1 and Cx activities were more in non buffered medium. Zaldivar et al (2001) observed that cellulase production by T.aureoviridae is best if pH doesn't fall below 3.5. Pei-Jun et al (2004) reported a pH of 6.5 for optimum cellulase production by T.koningii. Among the 4 temperature levels tested, 30°C was the optimum

temperature with C_1, Cx and C_B activities of 0.096, 0.104 and 1.27 U ml $^{-1}$ at 8, 8 and 6 days of incubation respectively (Table 3). The C_1 activity was most affected by temperature as at temperatures of 20 and 37°C, it was drastically reduced while C_1 and Cx activities didn't show much variation at the 5 temperatures tested. Zaldivar et al (2001) reported a temperature of 28°C by T.aureoviridae while Pei-Jun et al (2004) used two temperature zones of 32°C (first 30 h) and 27°C thereafter for cellulase production by T.koningii.

Three inoculum sizes were tested and it was found that a higher initial inoculum of 10 ^{8} spores ml $^{-1}$ significantly increased cellulase activities as optimum C_1, and Cx activities of 0.103 and 0.121 respectively after 8 days of incubation and C_B activity of 1.687 U ml $^{-1}$ after 6 days of incubation were reported (Table 3). While Zaldivar et al (2001) reported optimum cellulase production with 5 X 10 ^{6} spores ml $^{-1}$ of T.aureoviridae, a 2% (v/v) spore suspension of T.koningii was used by Pei-jun et al (2004). Ray et al (2007) optimized 3% (v/v) inoculum of Bacillus circulans TP3 for optimum cellulase production.

Detergents like Tween 80, SDS etc. have been reported to enhance cellulase activities by increasing availability of nutrients (El-Hawary and Mostafa ,2001). Among the 4 concentratiosn of Tween 80 used in this study, a concentration of 0.10% (v/v) was found to enhance the C_1 and Cx activities to 0.127 and 0.15 respectively while C_B activity (1.65 U ml $^{-1}$) was not affected

Figure 3

Table 3: Effect of fermentation conditions on glucoamylase production by MTCC 8230.

Cultural parameters	Cellulase production (IUml^{-1} of enzyme filtrate) at Days											
	C_1				Cx				C_B			
Days	pH values											
	4	5	6	7	4	5	6	7	4	5	6	7
2	0.004	0.004	0.004	0.004	0.031	0.051	0.026	0.045	0.03	0.05	0.03	0.01
4	0.023	0.046	0.010	0.008	0.036	0.059	0.063	0.051	0.04	0.06	0.05	0.03
6	0.052	0.051	0.040	0.006	0.039	0.073	0.064	0.064	0.39	0.39	0.24	0.05
8	0.010	0.060	0.056	0.006	0.053	0.10	0.06	0.03	0.61	1.21	0.77	0.30
10	0.010	0.053	0.005	0.005	0.040	0.093	0.039	0.03	0.30	0.57	0.39	0.08
	Temperature (°C)											
	20	25	30	37	20	25	30	37	20	25	30	37
2	0.005	0.007	0.014	0.005	0.014	0.003	0.019	0.012	0.11	0.17	0.24	0.28
4	0.076	0.011	0.042	0.015	0.036	0.027	0.049	0.004	0.25	0.30	0.48	0.11
6	0.084	0.025	0.093	0.018	0.065	0.044	0.081	0.004	1.09	1.08	1.27	1.05
8	0.02	0.08	0.10	0.05	0.03	0.04	0.104	0.004	0.51	0.45	1.04	1.35
10	0.02	0.01	0.06	0.03	0.02	0.02	0.004	0.011	0.51	0.11	0.28	0.15
	Inoculum size											
	10^6		10^7	10^8	10^6		10^7	10^8	10^6		10^7	10^8
2	0.066		0.016	0.062	0.028		0.016	0.024	0.55		1.31	1.26
4	0.078		0.014	0.072	0.026		0.038	0.048	1.25		1.37	1.38
6	0.081		0.053	0.084	0.046		0.046	0.059	1.21		1.35	1.69
8	0.092		0.096	0.103	0.012		0.104	0.121	1.25		1.39	1.36
10	0.085		0.090	0.090	0.024		0.100	0.115	1.20		1.15	1.16
	Tween 80 (%, v/v)											
	0.05	0.10	0.20	0.30	0.05	0.10	0.20	0.30	0.05	0.10	0.20	0.30
2	0.019	0.058	0.040	0.021	0.040	0.009	0.007	0.072	0.92	1.06	0.97	0.99
4	0.034	0.061	0.043	0.029	0.080	0.057	0.013	0.080	1.05	1.65	1.05	1.04
6	0.051	0.077	0.044	0.031	0.050	0.088	0.032	0.012	0.91	0.99	0.98	0.90
8	0.082	0.127	0.055	0.022	0.040	0.150	0.044	0.014	1.10	1.02	1.07	1.10
10	0.067	0.096	0.043	0.020	0.040	0.125	0.032	0.014	1.05	1.02	0.85	0.95

- C_1 - Filter Paper Activity
- Cx - Carboxymethyl Cellulase activity
- C_B - Cellobiase activity

(Table 3). While Goshel (1992) and El- Hawary and Mostafa (2001) reported increase in cellulase activities with Tween 80, Pei-jun et al (2004) reported negative effect of Tween 80 on cellulase activities. The optimized cellulase activities if converted to per g of paddy straw, came out to be 12.7, 15.0 and 165 IU of C_1, Cx and C_B activities, respectively.) In literature, a number of agricultural materials have been used for cellulase production. Gashel (1992) reported wheat straw, Liming and Xueling (2004) used corn cobs and Pei-jun et al (2004) used rice straw for cellulase production by Trichoderma sp. A001, T. reesei and T.koningii respectively. The C_B activity of this strain was unusually high while Cx activity was comparatively low. Earlier, Khan et al (2007) reported C_1 and Cx activities of 1.43 and 2.4 U ml $^{-1}$ using rice straw by Phanerochaete chrysosporum under SSF. Wen et al (2005) reported C_1, Cx and C_B activities of 1.74, 12.22 and 0.0978 IU ml $^{-1}$ by Trichoderma reesei using dairy manure as substrate.

The cellulase complex of T.harzianum 8230 was concentrated by SAS precipitation followed by dialysis from 1150 ml to 50 ml with a C_1 activity of 3.89 U ml $^{-1}$ (Table 4). Elsewhere, commercial enzymes with a C_1 activity of around 30 U ml $^{-1}$ have been reported to be used at a concentration of 0.5 to 2%, v/v (Wati et al, 2007).The specific activity was 1.88 U mg $^{-1}$ of protein and total purification was 2.65 folds with a yield of 73.8% of activity. Though the C_1 activity of

our cellulase is around 10 times less than commercial cellulase, it can be used in its crude form (salt precipitated) at higher ratios. This cellulase has the potential and can thus be used to saccharify pretreated rice straw for bioethanol production. The work in this regard is in progress in our laboratory.

Figure 4

Table 4: Partial purification of the cellulase of

Purification step	Activity ($IUml^{-1}$)	Volume (ml)	Total protein (mg)	Total activity ($IUml^{-1}$)	Specific activity	Purification (folds)	Yield (%)
Crude	0.37	1150	995	425.5	0.428	1	100
*Salt precipitation	3.89	50	278.5	194.5	0.70	2.65	73.8

*Salt precipitation was conducted by saturated $(NH_4)_2SO_4$ precipitation (0-80%) followed by dialysis.

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Author Information

GS Kocher, Ph.D.
Department of Microbiology, Punjab Agricultural University

KL Kalra, Ph.D.
Department of Microbiology, Punjab Agricultural University

G. Banta, Ph.D.
Department of Microbiology, Punjab Agricultural University