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OPTIMIZATION OF PROCESS FOR THE PRODUCTION OF CELLULASES BY *GLIOCLADIUM VIRENS* MILLER *ET AL.*, AND *TRICHODERMA REESEI* QM 9414 UNDER SOLID STATE FERMENTATION CONDITIONS AND THEIR APPLICATION IN THE SACCHARIFICATION OF BARLEY

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Cellulases are being increasingly used to hydrolyse lignocellulosic materials (Okeke and Obi, 1995; Tomme *et al.*, 1995), however, due to the presence of lignin, the accessibility of the enzyme is reduced (Kuhad and Singh, 1993) and the alkali-pretreatment is known to remove much amount of the constituent lignin (Marsden and Grey, 1986). *Trichoderma reesei* has been widely used for the hydrolysis of lignocellulosic materials but due to its low levels of β-glucosidase, *Gliocladium virens* is being investigated as an alternate source for cellulases (Esterbauer *et al.*, 1983; Singh & Garg, 1995; 1996). The recent advancement in solid-state-fermentation technology (Chahal, 1985) has enabled the biotechnologists to use higher concentrations of the substrate, resulting in the yield of very high activity of cellulases. As per F.A.O. (1989) estimates, the total annual world production of lignocellulose from cereals alone accounts for 2946 million tons of which 1135 million tons is from Asia. Barley is the one of the major crop in India and its straw can be used as a source of lignocellulosic material.

In this paper, the authors have standardized various parameters like different fractions, temperature, pH, urea concentration in nutrient salt solution and incubation period (in days) for the optimum production of cellulases by *G. virens* on alkali pretreated barley straw under solid-state-fermentation conditions. The enzyme produced, has been used to solubilize the partially delingified barley straw. For comparison of the process, widely used strain of *Trichoderma reesei* QM 9414 has also been included in the study.

MATERIALS AND METHODS

Gliocladium virens Miller *et al.*, was obtained from Professor Dr. Hermann Esterbauer, Institute of Biochemistry, University of Graz, Austria. The fungus was cultured on Potato-Dextrose Agar (PDA) medium and maintained at 4°C.

Alkali-pretreatment of barley straw : Barley straw used in this study was obtained from the nearby agricultural field in C.C.S. University and washed thoroughly with water to remove extraneous materials and dried in oven at 50°C. 250 g of dried barley straw was treated with 1000 ml of 4% (w/v) NaOH and autoclaved at 15 Pa for 30 minutes. The resultant material was squeez filtered through four layers of cheese cloth and the filtrate was treated as lignin fraction. The fibrous residue was washed with tap water, squeez filtered and the supernatent was treated as "water solubles fraction". The fibrous residue was finally thoroughly washed under running tap water till neutrality and kept at 50°C until fully dried. The oven dried material was powdered in grinder (Mellert Germany M-82), packed in polythene bags and stored under dry conditions at room temperature until used. This alkali-pretreated residue is referred here as 'partially delignified'. The cellulose, hemicellulose and lignin contents in both untreated and partially delignified barley were determined as described by Moubasher *et al.* (1982).

Preparation of the enzyme : For solid-state-fermentation, four sets of different fractions in triplicate were designed. All sets contained 2 g of partially delignified substrate + 2 ml nutrient salt solution (Mandels and Weber, 1969; modified by Chahal, 1985). In addition to this, first set contained 10 ml of lignin fraction, second 10 ml of water solubles fraction and third set contained 5 ml of each of the two fractions, while fourth set contained 10 ml of double distilled water and treated as control. Prior to addition, pH of the lignin and water solubles fractions

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Table 1. Per cent content of cellulose, hemicellulose and lignin in barley (each figure is an average of three independent replicates).

Barley	Cellulose	Hemicellulose	Lignin
Untreated	47	20	22
Partially delignified	69	18	10

including that of control was adjusted to 5.6 with concentrated H_2SO_4. All contents were autoclaved at 15 Pa for 15 minutes and 2 ml of spore suspensions of *G. virens* (10^4 spores/ml) were inoculated aseptically. Each of the four sets had one control which contained all contents and treated similarly except the inoculum. Similar sets were prepared for *T. reesei* for comparison. The flasks were incubated at 25°C. The enzyme was harvested twice, each time with fresh 25 ml of sterile double distilled water by shaking on orbital shaker at 100 rpm for 30 min at room temperature and then centrifuged (Sigma 2 K 15) at 3,000 xg at 4°C for 10 min followed by 15,000 x g at 4°C for 1 h. The supernatant was treated as crude enzyme. The cellulase activity was determined in terms of CMCase (Miller, 1959) and FPase (Mandels *et al.*, 1976). The extracellular soluble proteins were estimated according to the method of Bradford (1976).

For optimization of temperature, pH, urea concentration in nutrient salt solution (NSS) and incubation period, similar experiments were designed by varying one of the parameters and keeping others as constant. For optimization of temperature 20, 25, 30, 35 and 40°C were used while for pH, the respective fractions were adjusted to pH 4.0, 4.5, 5.0. 5.5 and 6.0. For urea concentration 1, 2, 3, 4 and 5% urea (w/v) in NSS was used separately. For determination of optimum incubation period, each time flasks (3 with inoculum and 1 without inoculum as control) were harvested at an interval of 3 days upto 30 days. The results are expressed as per the recommendations of International Union of Pure and Applied Chemistry (Ghose, 1987).

Saccharification of partially delignified barley straw : 50 mg of alkali treated, partially delignified substrates of barley was added in 25 ml sterile universal screw capped tubes. 10 IU of the enzyme activity in terms of CMCase diluted with citrate buffer (0.05 M, pH 4.8) to obtain the final volume of 5 ml were added in each set of 6 tubes which were incubated separately at 30, 40, 50 and 60°C temperature simultaneously. Suitable controls in which enzyme or substrate were omitted, were routinely included. Three tubes from each set and two controls, one for enzyme and other for substrate were harvested after 24 and 48 h. The reaction was stopped by keeping the tubes on ice cooled water bath and the contents were centrifuged at 10,000 x g for 10 minutes at 4°C. The amount of reducing sugars released was measured in the supernatent by DNS reagent method (Miller, 1959). The rate of saccharification was determined by glucose release from per cent content of cellulose in partially delignified substrate.

Similarly, to determine the effect of pH on the standardized temperatue of 50°C, 0.05 M citrate buffer at pH 4.0, 4.8, 5.6 and 6.2 were used and the contents were harvested as desired.

RESULTS AND DISCUSSION

Effectiveness of alkaline pretreatment : The untreated lignocellulose of barley contained 47% cellulose, 20% hemicellulose and 22% lignin. The alkali pretreatment with 4% NoOH removed approximately 50% of the total constituent lignin from barley straw and the fibrous residue contained 69% cellulose, 18%, hemicellulose and 10% lignin (Table 1). Thus, the alkali-pretreatment removed lignin and a part of other carbohydrates like hemicellulose. The alkali pretreatment is reported to loosen the complex fibrous structure of lignocellulose which facilitate the biological process involving the microbes and/or their enzymes (Viikari *et al.*, 1993).

Solid-state-fermentation : Liquid-state-fermentation (LSF) has been widely used for the production

Table 2. Effect of different fractions on enzyme protein concentration (µg/ml) and its cellulase activity in terms of CMCase and FPase of *G. virens* and *T. reesei* on barley under solid state fermentation conditions.

Fraction	Enzyme protein µg/ml		CMCase IU/g		FPase IU/ml	
	G. virens	*T. reesei*	*G. virens*	*T. reesei*	*G. virens*	*T. reesei*
Distilled water (control)	2.62	4.88	5.735 ±0.185	12.197 ±0.283	0.041 ±0.004	0.085 ±0.003
Water solubles	3.85	7.24	9.127 ±0.283	17.095 ±0.185	0.0637 ±0.003	0.126 ±0.004
Lignin	5.22	5.54	14.306 ±0.649	13.813 ±0.106	0.102 ±0.007	0.097 ±0.004
Lignin+water	4.84	6.088	12.086 ±0.282	15.231 ±0.283	0.098 ±0.006	0.106 ±0.003

Table 3. Effect of temperature on extracellular secretion of enzyme protein (µg/ml) and its cellulase activity in terms of CMCase and FPase of *G. virens* and *T.reesei* produced on barley under solid-state-fermentation conditions.

Temperature °C	Enzyme protein (µg/ml)		CMCase (IU/g)		FPase (IU/ml)	
	G. virens	*T. reesei*	*G. virens*	*T. reesei*	*G. virens*	*T. reesei*
20	4.09	4.87	10.294 ±0.283	12.172 ±0.282	0.071 ±0.005	0.085 ±0.006
25	5.23	7.91	14.325 ±0.621	17.232 ±0.283	0.102 ±0.004	0.127 ±0.004
30	5.81	8.25	14.526 ±0.529	18.126 ±0.183	0.108 ±0.003	0.128 ±0.004
35	3.57	3.31	8.925 ±0.281	8.296 ±0.422	0.062 ±0.006	0.057 ±0.006
40	0.94	2.45	2.321 ±0.172	6.126 ±0.282	0.016 ±0.005	0.042 ±0.005

Table 4. Effect of Urea concentration (% w/v) in NSS on production of enzyme protein (µg/ml() and its cellulase activity in terms of CMCase and FPase of *G. virens* and *T. reesei* on barley under solid state fermentation conditions.

Urea concentration (w/v)	Enzyme protein (µg/ml)		CMCase (IU/g)		FPase (IU/ml)	
	G. virens	*T. reesei*	*G. virens*	*T. reesei*	*G. virens*	*T. reesei*
0	4.83	4.83	11.470 ±0.832	12.095 ±0.185	0.082 ±0.007	0.086 ±0.002
1	5.72	7.32	13.975 ±0.667	17.513 ±0.107	0.097 ±0.009	0.136 ±0.005
2	5.86	8.32	14.661 ±0.486	18.581 ±0.534	0.110 ±0.011	0.138 ±0.004
3	2.69	6.48	6.598 ±0.931	16.280 ±0.185	0.046 ±0.004	0.114 ±0.006
4	1.72	5.64	4.378 ±0.770	14.156 ±0.283	0.033 ±0.002	0.098 ±0.005
5	1.27	4.84	3.219 ±0.628	12.289 ±0.282	0.023 ±0.002	0.086 ±0.004

of cellulases by several microbes including fungi, however, the use of limited substrate concentration (0.5 to 6% depending upon the density of the substrate) in LSF has forced the scientists to standardize solid-state-fermentation (SSF) technology which allows the use of high substrate concentration and reduces the cost of maintenance of sophisticated equipments required in LSF.

Standardization of parameters : The lowest activity was obtained in control with distilled water. *G. virens* yielded high concentration of enzyme protein and maximum cellulase activity in terms of CMCase as well as FPase on barley under SSF conditions when the medium was supplemented with lignin fraction, however, when the lignin fraction was supplemented with water solubles, the enzyme activity was not of that order as with the former alone indicating that the lignin fraction enhanced the production of cellulases by *G. virens* on barley. The enzyme activity was approximately 3 times greater than the control (with distilled water) in case of *G. virens* on barley. It seems that lignin and water solubles fraction might have supported higher growth of the fungus, and therefore, produced greater amount of cellulases than the control (Table 2).

On the other hand, *T. reesei* produced more enzyme protein and greater cellulase activity in the medium supplemented with water solubles. It suggests that the water solubles fraction in *T. reesei* enhanced the extracellular secretion of proteins, thus, exhibiting greater enzyme activity (Table 2). This supports the earlier observations of Hahn-Hagerdal *et al.* (1991).

After the standardization of the fraction, the temperature was optimized and *G. virens* yielded highest enzyme protein concentration when incubated at 25-30°C temperature while at 40°C it yielded very low and almost negligible activity. *T. reesei* also showed highest activity as well as enzyme protein concentration at 25-30°C temperature but comparatively good amount of activity at 40°C, when compared with *G. virens* at 40°C (Table 3). Thus, *G. virens* strain was remarkably fast growing but very sensitive to the temperature. The maximum temperature for mycelial growth of the fungus has been reported as 38°C by Labudova *et al.* (1989) which is in confirmation of our observations. Gomes *et al.* (1989) also made similar observations. Hence, the little or no enzyme activity of *G. virens* at 40°C is due to its temperature sensitiveness. On the other hand, greater adaptibility of *T. reesei* to the temperature is well documented in the literature (Gomes *et al.*, 1989; Maheshwari *et al.*, 1990, 1993).

Temperature is known to determine the amount and rate of growth of an organism (Garg *et al.*, 1985) and the increasing temperature has the general effect of increasing enzyme activity (*see* : Moorelandecker, 1990), but enzyme begin to suffer thermal inactivation at higher temperatures. It is also known to influence the growth of the microbes as well as the secretion of the secondary metabolities.

Table 5. Effect of pH on enzyme protein concentration (μg/ml) and its cellulase activity in terms of CMCase and FPase of *G. virens* and *T. reesei* on barley under solid state fermentation conditons.

pH	Enzyme protein (μg/ml)		CMCase (IU/g)		FPase (IU/ml)	
	G. virens	*T. reesei*	*G. virens*	*T. reesei*	*G. virens*	*T. reesei*
4.0	4.89	5.90	12.210 ±0.806	14.747 ±0.107	0.087 ±0.007	0.103 ±0.008
4.5	5.44	7.30	13.690 ±0.489	18.240 ±0.370	00.085 ±0.007	0.134 ±0.007
5.0	6.42	7.54	16.033 ±0.427	18.610 ±0.370	0.131 ±0.011	0.138 ±0.006
5.5	5.88	8.97	14.663 ±0.320	18.685 ±0.185	0.113 ±0.009	0.139 ±0.005
6.0	4.32	7.14	10.733 ±0.375	17.822 ±0.283	0.084 ±0.006	0.125 ±0.006

Table 6. Effect of temperature on glucose release (mg) from 50 mg alkali treated partially, delignified substrate using 10 IU of crude enzyme preparations of *G. virens* and *T. reesei* separately in barley at pH 4.8.

Tempe rature °C	*G. virens*		*T. reesei*	
	24 h	48 h	24 h	48 h
30	3.92±0.258 13.0%	4.11±0.283 13.7%	3.33±0.248 11.1%	3.41±0.282 11.3%
40	5.86±0.422 18.5%	6.26±0.422 20.8%	5.84±0.422 19%	6.18±0.418 20.6%
50	8.24±0.285 27%	8.92±0.283 29%	7.94±0.282 26%	8.56±0.283 28.5%
60	1.86±0.283 6.2%	2.08±0.248 6.93%	2.52±0.283 8.4%	2.86±0.282 9.5%

Table 7. Effect of pH on glucose release (mg) from 50 mg alkali treated partially delignified substrate using 10 IU of crude enzymes preparation after 24 h at 50°C.

pH	*G. virens*	*T. reesei*
4.0	3.72±0.282 12.4%	3.33±0.283 1%
4.8	8.92±0.183 29.7%	8.62±0.176 28.7%
5.6	6.26±0.247 20.7%	6.18±0.238 20.6%
6.2	6.21±0.242 20.6%	6.16±0.238 20.5%

Nitrogen is the essential nutrient for the synthesis of microbial protoplasm, therefore, an additional supply of nitrogen has been reported by various workers to enhance the growth of microbes and their various physiological functions (Garreett, 1971, 1976; Garg, 1990).

Table 8.Glucose released (mg) from 50 mg of alkali treated partially delignified substrate using 10 IU of crude enzyme preparation of *G. virens* and *T. reesei* (5 IU of each) after 24, 48 and 72 h at 50°C.

Time h	barley
24	12.28±0.422 40.9%
48	13.41±0.283 44.7%
72	13.58±0.249 45.2%

With this view, after standardization of fraction and temperature, we decided to optimize the urea concentration (% w/v) in NSS. On barley, *G. virens* showed highest activity in 2% urea in NSS. Higher concentration of urea (>3%) reduced the production of enzyme protein as well as the enzyme activity (Table 4), while *T. reesei* showed little differences at 1-3% urea in NSS on barley. Controls exhibited almost similar amounts of enzyme protein as well as enzyme activity in both the test species indicating no substrate specificity by the fungus. Thus, *T. reesei* is more tolerant to nitrogen concentration than *G. virens*. Yadav (1987 has reported maximum fermentation of wheat straw when supplemented with 1-5% of urea and the adverse effects of higher concentration of urea confirms our findings. These studies, thus, confirm that additional supply of urea in NSS is beneficial but only upto a certain limit and should be standardized for each test organism separately for each substrate.

On optimization of pH, it was found that *G. virens* produced highest concentration of extracellular soluble proteins at pH 5.0. At pH 4.0 and 6.0 there was significant reduction in enzyme protein as well as cellulase activity (Table 5). In case of *T. reesei* on barley interestingly, the fungus exhibited no major changes between pH 4.5 to 6.0. It may, therefore, be concluded that pH seems to control the secondary metabolism of *G. virens* while *T. reesei* is more tolerant to pH. The effect of pH on cellulase production in SSF has been very little studied and various workers have reported the change of pH during the growth in LSF (Grajek, 1987; Kim *et al.*, 1988).

The incubation period (in days) greatly affected

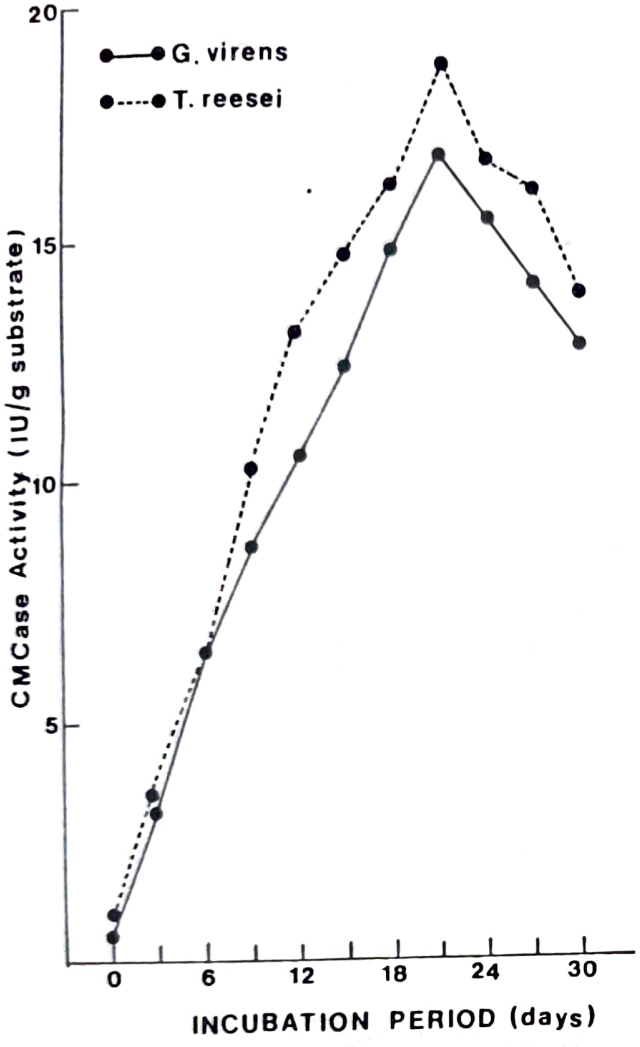

Figure 1. Production of cellulases in terms of CMCase activity by *G. virens* and *T. reesei* on barley at different periods of incubation.

the secretion of enzyme by the fungus. Therefore, after optimization of different fractions, pH, temperature and urea concentration in nutrient salt solution, the incubation period was standardized. The highest enzyme activity in terms of CMCase as well as FPase on partially delignified barley by *G. virens* was produced after 21 days of incubation at 30°C which decreased on longer incubation (Fig 1). *T. reesei* also exhibited almost similar results (Fig. 2). Thus, both *G. virens* and *T. reesei* showed linear relationship in between the incubation period and the cellulase production and yielded highest concentration of the enzyme after 21 days of incubation.

Biotechnologists have also not given much emphasis on the standardization of incubation period under SSF conditions and very few studies were encountered by the authors on the effect of incubation

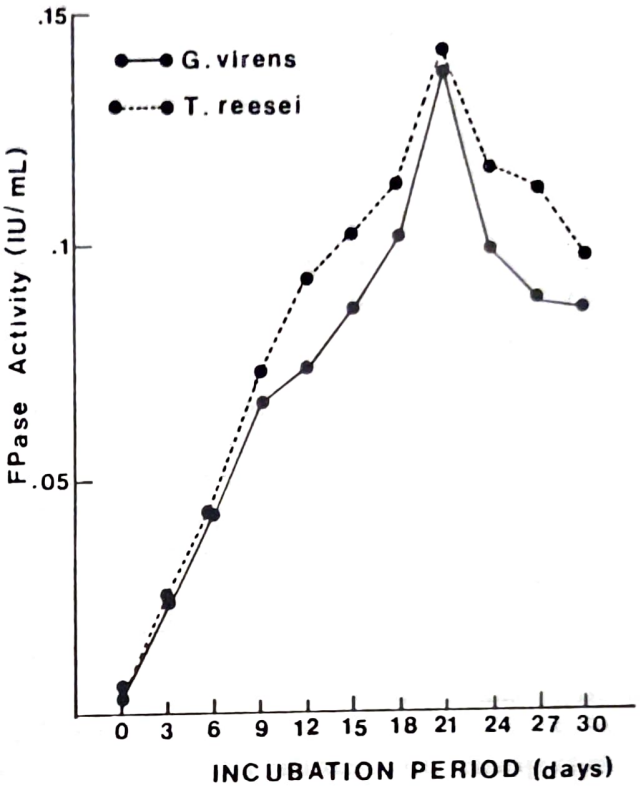

Figure 2. Production of cellulases in terms of FPase activity by *G. virens* and *T. reesei* on barley at different periods of incubation.

period for the production of cellulases however, Chahal (1985) recommended 21 days incubation for the production of cellulases by *T. reesei* on wheat straw under SSF. It confirms our findings.

Saccharification :

Effect of temperature : Using the optimized enzyme preparation we found that cellulases of both *G. virens* and *T. reesei* exhibited highest release of glucose at 50°C temperature after 24 h of incubation. At greater than 50°C, the rate of saccharification was greatly reduced in both the test species (Table 6). Longer incubations of 48 h did not sufficiently enhance the release of glucose which may possibly be due to the accumulation of glucose and/or cellooligomers, exhibiting the phenomenon of feedback inhibition. During 24 h period of incubation approximately 27% of partially delignified barley was hydrolysed by the enzymes of *G. virens*. Comparatively, the cellulases of *G. virens* were little more active on the barley than *T. reesei* at all temperatures of incubation, however, both cellulases demonstrated highest rates of saccharification at 50°C (Table 6).

Manomani and Sreekantiah (1987) also found highest rate of saccharification at 50°C when incubated for 24 h on alkali treated bagasse with fungal cellulases. More than 50°C temperature of incubation resulted in marked decrease rate of saccharification which may possibly be due to the thermal inactivation of the enzyme itself.

Effect of pH : At pH 4.8, both cellulases exhibited maximum hydrolysis of partially delingified barley and lowest at pH 4.0 (Table 7). At pH 5.6 and 6.2, the enzyme activity of both species decreased and showed little differences with each other. Eassentially each enzyme operates maximally at optimum pH and the dependence of cellulase activity on pH has been demonstrated by various workers (Chahal, 1985; Kim *et al.*, 1988; Maheshwari *et al.*, 1993). Sternberg *et al.* (1977) demonstrated optimum cellulase activity of *Trichoderma viride* at pH 4.8 and 50°C temperature. Manomani and Sreekantiah (1987), while working on *Aspergillus ustus* and *T. viride* found maximum rate of saccharification on bagasse at pH 4.8. On close comparison of enzyme activities of both species, it is clear that there are little differences between them. However, it was found that when both enzyme preparations were mixed together (1:1 ratio) so as to give 10 IU of the crude enzyme to hydrolyse 50 mg substrate under similar condition, the rate of saccharification enhanced markedly (Table 8). It suggests that cellulases of *G. virens* act synergistically with *T. reesei.* It may possibly be due to the high activity of β-glucosidases exhibited by *G. virens* than *T. reesei* as demonstrated in our laboratories.

From the present investigation, it may, therefore, be concluded that *G. virens* produces high activity cellulases which can effectively hydrolyse the alkali-treated barley and the process has great potential for commercial exploitation, however, further studies are recommended.

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