

A Novel Cellulase from an Endophyte, *Penicillium* Sp. NFCCI 2862

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Abstract An endophytic fungus identified as *Penicillium* sp. CPF2 (NFCCI 2862) was used to evaluate the activity of its cellulolytic enzymes to degrade pretreated sugarcane bagasse and characterize the cellulase enzymes. Different substrates were evaluated for optimum cellulase production by CPF2. The best activities for FPase (1.2 IU/ml), endocellulase (19 IU/ml), xylanase (40 IU/ml) and β -glucosidase (2.8 IU/ml) with a protein content of 0.86 mg/ml were observed when cellulose (1.5 % w/v) was used in combination with peptone (0.2 % w/v) in the growth medium. Optimum temperature and pH for the extracellular cellulase production were 28°C and 5.5, respectively. Furthermore the hydrolysis performance of *Penicillium* cellulase was compared with *Trichoderma reesei* cellulase (celluclast). Concentrated filtrate (~20 fold) from the fermented broth of CPF2 was able to bring about > 90 % and >63 % hydrolysis of cellulose and steam exploded bagasse (SEB) respectively at 5 % (w/v) substrate concentration in 24 h which was significantly higher than hydrolysis yield obtained with the commercial enzyme Celluclast.

Keywords: *Penicillium* sp., cellulase, endophyte, extracellular, sugarcane bagasse

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1. Introduction

Cellulases have been commercially available for more than 30 years, and these enzymes have represented a target for both academic as well as industrial research [1]. Cellulolytic enzymes have demonstrated their biotechnological potential in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textile, and laundry [2]. Cellulolytic microorganisms produce an array of cellulases which act synergistically to degrade cellulose [3]. The degradation of cellulose to glucose is effected by the cooperative action of endocellulases, exocellulases and β -glucosidases [4]. Endocellulases and exocellulases act synergistically upon cellulose to produce cellobiose, which is then cleaved by β -glucosidase (BGL) to glucose [5].

Most commercial cellulases are produced by the filamentous fungus *Trichoderma reesei* and *Aspergillus niger*. However conversion of waste cellulose to glucose is not yet commercially feasible due to low specific activity and end product inhibition of these enzymes. Many hyper-producing strains of *Trichoderma* produce low BGL activity relative to the total cellulase activity (filter paper activity), which impairs the performance of the cellulase preparation [6,7]. Extra BGL activity is, therefore, frequently supplied from other sources, e.g., *Aspergillus*. Different species belonging to the genus

Penicillium have previously shown the ability to produce a complete cellulase system and that the *Penicillium* species generally produced an enzyme mixture with a better ratio between filter paper activity and BGL activity than *Trichoderma* [8,9]. The objective of this work was to evaluate and optimize the production of cellulolytic enzyme mixture by an endophytic fungus identified as *Penicillium* sp. CPF2, isolated from *Chlorophytum* leaf, as well as to investigate their application for the saccharification (*in vitro*) of cellulose and pretreated sugarcane bagasse.

2. Materials and Methods

Carboxymethylcellulose sodium salt (CMC), p-nitrophenyl- β -D-cellobioside (pNPC), p-nitrophenyl- β -D-glucopyranoside (pNPG), commercial cellulase from *Trichoderma reesei* QM 9414 (Celluclast) was purchased from M/s. Sigma. BGL (Novo-188) from *Aspergillus niger* was purchased from M/s Novozymes. Ultramembrane UM~10 and UM~30 were purchased from M/s Millipore. Peptone and yeast extract was procured from M/s. Difco. All other reagents of analytical grade were procured from M/s. Himedia. Crystalline cellulose, α -cellulose and pretreated sugar cane bagasse was kindly supplied by National Chemical Laboratory (NCL) Pune, India.

2.1. Isolation of Endophytic Fungi

Fresh tissues of various plants collected from Kashmir valley were used for the isolation of endophytic fungi. Surface sterilization of small fragments of plant parts of (10 mm x 5 mm) in size was done by sequential immersion in 90 % ethanol for 1 min and 1.3 M sodium hypochlorite for 1 min and evenly placed in petri dishes containing water agar (WA) amended with Streptomycin (100 µg/ml) to eliminate any bacterial growth. Sealed petri dishes were incubated at 28°C ± 2°C until fungal growth started [10]. The hyphal tips, which grew out from sample segments over 4-6 weeks, were isolated and subcultured onto Sabouraud dextrose agar (SDA). Fungal isolates were cryopreserved at -70°C in the microbial repository of our institute.

2.2. Screening of Fungal Isolate for Cellulase Activity

A plate assay method with 1% (w/v) CMC in MQH₂O with 1.0 % (w/v) agar was used for screening of cellulase positive fungal isolates. The plates were incubated at 37 °C for 16 h and the cellulase activity was indicated as clear orange halos around the inoculated wells after staining with 1 % Congo red solution for 30 min and washing several times with 1 M NaCl [11]. The magnitude of the activity was calculated by measuring the diameter of the zones.

2.3. Identification of the Cellulase Producer Fungal Isolate

Phylogenetic analysis of CPF2 was carried out by the acquisition of the ITS1-5.8 S-ITS2 ribosomal gene sequence. The fungus was grown for 7 days on PDA, and DNA template was prepared by CTAB method [12]. The ITS regions of the fungus were amplified with the universal ITS primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') using PCR. Amplified PCR product was purified and sequenced by an automated DNA sequencer (377 Genetic Analyser; Applied Biosystems, CA, USA). Comparative sequence analysis was carried out using the BLASTN programme to retrieve homologous nucleotide sequences from database at NCBI (www.ncbi.nlm.nih.gov/BLAST/). The closely related sequences were downloaded and aligned with the CPF2-ITS sequence by ClustalW and a phylogenetic tree and distance matrix were prepared using the DNASTAR (Lasergene) sequence analysis software.

2.4. Optimization of Culture Conditions for Enzyme Production

A step by step optimization procedure regarding the effect of type and concentration of carbon and nitrogen source, growth temperature, pH and incubation time on the growth and enzyme production was employed. Strain presenting large clearing zones in Congo red assay was used for enzyme production on MS medium [13]. (Mandels and Sternburg 1976), pH 5.5 containing, per liter, 15 g of crystalline cellulose, 7 g of (NH₄)₂SO₄, 1.5 g of Urea, 2.0 g of KH₂PO₄, 0.3 g of CaCl₂, 0.3 g of MgSO₄ 7H₂O, 2 g peptone, 10 ml (10 % v/v) of Tween 80, and 1 ml of Vogel's trace element solution. The culture medium (100 ml) in 500 ml shake flasks was inoculated with two

discs (7 mm diameters) cut from 4-day old culture from PDA plates and the cultivation was performed at 28°C ± 2°C with shaking at 180 rpm. After 6 days of growth, the mycelium was filtered off, the culture filtrate was centrifuged at 10,000 × g for 10 min at 4°C, and the resultant supernatant was filtered through 10 kDa ultra-membrane (Pall filtration unit). Sample of crude cellulases was stored at 4°C.

2.5. Enzyme Assay

Filter paper activity (FPase), endoglucanase, xylanase and BGL activities were determined using Whatman No. 1 filter paper, CMC, birch wood xylan and PNPG as substrates, according to previously described standard conditions [14]. Appropriately diluted 0.5 ml enzyme along with 0.5 ml of 0.05 M sodium acetate buffer, pH 4.8 was added to a test tube of 25 ml volume. Temperature was adjusted to 50°C and substrate (filter paper strip/CMC/ birch wood xylan) was added and mixed. Samples were incubated at 50°C for 60 minute. 3.0 ml DNS was added mixed and kept for 5 min in boiling water bath followed by cooling in a cold bath. Enzyme blank, substrate blank, spectra zero and glucose standards were run simultaneously and each experiments were run in triplicates. 20 ml of MQH₂O was added to each tube, mixed by inverting the tube several times. The color formed was measured against the spectra zero at 540 nm after 20 min. The color formed in substrate blank and enzyme blank were subtracted from sample reading. Absorbance of the sample was translated into reducing sugar production during the reaction using a glucose/xylose standard curve. β- glucosidase was assayed in a reaction mixture containing 10 mM of p-nitrophenyl-glucopyranoside (pNPG) in 50 mM sodium acetate buffer, pH 4.8 and 20-50 µl of appropriately diluted enzyme solution in triplicate. The mixture was incubated at 50°C for 10 minute. The reaction was terminated by adding 2 ml of 1 M Sodium carbonate, and the developed yellow color was read at 405 nm. One unit of β-glucosidase activity was expressed as the amount of enzyme required to release 1 micromole of p-nitrophenol (pNP) from the substrate per min under assay conditions.

Protein concentrations were determined with the Bradford method [15] with bovine serum albumin as the standard.

2.6. Enzymatic Saccharification

Cellulose and pretreated sugarcane bagasse (cellulose ~52 %, hemicelluloses ~11 %, lignin ~24 % and miscellaneous ~2 %) was used for saccharification. The hydrolysis was carried out at 5 % (w/v) substrate concentration using various enzyme concentrations from CPF2 (5-25 IU FPA/g of substrate). The saccharification experiments were carried out by incubating 1 g substrate in appropriate amount of enzyme and 0.05 M Citrate buffer pH 4.8 in a final volume of 20 ml in Erlenmeyer flasks (100 ml) fitted with stoppers. The mixture was incubated at 50°C in a rotary shaker at 150 rpm for 24 hours. The commercial cellulase preparation Celluclast was used as reference. The samples were collected after every 3 h and heated to 100°C immediately to denature the cellulase, then cooled at 4°C and centrifuged at 10,000 × g for 10 minutes. Saccharification yield was calculated

by total sugar quantification and calculated by the method of [16].

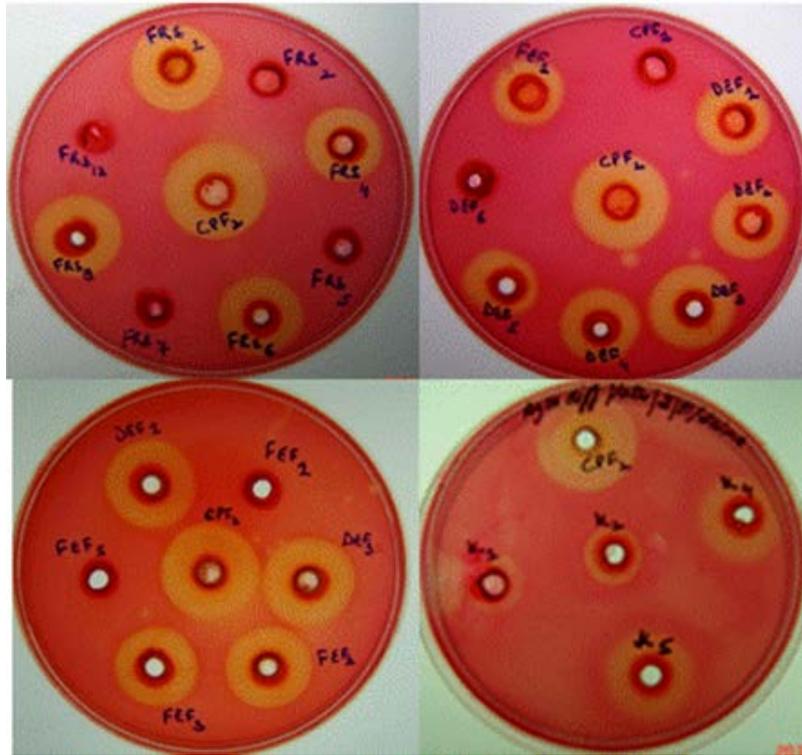
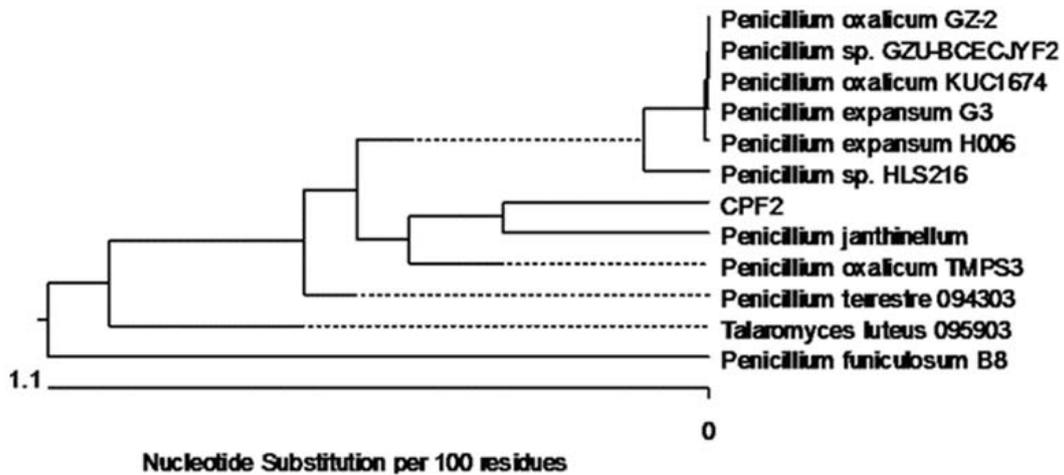


Figure 1. Screening of endophytic fungal isolates for cellulase activity



a

Percent Identity												
	1	2	3	4	5	6	7	8	9	10	11	12
1	█	99.0	99.2	98.5	99.8	99.0	99.8	99.0	99.2	99.8	99.8	1
2	1.0	█	100.0	98.7	100.0	100.0	100.0	100.0	99.8	100.0	100.0	2
3	0.8	0.0	█	98.7	100.0	100.0	100.0	100.0	99.8	100.0	100.0	3
4	1.5	1.3	1.3	█	98.5	98.7	98.7	98.7	98.5	99.8	100.0	4
5	0.2	0.0	0.0	1.5	█	100.0	100.0	100.0	99.8	100.0	100.0	5
6	1.0	0.0	0.0	1.3	0.0	█	100.0	100.0	99.8	100.0	100.0	6
7	1.0	0.0	0.0	1.3	0.0	0.0	█	100.0	99.8	100.0	100.0	7
8	0.2	0.0	0.0	1.3	0.0	0.0	0.0	█	100.0	99.8	100.0	8
9	1.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	█	99.8	100.0	9
10	0.8	0.2	0.2	1.5	0.2	0.2	0.2	0.2	0.2	█	99.8	10
11	0.2	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.2	0.2	█	11
12	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	█

b

Figure 2. a. Tree showing the Phylogenetic of the fungal isolate CPF2 based on 18S-ITS1-5.8S ribosomal gene sequence; b. Distance matrix generated by the ENASTAR software, showing the homology of 18S-ITS1-5.8S ribosomal gene sequence of sequence of CPF2 with its close relatives

3. Results

3.1. Isolation and Identification of CPF2

Twenty-one fungal isolates, belonging to fourteen different species were isolated from endemic and medicinal plants like *Cedrus deodara*, *Pinus roxburgii* and *Abies pindrow* and *Chlorophytum comosum* from a total of 55 segments (22 leaf, 15 stem and 18 root samples). Out of sixteen fungal isolates, fifteen belonged to the division ascomycetes and one to the division basidiomycetes (Table 1). Screening of the endophytes led to shortlisting of high cellulase producer fungus isolated from the leaf of *Chlorophytum* plant which was designated as CPF2. Extracellular cell free broth (CFB) of

CPF2 grown on cellulose was found to produce a largest zone of clearance on the CMC agar plates in comparison to the CFB from other endophytic fungi (Figure 1) CPF2 was grown on various types of rich mycological media. Colonies of CPF2 were 2.5-3 cm in diameter. The colonies were initially white and became grayish green in color after 6-7 days of growth on PDA. Microscopically the strain showed a mycelium of septate hyphae and stripes fruiting in penicilli structures with conidia catenulate, subspheroidal with smooth walls, a characteristic feature of genus *Penicillium*. The ITS sequence of CPF2 showed 99.8 % sequence similarity with *Penicillium oxalicum* (*P. oxalicum*) TMPS3, *P. janthinellum*, *P. terrestre* 094303 and *Talaromyces luteus* 095903 (Figure 2a, Figure 2b).

Table 1. Screening of fungal endophytes for cellulase activity

Endophytes isolated (GenBank Acc. no. of the ITS sequence)	Microrganism with the highest sequence identity and % sequence similarity	Plant host and Voucher number	Cellulase activity
CPF2 (HM440340.1)	<i>Penicillium</i> sp. ~98%	<i>Chlorophytum comosum</i>	+++
CPF1	<i>Aspergillus</i> sp. ~96%	-do-	-
DEF1 (JQ769260)	<i>Sordaria humana</i> , ~98%	<i>Cedrus deodara</i> ; RRL(H)1832)	++
DEF2 (JQ769261)	<i>Alternaria alternata</i> ~100%	-do-	++
DEF3 (JQ769262)	<i>Talaromyces trachyspermus</i> 93%	-do-	++
DEF4 (JQ769263)	<i>Cochliobolus spicifer</i> ~99%	-do-	+
DEF5 (JQ769264)	<i>Scleroconidioma sphagnicola</i> ~98%	-do-	-
FEF2 (JQ769265)	<i>Daldinia fissa</i> ~99%	<i>Abies pindrow</i> ; RRL(H)21551	-
FEF3 (JQ769266)	<i>Penicillium oxalicum</i> ~96%	-do-	+
FEF4 (JQ769267)	<i>Polyporus arcularius</i> ~98%	-do-	+
FEF5 (JQ769268)	<i>Apiosordaria otanii</i>	-do-	++
K1 (JQ769269)	<i>Petriella setifera</i> ~99%	<i>Pinus roxburgii</i> ; RRL(H)15011	+
K2 (JQ769270)	<i>Bipolaris tetramera</i> ~97%	-do-	-
K4 (JQ769271)	<i>Trichophaea abundans</i>	-do-	+
K6 (JQ769272)	<i>Penicillium expansum</i> ~95%	-do-	++
K7 (JQ769273)	<i>Ulocladium</i> sp. ~98%	-do-	-

-, not active; +++, highly active; ++, moderately active; +, least active

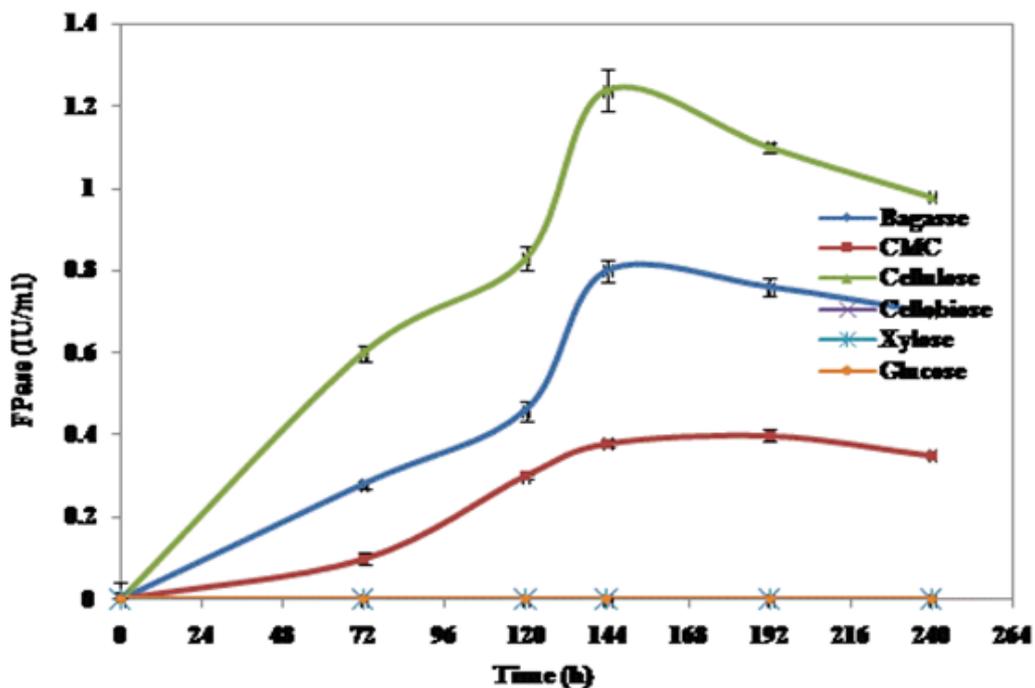


Figure 3. Total cellulase activity curves of CPF2 with different substrates

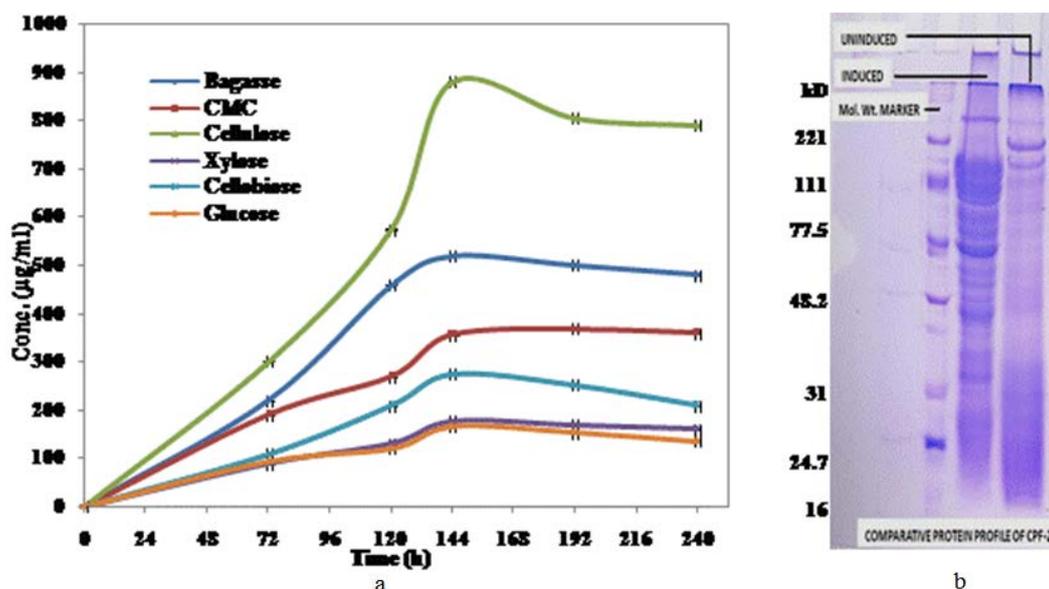


Figure 4. a. Total protein obtained from CPF2 grown in the presence of different substrates; b. SDS-PAGE showing total protein produced by CPF2 grown with and without cellulose

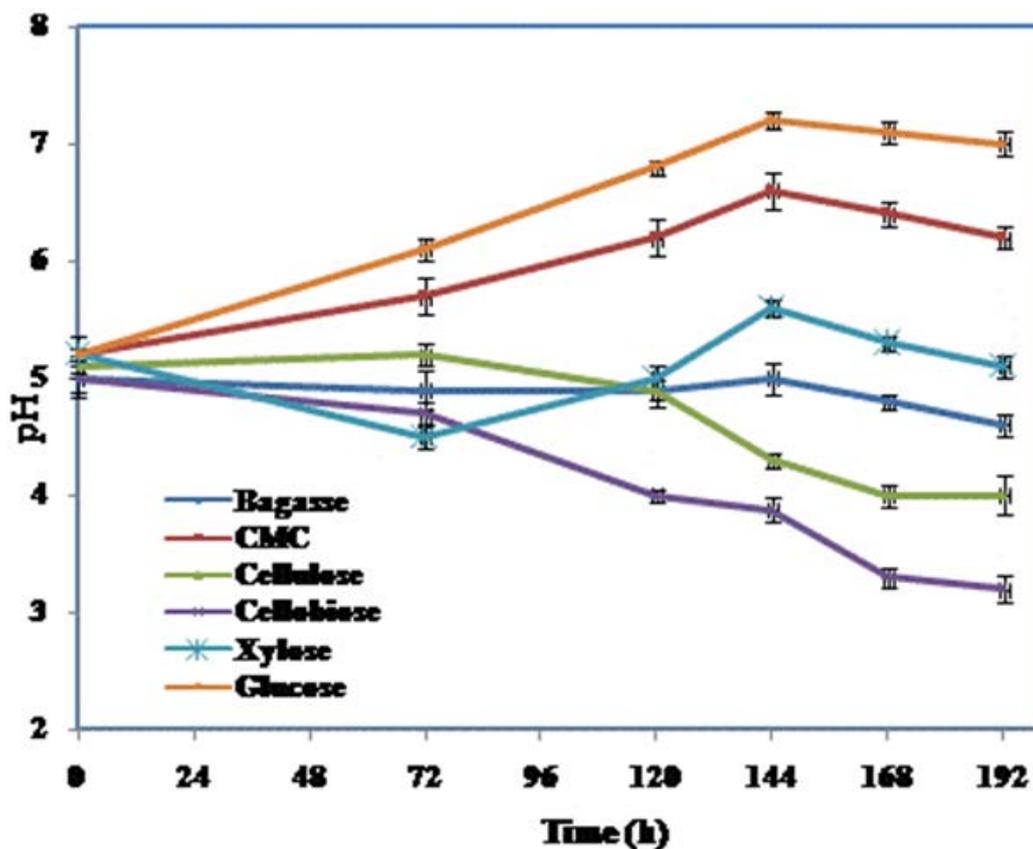


Figure 5. pH profile during the growth of CPF2 on various carbon source Over the incubation period

3.2. Optimization of Culture Conditions for Enzyme Production

Penicillium sp. CPF2 was characterized to measure the effect of carbon source on the specific growth rate and production of cellulolytic enzymes. Maximum specific growth rate of CPF2 was observed to be 0.15 h^{-1} on glucose and 0.11 h^{-1} on xylose respectively. Among the various carbon sources like crystalline cellulose, SEB, cellobiose, xylose, glucose and CMC tested, maximum production of total cellulase activity (FPase) was obtained by growing CPF2 in MS medium with 1.5 % (w/v)

crystalline cellulose. SEB (1.5 % w/v) was the second best carbon source for total cellulase production followed by CMC. Estimation of FPase activity indicated that activity began on day 70 h with a consistent rise in activity during the day 144 h period in cell free broth of CPF2 grown on cellulose and SEB, but CPF2 did not produce FPase during growth on glucose and xylose though very low activity was observed on cellobiose (Figure 3). The increase in total protein concentration through the 144-168 h period in the presence of glucose was found to be significantly lower as compared the total protein production in the presence of cellulose, SEB and CMC

(Figure 4a) with highest concentration of 0.50-0.86 mg/ml released with crystalline cellulose and SEB. Figure 4b shows SDS-PAGE profile of total protein produced by CPF2 grown under cellulose medium induced and uninduced conditions. Appearance of large number of additional proteins bands were observed in CPF2-broth grown under induced conditions. The media was adjusted to pH 5.5 on day 1 and subsequently the pH was recorded during the 8 day period for growth on the different substrates. There was an increase in pH during the growth of the culture in the presence of both CMC and glucose as substrates. However, cellulose and cellobiose containing

medium showed drop in the pH (Figure 5). Figure 6 shows the kinetics of the cellulases as well as the total extracellular protein production during the growth of CPF2 with crystalline cellulose. The maximum FPase, endoglucanase, xylanase and β -glucosidase activities observed were 1.2, 19, 40 and 2.8 IU /ml, respectively. FPase, CMCase and xylanase activities started emerging on day 2 and then gradually reached its peak on day 6 and then started declining. However, β - glucosidase activity started emerging from day 3 and reached its peak on day 6. Concomitant with this, a significant increase in total protein concentration was registered.

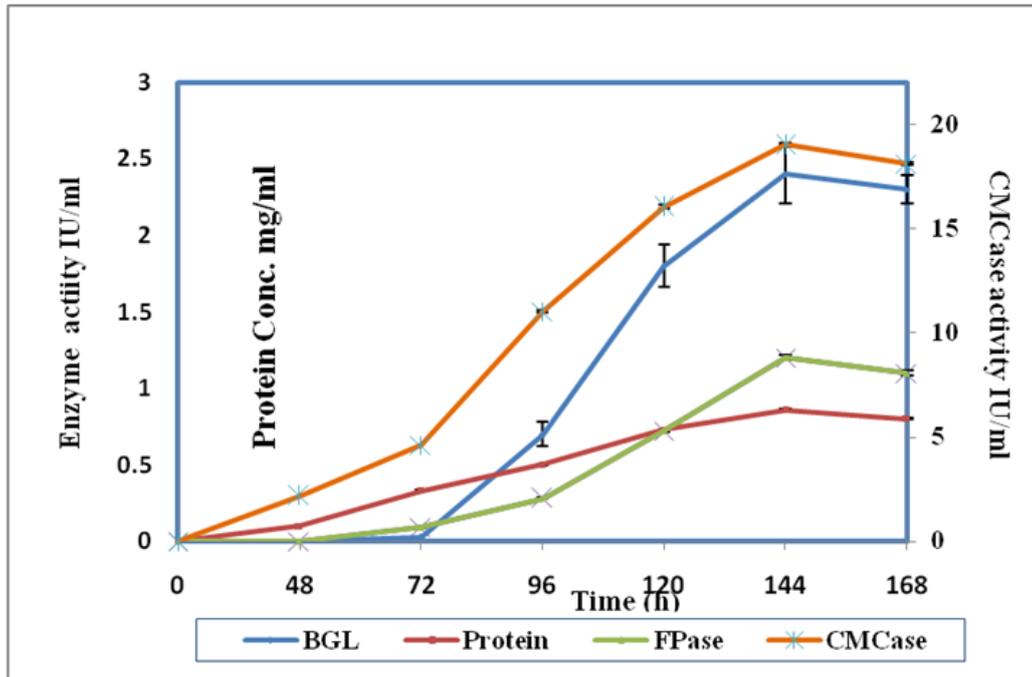


Figure 6. Kinetics of the enzyme and total extracellular protein production by CPF2 grown on cellulose

Table 2. Hydrolysis (%) of SEB by CPF2 at 5 % (w/v) substrate conc

Time (h)	Enzyme conc. FPU (CPF2)/g substrate					FPA (Celluclast)	FPA(Celluclast) +BGL (Novo188)
	5	10	15	20	25	25	25 : 8
6	3.86 ± 0.19	9.93 ± 0.75	17.83 ± 0.11	25.76 ± 0.15	30.24 ± 0.25	10.08 ± 0.2	29.80 ± 0.85
12	13.29 ± 0.6	26.86 ± 0.35	35.1 ± 0.85	46.3 ± 0.65	59.8 ± 1.05	20.02 ± 1.0	53.04 ± 1.97
24	20.59 ± 1.52	29.03 ± 0.31	38.51 ± 0.5	51.67 ± 0.62	63.79 ± 0.99	31.41 ± 1.3	60.66 ± 1.51

Table 3. Hydrolysis (%) of cellulose by CPF2 at 5 % (w/v) substrate conc

Time (h)	Enzyme conc. FPU (CPF2)/g substrate					FPA (Celluclast)	FPA(Celluclast) +BGL (Novo188)
	5	10	15	20	25	25	25 : 8
6	6.88 ± 0.21	15.09 ± 0.95	27.03 ± 0.25	38.76 ± 0.26	45.24 ± 0.38	12.28 ± 0.25	33.85 ± 0.88
12	18.293 ± 0.8	29.76 ± 0.45	45.15 ± 0.95	66.3 ± 0.75	82.8 ± 1.35	25.62 ± 1.0	59.94 ± 2.17
24	26.50 ± 1.72	32.06 ± 0.87	52.51 ± 0.59	78.67 ± 0.78	90.79 ± 1.49	38.48 ± 1.5	64.06 ± 1.1

3.3. Enzymatic Saccharification

The hydrolytic performance of the enzyme preparation obtained after the cultivation of the CFP2 on cellulose was evaluated on cellulose and steam exploded sugarcane bagasse. The hydrolysis was carried out at 5 % (w/v) substrate concentration and optimization of enzyme

loading in the hydrolysis of SEB was performed. A commercial cellulase preparation, Celluclast (FPase : CMCase : BGL; 1: 9.5: 0.34) was included in the study for comparison. Highest hydrolysis yield of ~90 % for cellulose and ~63 % for SEB was obtained using 25 FPU/g substrate concentrations after 24 h (Table 2, Table 3) which was significantly higher than hydrolysis yield

obtained using the commercial enzyme Celluclast (31 % saccharification) However, the incorporation of additional BGL activity (Novozyme 188) up to 8 IU BGL/g cellulose along with Celluclast resulted in a significantly improved saccharification to >60 %.

4. Discussion

Screening of the endophytic fungi for cellulases was attempted with a view that endophytes penetrate into the plant tissues through the lignocellulosic wall with the help of battery of hydrolytic enzymes, cellulases being the predominant among them [17,18]. However, a report on the production of stable cellulases in a proper ratio for polymer degradation from fungal endophyte is lacking. Therefore, a selective search for fungal endophytes was pursued with the ability to produce higher levels of stable cellulases. The ITS sequence of CPF2 showed 99.8 % sequence similarity with *P. oxalicum* TMPS3, *P. janthinellum*, *P. terrestre* 094303 and *Talaromyces luteus* 095903. Since the fungal isolate CPF2 could not be identified at species level, hence the isolate was designated as *Penicillium* sp. CPF2

The ITS sequence obtained has been deposited at the GenBank under accession number HM440340.1. Species from the genus *Penicillium* in addition to species from the genus *Aspergillus* and *Trichoderma* have been reported to possess potential for cellulase production [19,20,21]. nevertheless; sugarcane bagasse hydrolyzing cellulase with high specific activity from an endophytic *Penicillium oxalicum* CPF2 is reported for the first time. The fungal strain CPF2 has been deposited in the national repository, National Fungal Culture Collection of India with accession number NFCCI 2862.

A number of studies have shown that cellulase biosynthesis is higher when organisms are exposed to complex substrates as opposed to when they are grown in the simple substrates [22,23]. In most organisms, cellulase production is repressed in the presence of readily metabolizable carbon sources such as glucose but is induced by low molecular weight compounds such as cellobiose [3]. Among the various substrates used, cellulose (1.5 % w/v) was found to be the best carbon source to induce cellulase activity followed by sugarcane bagasse (1.5 % w/v). The inability of CPF2 to produce cellulases may be due to the repression of enzyme synthesis or activity in the presence glucose and xylose [24]. Among the various nitrogen sources like peptone, beef extract, yeast extract, ammonium nitrate, sodium nitrite and sodium nitrate tested at 0.2 % (w/v), peptone produced maximum cellulase followed by beef extract and ammonium nitrate. The optimum pH for fungal cellulases and crude protein production varies from species to species though in most cases, the optimum pH ranges from pH 3.0 to 6.0 [25]. The media was adjusted to pH 5.5 on day 1 and subsequently the pH was recorded during the 8 day period for growth on the different substrates. There was an increase in pH during the growth of the culture in the presence of both CMC and glucose as substrates. However, cellulose and cellobiose containing medium showed drop in pH but xylose containing medium showed no significant pH change over the course of the experiment. The drop in pH may arise as a result of

formation of cellobiose, oxidising to cellobionolactone by cellobiose dehydrogenase (CDH) (E.C.1.1.3.25), and cellobionolactone is subsequently hydrolysed to carboxylic acids [22]. High protein concentration was shown to be correlated with a high FPase activity with a specific activity of 1.39 IU/mg protein which is significantly higher than the specific activity of FPase reported for cellulases from other *Penicillium* sp. [19,20,21]. A steady increase in cellulases in the cultivation broth was recorded on 96-144 h of fermentation, which is significantly faster than reported for other species of *Penicillium* [21] with the time course of 170-230 h for optimum enzyme production. Endoglucanase was induced earlier than BGL since it acts in the polymeric cellulose, which is abundant at the beginning of fermentation. As this substrate was hydrolyzed into short chain oligosaccharides, BGL induction increased, even after the endoglucanase activity became stable. Strains of the genus *Penicillium* has been investigated previously for cellulase production [19,21]. A study of about twelve strains, cultivated with solka floc under submerged fermentation, demonstrated *P. brasilianum* (IBT 20888) producing the highest FPase activity (34 IU/g cellulose) [20]. Similarly maximum FPase activity of 0.35 IU/ml corresponding to 76 IU/g cellulose was reported to be produced by *P. funiculosum* when sugarcane bagasse partially delignified cellulignin was used as substrate [21]. However, increased levels of cellulase production by *P. echinulatum* strain 9A02S1 and *P. janthinellum* strain NCIM 1171 has been reported only after strain improvement by mutagenesis [26]. Comparing these results with those obtained in the present work, it is clear that CPF2 is a promising strain for cellulase production. The enzymatic saccharification using CPF2 cellulase (FPase) increased with increase in enzyme dosage from 5 IU-25 IU/g of substrate thereafter no significant increase in sugar yield was observed. Increasing the enzyme concentration from 5 IU to 25 IU of FPU/g each substrate increased the hydrolysis by 7-8 fold during initial period of 6 h. In hydrolysis studies, the presence of sufficient BGL activity is often critical as the accumulation of cellobiose can strongly inhibit the activity of the cellulases [27]. Celluclast and other cellulase preparations produced by *T. reesei* normally have a low BGL activity and extra BGL activity is frequently added to avoid temporary product inhibition caused by cellobiose [27]. Under these conditions, presence of much higher BGL activities in the cellulase preparations produced by CPF2 might be beneficial for the hydrolysis performance and final yields.

In conclusion, production of cellulases by endophytic fungi *Penicillium* sp. CPF2 in liquid state fermentation was optimized. Cellulose served as a best carbon source that enhanced FPase activity to 1.2 IU/ml corresponding to 80 IU/g cellulose in combination with 0.2 % peptone at pH 5.5. Cellulase produced from CPF2 under optimized conditions was capable of hydrolyzing cellulose (> 90 %) and SEB efficiently (> 63%) in comparison to the commercial enzyme Celluclast. This is due to the fact that CPF2 produces cellulases in a proper ratio with the advantage of containing high BGL activity that could potentially be used for hydrolysis of lignocellulosic materials. Conventional mutagenesis approach is being followed in order to increase the cellulase yields in CPF2.

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References

- [1] Singh, A., Kuhad, R. C. and Ward, O. P. "Industrial application of microbial cellulases," in *Lignocellulose Biotechnology: Future Prospects*, R. C. Kuhad and A. Singh, Eds., pp. 345-358, I.K. International Publishing House, New Delhi, India, 2007.
- [2] Kuhad, R. C., Gupta, R. and Singh, A., "Microbial Cellulases and Their Industrial Applications", 2011, *Enzyme Research*, Article ID 280696.
- [3] Lynd, L. R., Weimer, P. J., Vanzyl, W. H. and Pretorius, J. S., "Microbial cellulose utilization: fundamentals and biotechnology" *Microbiology and Molecular Biology Reviews*, 66. 506-577. 2002.
- [4] Bhat, M. and Bhat, S., "Cellulose degrading enzymes and their potential industrial applications," *Biotechnology Advances*, 15. 583-620. 1997.
- [5] Wood, T. M., "Mechanisms of cellulose degradation by enzymes from aerobic and anaerobic fungi. In: Enzyme systems for lignocellulose degradation" NY: Elsevier., pp. 1. 1989.
- [6] Tolan, J. S. and Foody, B., "Cellulases from submerged fermentation," *Advances in Biochemical Engineering Biotechnology*, 65. 41-67. 1999.
- [7] Duff, S. J. B., Murray, W. D., "Bioconversion of forest products industry waste celluloses to fuel ethanol: a review," *Bioresource Technology*, 55. 1-33. 1996.
- [8] Brown, J. A., Collin, S. A. and Wood, T. M., "Development of a medium for high cellulase, xylanase and β -glucosidase production by a mutant strain (NTG III/6) of the cellulolytic fungus *Penicillium pinophilum*," *Enzymes and Microbial Technology*, 9. 355-360. 1987.
- [9] Krogh, K. B. R., Mørkeberg, A., Jørgensen, H., Frisvad, J. C. and Olsson, L. "Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes," *Applied Biochemistry Biotechnology*, 116. 389-401. 2004.
- [10] Strobel, G. A., Daisy, B., Castillo, U. and Harper, J., "Natural products from endophytic microorganisms," *Journal of Natural Products*, 67. 257-268. 2004.
- [11] Shoseyov, O., Doi, R. H., "Essential 170-kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulose," *Proceedings of the National Academy of Sciences USA*, 87. 2192-2195. 1990.
- [12] Saghai-Marooof, M. A., Soliman, K. M., Jørgensen, R. A. and Allard, R. W., "Ribosomal spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics" *Proceedings of the National Academy of Sciences USA*, 81. 8014-8019. 1984.
- [13] Mandels, M., Sternburg, D., "Recent advances in cellular technology," *Journal of Fermentation Technology*, 54. 267-286. 1976.
- [14] Ghose, T. K., "Measurement of cellulase activities" *Pure and Applied Chemistry*, 59. 257-268. 1987.
- [15] Bradford, M. M., "A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, 72. 227-254. 1976.
- [16] Hari, K. S., Prasanthi, K., Chowdary, G. V. and Ayyanna, C., "Simultaneous saccharification and fermentation of pretreated sugar cane leaves to ethanol," *Process Biochemistry*, 33. 825-830. 1998.
- [17] Sieber, T. N., Sieber-Canavesi, F. and Dorworth, C. E., "Endophytic fungi of fungi alder (*Alnus rubra*) leaves and twigs in British Columbia," *Canadian Journal of Botany*, 69. 407-411. 1991.
- [18] Carroll, G. C., Petrini, G. C. and Petrini, O., "Patterns of substrate utilization by some endophytes from coniferous foliage," *Mycologia*, 75. 53-63. 1983.
- [19] Jørgensen, H., Mørkeberg, A., Krogh, K. B. R. and Olsson, L., "Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulase adsorption by capillary electrophoresis," *Enzymes and Microbial Technology*, 36. 42-48. 2005.
- [20] Jørgensen, H. and Olsson, L., "Production of cellulases by *Penicillium brasilianum* IBT 20888 : Effect of substrate on hydrolytic performance," *Enzymes and Microbial Technology*, 38. 381-390. 2006.
- [21] de Castro, A. M., de Albuquerque, de Carvalho, M. L., Leite, S. G., Pereira, N. J., "Cellulases from *P. funiculosum*: production, properties and application to cellulose hydrolysis," *Journal of Industrial Microbiology Biotechnology*, 37. 151-158. 2010.
- [22] Henriksson, G., Johansson, G. and Pettersson, G., "A critical review of cellobiose dehydrogenases," *Journal of Biotechnology*, 78. 93-113. 2000.
- [23] Wymelenberg, A. V., Sabat, G., Martinez, D., Rajangam, A. S., Teeri, T. T. and Gaskell, J., "The *P. chrysosporium* secretome: Database predictions and initial mass spectrometry peptide identifications in cellulose-grown medium" *Journal of Biotechnology*, 118. 17-34. 2005.
- [24] Spiridonov, N. A. and Wilson, D. B., "Regulation of Biosynthesis of Individual cellulases in *Thermomonospora fusca*," *Journal of Bacteriology*, 180. 3529-3532. 1998.
- [25] Garg, S. K. and Neelakantan, S., "Effect of cultural factors on cellulase activity and protein production by *Aspergillus terreus*," *Biotechnology and Bioengineering*, 23. 1653-1659. 1981.
- [26] Adsul, M. G., Bastawde, K. B., Varma, A. J. and Gokhale, D. V., "Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulose production," *Bioresource Technology*, 98. 1467-1473. 2007.
- [27] Brown, J. A., Collin, S. A., Wood, T. M., "Development of a medium for high cellulase, xylanase and β -glucosidase production by a mutant strain (NTG III/6) of the cellulolytic fungus *Penicillium pinophilum*," *Enzymes and Microbial Technology*, 9. 355-360. 1987.