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ISOLATION OF A NEW *PENICILLIUM CHRYSOGENUM* STRAIN BF02 FROM AGRICULTURAL SOIL OF RURAL INDIA PRODUCING A THERMOSTABLE LOW K_M CELLOBIASE

Samudra Prosad Banik, Swapan Bhattacharyya, Shakuntala Ghorai*

Address(es): Samudra Prosad Banik,
Maulana Azad College, Faculty, Department of Microbiology, 8 Rafi Ahmed Kidwai Road, Kolkata 700013, West Bengal, India, Office Phone Number +9133 2249 3737.

*Corresponding author: samudrapb@gmail.com

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ABSTRACT

A new strain of *Penicillium chrysogenum* producing a low K_m, thermostable cellobiase was isolated from agricultural soil of rural West Bengal, India. The culture filtrate showed strong antibacterial activity against gram positive organisms, gave two cellobiase activity bands on native PAGE zymography and two peaks on sephacryl S200 gel chromatography. The higher molecular weight peak had a higher specific activity and was characterised further. It gave three bands corresponding to 130 kDa, 65 kDa and 55 kDa in SDS-PAGE and showed a K_m of 0.196 mM against *p*- nitrophenyl β-D glucopyranoside. It was highly thermostable and retained full activity after one hour incubation at 55°C with temperature optima of 75°C. Its pH stability ranged between 4.5 and 8.5 with optimal activity at pH 5 and it retained more than 65% activity in presence of 100 mM guanidium hydrochloride and 2 M urea. Apart from Zn^{+2} and Hg^{+2}, enzyme activity was not affected by other metal ions and in presence of Mg^{+2} and Mn^{+2}, activity was boosted. It also retained more than 80% activity in presence of 0.5% glucose. This is one of the very few reports of an efficient cellobiase from the *Penicillium* genus which can be utilised for biotechnological applications.

Keywords: Filamentous fungi; *Penicillium chrysogenum*; antimicrobial potential; thermostable low K_m cellobiase

INTRODUCTION

Lignocellulosic raw materials derived from plant biomass are the most promising renewable energy resource in terms of their abundance (**Singh *et al.*, 2006**) and ecofriendliness. Accordingly, they have emerged as one of the most sustainable sources of fuel, animal feed and feed stock for chemical synthesis (**Bhat, 2000**). Fast depletion of global oil reserve has accelerated the research leading to utilization of cellulosic biomass (**Kuhad *et al.*, 1997**; **Gong *et al.*, 1999**). In this respect, the enzymatic conversion of cellulosic mass to fermentable sugars (which is subsequently converted into cellulosic ethanol) has been the most sought after technology in terms of feasibility and reduction of environmental pollution (**Dale, 1999**; **Lynd *et al.*, 1999**). Complete enzymatic hydrolysis of cellulose requires synergistic action of three enzymes, namely endoglucanase (EC 3.2.1.4 carboxymethylcellulase) which cuts at random internal sites of cellulose generating oligosaccharides and new chain ends, exoglucanase (EC 3.2.1.74 cellobiohydrolase) which cuts both at non reducing and reducing ends to generate glucose, cellobiose and higher cellooligosaccharides and β-glucosidase (E.C. 3.2.1.21 cellobiase) which cleaves cellobiose to release additional glucose units (**Bhat, 2000**). Cellobiase tends to be the rate limiting enzyme of this process since presence of traces of cellobiose can significantly inhibit activity of the other two enzymes. The enzyme cellobiose belongs to the broad family of β-glucosidases which catalyzes the hydrolysis of cellobiose into two glucose units. Apart from its role in cellulose utilisation, the enzyme has crucial roles in biotechnological applications (**Bhatia *et al.*, 2002**) like removal of aglycone moiety from flavanoids and isoflavanoidglucosides which are phenolic and phytoestrogen glucosides that occur naturally in fruits, vegetables, tea, red wine and soyabeans (**Bhatia *et al.*, 2002**), detoxification of cassava, aroma enhancement and removing bitter compounds from citrus fruit juices or unripe olives (**Bajaj *et al.*, 2009**) etc. Synthetic activity of cellobiase also has potential applications in preparations of agrochemicals and drugs (**Fischer *et al.*, 1996**).
Keeping in mind the huge potential of this enzyme, there has been a constant endeavour to screen for good producer organisms of this enzyme. Bacteria (**Gueguen *et al.*, 1997**), yeast (**González-Pombo *et al.*, 2008**) and fungi (**Inglin *et al.*, 1980; Dhake and Patil, 2005**) are well known producers of this enzyme. However due to high yield and ease of purification, filamentous fungi have been preferred to the other two microbial colleagues. Traditionally, strains derived from *Trichoderma reesei* and *Aspergillus niger* have been commercially utilised in industrial processes (**Chauve *et al.*, 2010**). *Trichoderma ressei* has been reported to produce extracellular (**Chirico and Brown, 1987**), cell-wall bound (**Umile and Kubicek, 1986**) and intra-cellular (**Inglin *et al.*, 1980**) β-glucosidases. However, the enzymes from these strains suffer from either low catalytic efficiency or low thermostability. Therefore, the industry is still in want of an optimal formulation from a novel producer organism. Over the recent years, a few of such alternative organisms had made their appearance; *Termitomycesclypeatus*, a filamentous fungus belonging to Basidiomycetes is a well known producer of cellulolytic and xylanolytic enzymes (**Ghorai*et al.*, 2010**). Enhanced cellobiase activity together with a higher catalytic efficiency and thermostability was reported from the fungus using 2-deoxy glucose as a glycosylation inhibitor in the culture medium (**Ghorai *et al.*, 2010**). Reports of *Penicillium* as producers of cellulolytic enzymes are few (**Zorov *et al.*, 2001; Morozova *et al.*, 2010**); they have been less explored as industrially viable producer organisms. Nevertheless, their importance is being recognised by the scientific community in recent times. In terms of thermostability, cellobiases from *Penicillium* seem to have better potential than *Trichoderma, Aspergillus, Termitomyces* etc. (**Hidalgo *et al.*, 1992**). The high antibacterial activities in the culture filtrates of these organisms also provide an additional advantage in terms of protection of the fermentate from microbial contamination. Recently, a *Penicillium chrysogenum* strain has been co-cultured with *Aspergillus niger* to obtain efficient cellulolytic enzyme titer (**Jayant *et al.*, 2011**). The present studies report the isolation of a novel *Penicillium chrysogenum* strain from rural West Bengal, India, which produces an efficient low K_m and highly thermostable cellobiase fit for industrial use.

MATERIAL AND METHODS

Growth conditions

In order to screen the cellulolytic fungal strains, 100 times diluted soil samples from different agricultural fields were spreaded on CMC- agar plates containing 0.2% peptone along with $NaNO_3$, $Mgcl_2$, Kcl, and KH_2PO_4 and grown at 28°C for 4-6 days. After six days the plates were flooded with Gram's iodine according to Kasana (**Kasana *et al.*, 2008**) and production of cleared zones indicating CMC utilisation were noted. The respective organisms were isolated, subcultured individually on separate CMC-agar plates in five replicates and diameters of zones were noted. For enzyme production, spore suspensions (1×10^6 cells ml^{-1}) of the organism in sterile water was inoculated in 25 ml of the corresponding

liquid medium and grown in 100 ml shake flasks at 28˚C. Growth was terminated on 4th day and culture filtrate was separated from mycelia. For media optimization, similar growth conditions were used with 1% respective carbon and 0.2% respective nitrogen sources. Media components were purchased from Sigma and Himedia. Antibiotics were purchased from Himedia. All other chemicals were of analytical grade and purchased from SRL unless otherwise stated.

Strain identification

Strain identification was carried out by Merck's fungal identification service (Cat No. 116702 GB) based on partial 18s rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene. The PCR product was sequenced using forward, reverse and internal primers. Sequence data was aligned and analysed for finding the closest homologues of the fungus. It was subsequently deposited at the GenBank (KC 469896).

Assessment of antibacterial activity of culture filtrate

The antibacterial activity of the culture filtrate was determined by a modification of Carter's method (**Carter, 1977**) as described below. Briefly, four cups were cut out from each 2% LB agar plate with a sterile standard cork borer (3 mm id) preadsorbed with 200 µl of an uniform layer of overnight grown bacterial cell suspension (cell density 10^8 cells/ml). On each plate, two cups were filled with the reference dose of standard antibiotic solutions made in sterile water. The third cup was filled with the crude undialysed culture filtrate concentrated to desired range by lyophilisation and the fourth cup was filled with sterile uninoculated culture medium which served as control. The plates were incubated overnight at 30°C and the diameters of the zones of inhibition were measured.

Enzyme, protein and sugar assays

Cellobiase (EC 3.2.1.21) assay was carried out spectrophotometrically by using *p*NPG Sigma) as substrate **(Mukherjee and Khowala, 2002)**. The reaction mixture (1 ml) contained 4 mM *p*NPG in 0.1 Msodium acetate buffer, pH 5.0 and an appropriate amount of the enzyme. Incubation was carried out at 50°C for 30 min. Reaction was terminated by the addition of 0.25 ml Na_2CO_3 (0.5 *M*). Intensity of the yellow colour developed by liberation of *p*NP was measured at 400 nm. One unit (U) of enzyme activity was expressed by the enzyme that produced 1 µmole of *p*NP per min under the assay conditions. Endoglucanase (3.2.1.4) assay was performed using CMC (Sigma) as substrate **(Ghosh, 1987)**. Culture filtrate samples were incubated with a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.05 M citrate acetate buffer (pH 5.0) at 50°C for 1 h and the reducing sugar produced was determined by DNS method (Miller 1979). One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min under assay conditions. Exoglucanase assay was performed using filter paper as substrate according to Mandels **(Mandels *et al.*, 1976)**. Aliquots of appropriately diluted culture filtrate as enzyme source was added to Whatman No.1 filter paper strip 1 X 6 cm; 50 mg) immersed in one millilitre of 0.05 M sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method **(Miller, 1959)**. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min. All other enzyme assays which involved determination of free glucose as end product were carried out by the DNSA method as described above. Protein content in crude culture filtrate was ascertained after acetone precipitation and resolubilisation **(Banik *et al.*, 2009)** by the Bradford method **(Bradford, 1976)** whereas that of sephacryl preparation was measured directly by Bradford method. Free glucose in the medium was ascertained by GOD-POD Glucose kit, Span Diagnostics.

Partial purification of cellobiase

The culture medium was freed from suspended mycelia and/or spores by centrifugation at 6,000 g for 10 minutes and subsequent filtration of the supernatant with a 0.22 µm membrane (Millipore) and was dialysed in a 3 kDa dialysis bag (Sigma) against 0.1 M acetate buffer (pH 5). The retentate was concentrated by lyophilisation and loaded on a Sephacryl S-200 gel permeation column (20 cm x 1.8 cm) in batches of 0.5 ml. Protein fractions, eluted at the rate of 8.0 ml h^{-1}, were monitored for protein (A_{280}) and cellobiase activities, and pooled as pool I (fractions 9-11) and pool II (fractions 13-15). They were desalted against 0.01 M of the same buffer by dialysis and concentrated by lyophilisation for use as partially purified extracellular cellobiase. For characterization of the crude enzyme, culture filtrate was extensively dialysed in order to eliminate interference by any other fungal metabolite.

Stability in presence of urea, guanidium hydrochloride and SDS

Stability in presence of the denaturants was measured by incubating the enzyme (100 ng) with 0.2-3 M urea and 0.1-1.5 M guanidium hydrochloride solution respectively for 1 h at room temperature and residual cellobiase activities were determined subsequently. Stability in presence of SDS was determined by incubation of enzyme preparations (50 ng) in presence of varying concentrations of SDS ranging between 0.1 and 1.5 % at room temperature for 1 h and subsequent elucidation of enzyme activity.

Effect of reducing agents

The effect of DTT on cellobiase activity was investigated by assaying the crude dialysed enzyme preparations. (10 µg aliquots of extracellular and intracellular) and the partially purified enzyme preparations (100 ng aliquots of extracellular and intracellular) in presence of DTT (0- 500 mM).

Glucose stability

The effect of glucose inhibition on the enzyme activity was ascertained by incubating measured aliquots of the enzyme with glucose concentrations ranging between 0.1% and 2% for 1 hr at room temperature and subsequently assaying for residual cellobiase activity.

Determination of enzyme kinetics

K_m and Vmax of cellobiases were determined under standard assay conditions using substrate *p*NPG) concentration between 0.025 and 0.4 mM from the respective Lineweaver–Burk plots.

Activity staining

Zymography or activity staining for in gel detection of cellobiase activity was performed by the method of Kwon **(Kwon *et al.*, 1994)**. Briefly, after electrophoresis (5% Native PAGE), the gel was soaked in a 100 mM sodium citrate buffer (pH 5.0) containing esculin ferric chloride for 5.0 min. It was then incubated at 50 °C for several minutes until a black band appeared.

SDS – PAGE

SDS polyacrylamide gel electrophoresis under reducing condition was carried out according to the method of Laemmli **(Laemmli, 1970)**. 10% gel containing 0.1% w/v) SDS appendix) was used. The protein samples 10-30 µg) were dissolved in the 1.25% SDS sample buffer, denatured by keeping the samples on boiling water bath for 5 mins and loaded into the 10% polyacrylamide slab gels containing 0.1% SDS. SDS–PAGE molecular weight standards (Broad range, Fermentas SM0671), were run alongside the samples. The gel was run at 20 mA till the tracking dye reached its lower edge. Thereafter it was immediately subjected to silver staining using ProteoSilver™ silver stain kit of Sigma according to the manufacturer's instructions. Molecular weights of the partially purified protein bands were determined from the distance vs log MW plot of standard molecular weight markers.

Statistical analyses

All experiments were done in several replicates and Microsoft Excel 2007 software was used for statistical treatment of the graphs plotted.

RESULTS AND DISCUSSION

Identification of the producer organism

Soil samples were diluted and spreaded on CMC agar plates. Organism showing maximum zone of clearing on CMC agar plate was further subcultured on 1% cellobiose liquid medium, and subsequently transferred to a fresh CMC slant for characterisation. Preliminary microscopic examination of the soil isolate with matty greenish colonies revealed distinct exogenously produced conidia growing basipetally in chains on sterigmata. Profuse production of ramus like branched conidiophores were also clearly identifiable (Figure 1). The strain was maintained on 1% CMC agar slants and its molecular phylogenetics was determined. Sequencing and subsequent alignment of the 1200 bp genomic DNA fragment coding for the 18s rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene identified the organism to be *Penicillium chrysogenum* (GenBank Accession Number JF922035) with an alignment score of 0.99 and the nearest homologue was found to be *Penicillium glabrum* (GenBank Accession Number JF922034) with an alignment score of 0.95. The sequence was subsequently deposited at the GenBank (Accession Number KC 469896).

Antibacterial activity of culture filtrate

Since the identified strain was *Penicillium chrysogenum*, antibacterial activities of the culture media were investigated and compared with known doses of standard antibiotics (ampicillin and streptomycin) by cup plate method against two gram positive (*Staphylococcus aureus*, *Bacillus subtilis*) and two gram negative (*Escherichia coli*, *Klebsiella pneumoniae*) organisms. The culture

filtrate was found to possess significant antibacterial activity over gram positive organisms. 100 µl of the culture filtrate (containing 100 µg protein equivalent) gave a 2 cm inhibition zone across the well in a *B. subtilis* plate as compared to a 3.6 cm corresponding to 100 µg ampicillin and 2.5 cm corresponding to 100 µg streptomycin (Figure 2A). Against *Staphylococcus aureus*, 1.4 cm zone diameter was observed corresponding to both 100 µg ampicillin and same volume and concentration of the culture filtrate as previously, whereas 2.4 cm was observed corresponding to 100 µg streptomycin (Figure 2B). However, no detectable inhibition zone was observed for the gram negative organisms (figure not shown).When the culture filtrate was dialysed in a 3 kDa cutoff dialysis bag and the retentate was loaded in the cups, no zones of inhibition were obtained for either of the organism tested (Data not shown). This indicated that the antibacterial activity of the culture filtrate owed to small molecules which were able to pass outside the dialysis membrane.

Figure 1 Bright field image of *Pencillium chrysogenum* BF02 (40X); branched conidiophores are shown by arrowhead

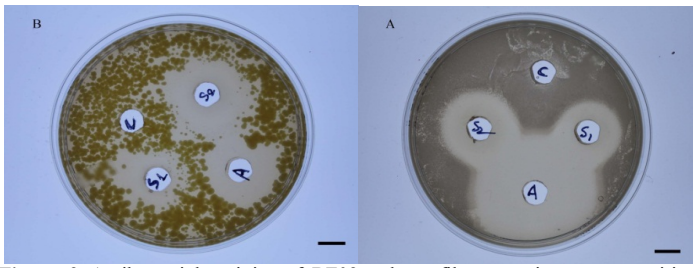

Figure 2 Antibacterial activity of BF02 culture filtrate against gram positive bacteria**;** 100 µl culture filtrate (S_1), 100 µg ampicillin (A) and 100 µg streptomycin (S_2) were applied against *Bacillus subtilis* (Figure 2A) and *Staphylococcus aureus* (Figure 2B). Bar denotes 1cm.

Partial purification of the enzyme from culture filtrate

The culture filtrate was dialysed extensively to get rid of free sugar, media components and other secondary metabolites. Total units of cellobiase inside the dialysis membrane were same before and after dialysis (Table 1). However, total protein distribution in retentate and filtrate was 7:3 leading to an increase in specific activity from 4.24 U/mg to 6.08 U/mg. The retentate was concentrated by lyophilisation and subjected to sephacryl S200 gel filtration chromatography. Two activity peaks of cellobiase were obtained corresponding to fractions 10 and 14. Accordingly they were pooled in two cumulative fractions; pool I (fraction 9-11) and pool II (fraction 13-15). However none of them coincided with the OD_{280} peaks (Figure 3) indicating that the cellobiases contained significantly low percentages of aromatic amino acids. Specific activity of pool I was significantly higher (930.46 U/mg) than that of pool II (7.75 U/mg). Therefore pool I indicated a better purified enzyme preparation and was chosen for further characterisation.

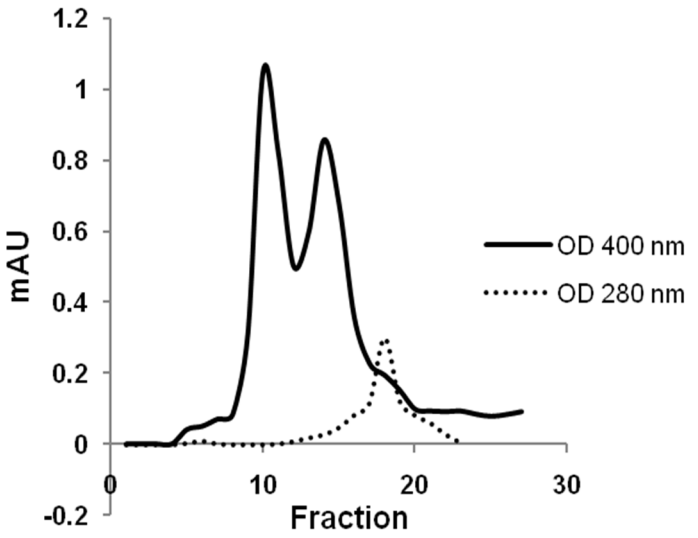

Figure 3 Elution profile of BF02 culture filtrate in Sephacryl S200; 740 µg dialysed protein from 3rd day culture filtrate was loaded and eluted at the rate of 8 ml hr^{-1}

Table 1 Partial purification of extracellular cellobiase

Stage		Protein	Cellobiase		
		Total mg (%)	Total Units (%)	Specific activity (U/mg)	Fold purification (X specific activity)
Culture filtrate		1.06 (100)	4.5 (100)	4.24	-
Dialysis	Retentate	0.74 (70)	4.5 (100)	6.08	1.43
	Filtrate	0.32 (30)	-	-	-
Gel filtration Of retentate	Pool I	0.065 (8.8)	1.98 (44)	30.46	7.18
	Pool II	0.116 (15.68)	0.9 (20)	7.75	1.83

Activity staining of culture filtrate cellobiase

In order to substantiate the presence of two bands in sephacryl gel filtration chromatography, the dialysed culture filtrate (retentate) was lyophilised and subjected to activity staining (zymography) with esculin hydrate after separation in a 5% Native PAGE. Two cellobiase activity bands were obtained near the lower half of the gel thus establishing the results of gel filtration chromatography. A parallel lane was coomassie stained; an intense band was obtained at a position corresponding to the second activity band (Figure 4A), however, no detectable protein band was seen corresponding to the first activity band owing to its higher specific activity (Table 1).

SDS PAGE of partially purified cellobiase

In order to determine the approximate molecular weights of the cellobiases corresponding to pool I and pool II, 5 µg protein from pool I and pool II was subjected to 10% SDS PAGE and subsequently silver stained (Figure 4B). Pool I gave three distinct bands corresponding to 130 Kda, 65 kDa and 55 kDa and a faint band near the MW region of 50 kDa. In case of Pool II, there were additional bands near 80, 45, 42 and 30 kDa regions respectively.

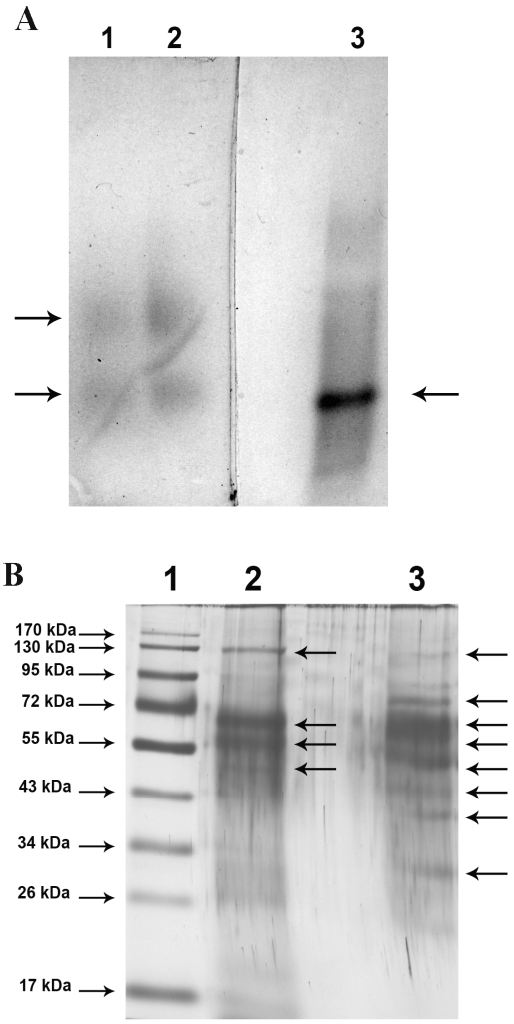

Figure 4 Native PAGE Activity staining and SDS PAGE of cellobiase;Activity staining of culture filtrate cellobiase (lanes 1 and 2) with a parallel coomassie blue stained lane (lane 3) of the same preparation (4A).SDS PAGE of sephacrylcellobiase; pool I (lane 2) and pool II (lane 3) against standard molecular weight marker (4B). Bands are marked by arrowheads in both figures

Determination of kinetic parameters:

Kinetic attributes of cellobiase were elucidated both in its crude form (culture filtrate) and partially purified form (Sephacryl pool I) (Table 2). The crude cellobiase showed a better substrate affinity (0.126 mM) than the partially purified form (0.196 mM). However, due to increase in specific activity, V_{max} of the sephacryl enzyme was increased substantially from 0.8 U/mg to 2.35 U/mg. As a result its catalytic efficiency also increased by 5.64 units.

Table 2: Determination of kinetic parameters

State	Reaction linearity (mins)	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg/mM)
Crude	20	0.126 ± 0.008	0.8 ± 0.006	6.35 ± 0.22
Sephacryl	30	0.196 ± 0.010	2.35 ± 0.12	11.99 ± 0.78

Legend: Values obtained are means of five independent measurements ± S.D.

Substrate specificity:

The crude culture filtrate showed moderate exoglucanase (0.8 U/mg) and endoglucanase (1.6 U/mg) activities apart from cellobiase (2.1 U/mg) as measured with filter paper, CMC and *p*NPG as substrates respectively. However it was devoid of any activity against *o*NPG, methyl α-D-galactopyranoside, starch, maltose, sucrose and trehalose. The partially purified enzyme retained activity only against *p*NPG (30.46 U/mg) and cellobiose (38 U/mg).

Effect of temperature and pH

Thermal stability and resistance to pH changes are two of the most important criteria for industrial efficacy determination. Accordingly, both the crude and sephacryl forms of the enzyme were assessed for their temperature and pH dependence. The crude enzyme showed optimal activity at 60°C (Figure 5A). It was completely stable upto 55°C and retained more than 50% activity at 60°C (Figure 5B). Thereafter, the enzyme activity decreased drastically. In comparison, the sephacryl enzyme (pool I) was more thermostable and retained 64% activity at 65°C and 40% activity at 70°C (Figure 5B). It was also found to possess higher temperature optima of 75°C (Figure 5A). This indicated that at a crude state some associated enzyme/factor was responsible for the enhanced thermal denaturation of the enzyme. Both the enzyme forms were optimally active at pH 5 (Figure 5C) which indicated that a protonated state of the active site was vital for catalysis. However, the pH stability of the crude enzyme was however found to be higher than the sephacryl form (Fig 5D). It was stable over a broad pH range of 4 to 9 and retained close to 75% activity at pH 3.5 and pH 10 respectively. In contrast, the sephacryl enzyme was tolerant to a confined pH range of 5 to 8 and showed close to 65% activity at pH4.5 and 8.5 respectively.

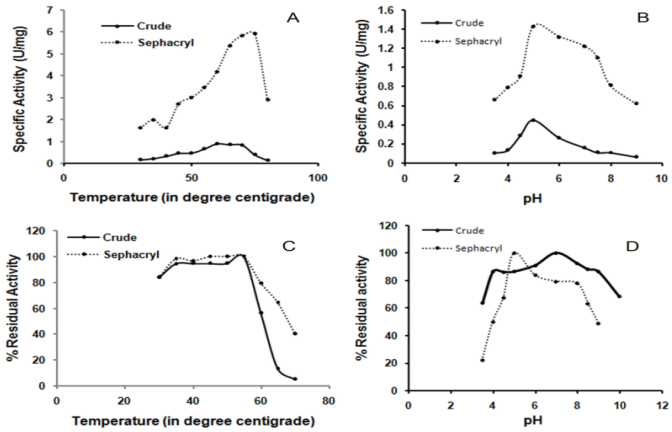

Figure 5 Temperature optima (5A) and temperature stability (5B), pH optima (5C) and pH stability (5D) of the crude and sephacryl cellobiase. Three independent sets of assays were performed in duplicates and mean values were plotted

Effect of chaotropes, reducing agents and detergents

In industrial formulations, substrates often undergo chemical pretreatments before actual enzymatic conversion. However, most of these are protein denaturants and their traces frequently remain in the reaction mixture exposing the enzymes to harsh conditions. Therefore for viable industrial candidature, an enzyme must be tolerant to low to moderate concentrations of these reagents. In order to investigate the effect of these denaturants, the cellobiase preparation from both crude and sephacryl stage were incubated with varying concentrations of the denaturants before assessing residual enzyme activity in presence of the denaturants. Both the crude and sephacryl forms were found to moderately stable to the destabilising effect of Guanidium Hydrochloride (GdHcl), a potent chaotrope and retained close to 70% activity in presence of 100 mMGdHcl (Figure 6A). The enzyme was however found to be significantly resistant to urea, a weaker chaotrope in comparison to GdHcl, and retained about 75% activity in presence of 1 M urea (Figure 6B). The effect of an anionic detergent and a strong protein denaturant, SDS, was also determined. The crude form retained close to 70% activity in presence of 0.2% SDS. Enzyme activity of the sephacryl form was found to increase by an additional 34% in presence of 0.1% SDS. However, it decreased drastically to 50% of original activity in presence of 0.2% SDS (Figure 6C). Reducing agents like dithiothreitol (DTT) and β-mercaptoethanol are known to perturb proteins' structure/activity by breaking disulfide bonds at or near the vicinity of structurally important domains and/or active sites. In contrast to this general notion, DTT significantly boosted activity of cellobiase. In presence of 50 mM DTT, activity of the crude cellobiase increased slightly by 13% and the enzyme retained about 80% activity when DTT concentration was increased to 250 mM. The sephacryl enzyme however observed an unprecedented rise in activity to twice of its original in presence of 20 mM DTT. The enzyme was also found to completely stable and active upto a concentration of 200 mM DTT (Figure 6D). Similar observations were found in presence of β-mercaptoethanol which indicated that the increase in activity probably owed some structural changes in the enzyme due to reduction of disulphide bonds (data not shown).

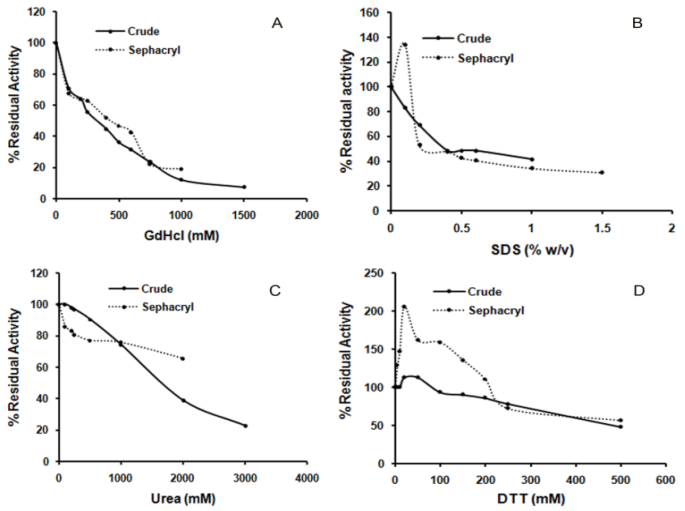

Figure 6 Effect of GdHcl (6A), Urea (6B), SDS (6C) and DTT (6D) on the stability of crude and sephacryl cellobiase of BF02. Three independent sets of assays were performed in duplicates and mean values were plotted

Effect of metal ions and glucose

Presence of metal ions may be either beneficial or detrimental for enzyme's activity. In order to screen the potential metal activators and inhibitors, effect of six different metal ions along with a chelator EDTA on cellobiase activity was investigated. In presence of the divalent cataions Mg^{+2}, Cu^{+2} and Zn^{+2}, cellobiase activity was partially increased (19%, 18% and 9% respectively). However in presence of Hg^{+2}, enzyme activity was significantly curbed by about 50%. In presence of Kcl and EDTA, activity was unaffected (Figure 7A). In case of the sephacryl enzyme, Zn^{+2} and Cu^{+2} inhibited enzyme activity by 24% and 14% respectively. K^{+} and EDTA on the contrary boosted enzyme activity by 17% and 15% respectively. Similar trends as those observed for the crude enzyme were seen in case Mg^{+2} and Hg^{+2}. Additionally, Mn^{+2} increased the activity of the sephacryl sample by 14% (Figure 7B). However, its effect on the crude enzyme could not be studied due to some interference in the assay.

Feedback inhibition by product often is a major cause of decline in enzyme activity. This effect is especially applicable for those enzymes which generate glucose as the end product. Therefore, glucose inhibition is considered as a serious bottleneck in increasing the performance of cellulolytic and amylolytic enzymes. Accordingly, effect of extraneously added glucose was added in enzyme reaction mixture along with the substrate. The culture filtrate was exhaustively dialysed and checked for presence of any residual free glucose which would lead to false interferences. The crude enzyme was found to retain almost complete activity upto a 1% w/v glucose concentration in the assay mix. However, it significantly went down to 40% of initial activity in presence of 2% glucose. The sephacryl enzyme, however, was found to be less tolerant to the inhibitory action of glucose and 82% activity was retained in presence of 0.5% glucose. Subsequently, activity loss was drastic and the enzyme retained only 46% activity in presence of 1% glucose (Figure 7C).

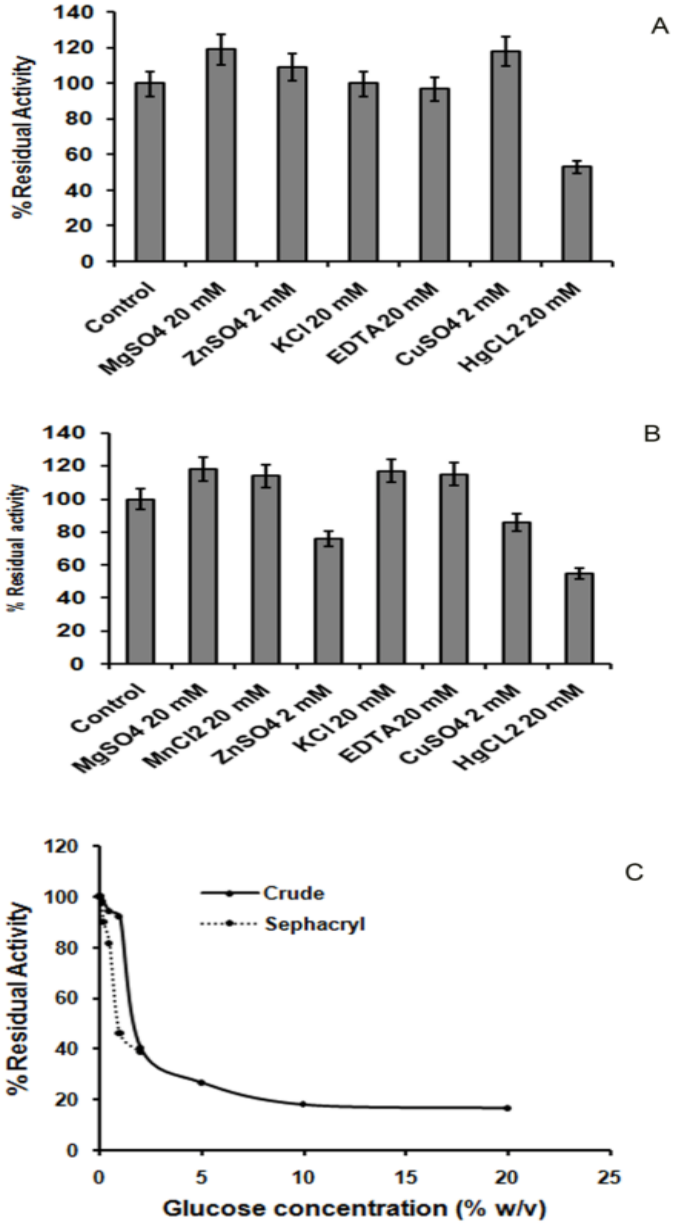

Figure 7 Effect of metal ions on crude (7A) and sephacryl (7B) cellobiase and effect of glucose inhibition (7C) on cellobiase catalysis. Three independent sets of assays were performed in duplicates and mean values were plotted.

Cellobiases from filamentous fungi are secreted in the culture medium as homoaggregates **(Roy *et al.*, 1994)** or in large heteroaggregates along with other glycosidases **(Mukherjee *et al.*, 2001)**. In most cases this aggregation results in enhanced stability and activity of the enzyme **(Mukhopadhyay *et al.*, 2003; Saha *et al.*, 2002: Mukherjee *et al.*, 2001)**. Aggregation with other enzymes also plays a pertinent role in guiding the secretion of cellobiases **(Mukherjee and Khowala, 2002)**. However, it might hamper purification of the enzyme subunits. In the Basidiomycota *Termitomyces clypeatus*, two cellobiase peaks were obtained on subjecting the culture filtrate to gel filtration and Native Page and they were identified to be a larger and smaller aggregate of the same enzyme **(Banik *et al.*, 2012)**. In the present studies also, extracellular cellobiase yielded two active fractions in gel filtration and Native PAGE. However, no other enzyme activity was found to be associated with either of the peaks. Molecular weight of cellobiases from different filamentous fungi has been found to vary between 70 kDa and 120 kDa **(Abdel-Naby *et al.*, 1999; Chauve *et al.*, 2010; Banik *et al.*, 2011)** with more than one cellobiase band detectable in a single organism **(Riou *et al.*, 1998)**. However, contrasting reports have been available regarding their nature; some had suggested that they are different isoforms **(Abdel-Naby *et al.*, 1999)** whereas others have evidenced that they are different aggregated forms of the same enzyme **(Pal *et al.*, 2010)**. In the present study also, similar molecular weights for the partially purified cellobiase were found. It will be worthwhile to explore the properties of the second activity peak along with

immunoblotting and amino acid analysis of the two peaks to identify whether they belong to same or different enzyme.

Catalytic efficiency of cellobiase is of pivotal importance in governing its industrial candidature. Cellobiase is an inhibitor of both endo- and exo-glucanases, and it must be removed to allow efficient and complete saccharification of cellulose **(Bhat and Bhat, 1997)** by increasing the rate of cellulose hydrolysis **(Barnett *et al.*, 1991)**. Under the circumstances, a low K_m cellobiase is of extreme suitability for industrial processes. Most of the strains in current industrial usage derive from *Trichoderma ressei* and the K_m values (*p*NPG as substrate) of cellobiases from those strains range between 0.182 mM to 0.102 mM **(Chauve *et al.*, 2010)**. Other commercial cellobiases used in industries are derived from *Aspergillus niger* range between 0.46 mM to 0.64 mM. Recently, an efficient underglycosylated cellobiase has been reported from *Termitomyces clypeatus* with a K_m of 0.187 mM. In this respect, cellobiase from the *Penicillium chrysogenum* in the present study seems to be a prospective industrial candidate with K_m of the crude enzyme being 0.126 mM and that of the sephacryl preparation being 0.196 mM. Glucose inhibition is a common bottleneck in improving catalytic efficiency of cellobiase. However, high glucose tolerant cellobiases have been reported from *Aspergillus niger* **(Riou *et al.*, 1998)**. Glucose tolerance of the cellobiase in the present study was found to be moderate. However, further kinetic studies in presence of glucose needs to be carried out using both cellobiose and *p*NPG individually as substrates to determine the K_i values.

Cellulose pretreatment during saccharification involves steam circulation to make the substrates more suitable for enzymatic hydrolysis **(Liu *et al.*, 2011)**. Under the circumstances, thermostable enzymes can be used simultaneously and directly in the saccharification procedures as they provide the following clear cut advantages; 1) Lower production costs due to cut down of a precooling step; 2) Temperature induced decreased fluid viscosity facilitating mass transfer 3) Reduction of contamination risks 4) Easier volatilization of products such as ethanol at higher temperatures and 6) Higher shelf life and room temperature purification and storage of the enzymes. In both thermophilic bacteria and fungi, cellobiase is the thermostability-limiting enzyme of the cellulolytic system **(Fahnrich and Irrgang, 1982; Aguirre *et al.*, 1986)**. Therefore, obtaining an efficient and thermostable β-glucosidase has become a universally sought after goal. Thermostable cellobiases have been reported by few researchers **(Woloswoska *et al.*, 2004; Liu *et al.*, 2012)**. A high thermostability of the *Penicillium* cellobiase therefore seems to be a promising alternative for the industry.

Reducing agents like DTT were found to significantly increase cellobiase activity. This trend was newly observed for cellobiases where in a separate study with the extracellular cellobiase of *Termitomyces clypeatus*, DTT was found to decrease the K_m of the enzyme suggesting that reduction of disulphides at or near the vicinity of the active site may allow better substrate exposure for these enzyme aggregates **(Banik *et al.*, unpublished results)**. The enzyme was found to be moderately stable in presence of other potent chaotropes like GdHcl and urea which act by disrupting the hydrogen bonding network within water molecules. In order to attain stabilization along the solvent exposed interfaces, proteins try to maximize the presentation of their hydrophilic surface **(Moelbert *et al.*, 2004)**. This leads to their gradual unfolding and subsequent departure from the native state. The extent of resistance showed by the cellobiase was probably attributable to its native aggregated state so that the active dissociated smaller units were still able to present a considerable amount of hydrophilic surface. Similar observations are known from other cellobiases **(Banik *et al.*, 2012);** however, for the present study, this needs to be substantiated by other experiments such as determination of aggregation status and study of chaotrope induced unfolding dynamics. The effect of enzyme aggregation was also revealed on SDS treatment. The anionic detergent caused an initial increase in activity which might be accounted for by the opening up of the aggregated sephacryl enzyme for better substrate exposure. Increasing SDS concentration however perturbed the enzyme structure significantly thus diminishing its activity.

Various metal cations and potential inhibitors modified the activity of the enzyme. Zn^{+2}, Hg^{+2} and Cu^{+2} was found to inhibit activity of the partially purified enzyme which was in accordance with other such reports from cellobiases **(Riou *et al.*, 1998; Ghorai *et al.*, 2010)**. However, in case of the culture filtrate enzyme, activity was unaffected in presence of Zn^{+2} and Cu^{+2}. This may be accounted for by the presence of some shielding agent in the culture filtrate which sequestered these metal ions effectively. Mg^{+2} and Mn^{+2} were found to boost enzyme activity which is also a known feature of these fungal cellobiases **(Riou *et al.*, 1998; Liu *et al.*, 2012)**. However, the chelating agent EDTA did not inhibit cellobiase activity, indicating that divalent cations were not absolutely essential for enzyme activity.

The culture filtrate of *P. chrysogenum* BF02 exhibited significant antibacterial effects against gram positive organisms. *S. aureus* and *B. subtilis*. This is consistent with the fact that most strains of *Penicillium chrysogenum* produce β-lactam antibiotics, mainly Penicillins **(de Hooge*t al.*, 2000)**. The fact that the antibacterial effect is comparable to that obtained with ampicillin, a derivatized penicillin and a broad spectrum antibiotic streptomycin) indicate the prospect of this strain as potential source of commercial antibiotics. Strong antibacterial activity was also an added advantage with respect to industrial usability of the crude culture filtrate since fermentation processes often suffer from the risk of bacterial contamination. These have been extensively reviewed by Brethauer **(Brethauer and Wyman, 2010)**. Therefore, development of a self-sufficient formulation as provided by *Penicillium chrysogenum* BF02 is of utmost significance for the biotechnology industry.

CONCLUSION

Strains of *Trichoderma* and *Aspergillus* have dominated the world market for supply of cellulolytic enzymes. However, to minimise production bottleneck with least economical burden, there has been a continuous strive for the discovery of new multipotent strains which can supply a steady source of these enzymes, especially cellobiase to the industry. Under the circumstances, the identification of a new *Penicillium chrysogenum* strain with a catalytically superior and thermostable cellobiase garnished with strong antibacterial activity is believed to be a promising alternative to the industry.

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