Advances in Bioscience and Biotechnology, 2015, 6, 213-222
Published Online March 2015 in SciRes. http://www.scirp.org/journal/abb
http://dx.doi.org/10.4236/abb.2015.63021

Molecular Cloning and Expression of a Family 6 Cellobiohydrolase Gene *cbhII* from *Penicillium funiculosum* NCL1

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Received 10 March 2015; accepted 24 March 2015; published 30 March 2015

Abstract

Aim: Lignocelluloytic enzymes are the largest class of hydrolase enzyme which utilizes the plant biomass to produce renewable sources. Hence practices for larger production of these enzymes at lower cost received much attention for industrial use. Hence this paper deals with expression and purification of cellobiohydrolase gene from *Penicillium funiculosum* NCL1. Methods & Results: A cellobiohydrolase gene, *cbhII* of *Penicillium funiculosum* NCL1 was cloned and expressed in *Pichia pastoris* X33. Two exons of the *cbhII* gene were amplified separately and fused by overlap extension PCR. The fused product was cloned in yeast expression vector pPICZ*α*A and expressed in *P. pastoris* under the control of the AOX1 promoter. *P. pastoris* transformants expressing recombinant cellobiohydrolase were selected on CMC agar plate and their ability to produce the cellobiohydrolase was evaluated in flask cultures. *P. pastoris* X33 (pPICbh6) efficiently secreted the recombinant cellobiohydrolase into the medium and produced the cellobiohydrolase activity (5 U/ml) after 96 h of growth. The recombinant cellobiohydrolase produced by *P. pastoris* (pPICBH6) showed maximum activity at pH 4.0 and temperature 50˚C and higher specificity in hydrolysis of filter-paper.

Keywords

***Penicillium funiculosum*, Cellulase, Cellobiohydrolase, Molecular Cloning, Affinity Chromatography, *P. pastoris*, Exon Fusion**

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How to cite this paper: Chinnathambi, V., Balasubramanium, M., Gurusamy, R. and Paramasamy, G. (2015) Molecular Cloning and Expression of a Family 6 Cellobiohydrolase Gene *cbhII* from *Penicillium funiculosum* NCL1. *Advances in Bioscience and Biotechnology*, **6**, 213-222. http://dx.doi.org/10.4236/abb.2015.63021

1. Introduction

Lignocellulosic biomass is an important source of the renewable energy for production of bioethanol. Cellulose and hemicelluloses can be hydrolyzed into glucose. Cellulase is an enzyme complex capable of hydrolyzing cellulose into glucose molecules. The complete degradation of cellulose to glucose requires the action of at least three types of enzymes: Endo-*β*-1, 4-glucanase, Exo-*β*-1, 4-glucanase (cellobiohydrolase) and *β*-glucosidase [1]. Cellobiohydrolase is the essential component of the cellulase system to hydrolyze cellulose, consisting of both crystalline and amorphous cellulose. However, fungi are the most studied organisms because of their higher yields and capacities to produce complete cellulase complex. Recently, research has been focused on microbial cellulases due to the large scale production in the industries and their use in biomass degradation biomass for several industrial applications including biofuel production [2] [3]. The most abundant enzymes are two cellobiohydrolase, Cel7A and Cel6A, also called CBHI and CBHII, respectively. These are also the most efficient enzymes on highly crystalline cellulose [4]. Cellulases have proved to be commercially useful in the textile industry, substituting conventional stonewash methods (biostoning).The fungus *Penicillium funiculosum* is a filamentous fungus an efficient producer of cellulase [5] [6]. The *P. funiculosum* have been reported to produce high amount cellulases such as CMCase (13 - 15 U/ml), pNPGase (10 - 12 U/ml) and cellobiase (7.5 U/ml). Genes coding cellobiohydrolase GH6 have been cloned and characterized from a variety of fungal sources, including *Trichoderma reesei* [7], *Chaetomium thermophilum* [8], *Irpex lacteus* MC-2 [9], *Chaetomium thermophilum* [10]. This is the first report on the expression of cbhII from *Penicillium funiculosum* in *pichia pastoris*. In the present study, we report the cloning and expression of *cbhII* gene in pichia expression system.

2. Materials and Methods

2.1. Strains, Reagents and Culture Media

Penicillium funiculosum NCL1 was obtained from National Chemical Laboratory, Pune, India. Stock cultures were kept on potato dextrose agar and subcultured monthly. *P. funiculosum*NCL1 spores were inoculated in Reese Basal medium as described by [11] at a final concentration of 10^8 spores/ml. Flasks were incubated in an orbital shaker (220 rpm) at 30˚C for 96 h. The mycelia were recovered by filtration on a nylonfilter (30 µm spore) washed with 0.9% (w/v) NaCl and dried by pressing between two filter papers. *Escherichia coli* DH5*α* was used as a host for plasmid propagation. *P. pastoris* X33 (Invitrogen, USA) was used as a host for expression of the recombinant *cbhII*.

2.2. PCR

PCRs were performed in a PTC-200 programmable thermal cycler (MJ Research, Massachusetts, USA) with one cycle of 94˚C for 5 min followed by 35 cycles of denaturation (60 s at 94˚C), annealing (60 s at 50˚C - 60˚C) and extension (60 s at 72˚C), with a final extension of 72˚C for 10 min. For analysis, 10 µl of reaction product was electrophoresed on a 1% agarose gel and stained with ethidium bromide (5 µg/ml). Primers used in this study are listed in **Table 1**.

Table 1. Oligonucleotide primers used in this study.

Primer name	Sequence (5'-3')	Length (bp)
ORFCBH6F	CCTAGCTAGCATGTTGCGATATCTTTCCATCGTTG	35
ORFCBH6R	TCTTAGGCCGGCCCTAGACCAAAGCTGGGTTGGCA	35
CBH6F	CCNGAYCGYGAYTGYGCYGC	20
CBH6R	TKRTARTTGCYRTCRAANAGCA	22
Cbh6e12R	TACCAGGGATACATTGTGCGTAGTAAGGGTTTAGAGTGCTGCA	43
Cbh6e345F	ACGTGCAGCACTCTAAACCCTTACTACGCACAATGTATCC	40
pPICBH6F	CCTAGCTAGCATGTTGCGATATCTTTCCATCGTTG	35
pPICBH6R	CATGCGGCCGCTACAAACATTGAGAGTAGTAAGGGT	36

2.3. Cloning of CBHII Cellobiohydrolase

Genomic DNA of *P. funiculosum* NCL1 was isolated from mycelia by the method of Murray and Thompson. DNA from agarose gel was also extracted using Prefect prep gel extraction column (Eppendorf, Germany) according to the manufacturer's instructions. Exon I of *cbhII* gene was amplified with overlapping primer sets [Cbh6e345F-ORFCBH6R] and [ORFCBH6F-Cbh6E12R]. Both amplified exon I and exon II were purified and mixed in 1:1 molar ratio. These fragments were joined by the second round of PCR with no primers. Final round of PCR was made with ORF specific primers (ORFCBH6F and ORFCBH6R) specific for *cbh6* gene. On the basis of the nucleotide sequence of cbh6 primers were designed to facilitate further cloning into pPICZαA vector. The fusion product cbh6 with its own signal sequence was digested with *EcoRI* and *NotI* and cloned into pPICZαA, the resulting plasmid pPICbh6 which was linearized by digestion with *SalI* to facilitate integration via homologous recombination at the AOX1 locus in *P. pastoris* X33 strain (Invitrogen, California, USA).

2.4. Transformation of *P. pastoris*

P. pastoris X33 was grown overnight (30˚C at 250 rpm) in YPD broth (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose) and transformed with the recombinant construct, according to the manufacturer's instructions (Invitrogen, California, USA). *P. pastoris* X33 (mut+) was transformed with linearized construct (pPICbh6) by electroporation [BTX (ECM399), Germany]. Transformed cells were selected on YPD agar medium Supplemented with 2 M sorbitol and zeocin (100 µg/ml).

2.5. Expression of Cellobiohydrolase in Recombinant *P. pastoris*

The yeast culture medium (BMG medium) consisted of (g/l) 13.4 g of YNB, 4×10^{-4} g of Biotin, 5 ml of glycerol and 100 mM phosphate buffer pH 6.0. *P. pastoris* was grown in BMG medium in an orbital shaker (250 rpm) at 30˚C to an OD_{600} nm of 1.3 to 1.6. The cells were harvested by centrifugation 1500 × g for 10 min and the pellet was resuspended in 25 ml of BMMY medium (BMG medium in which glycerol was replaced by methanol (5 ml/l) and further supplemented with yeast extract 10 g/l and peptone 20 g/l in 250 ml of Erlenmeyer flasks kept at the similar conditions. Methanol (0.5%) was fed to the culture every 24 h for induction and samples were withdrawn at intervals. The cells were removed by centrifugation (10,000 × g for 10 min) and the supernatant was assayed for cellobiohydrolase activity.

2.6. Characterization of Cellobiohydrolase

To determine the specificity of the recombinant cellobiohydrolase, the enzyme was assayed with different substrates (Sigma, St Louis, MO, USA) such as avicel, CMC, pNPG and xylan. Reducing sugars were measured by the 3, 5-dinitrosalicylic acid method with glucose and as the standard. The activities were compared with the highest one obtained for any of these substrates. The effect of pH on the cellobiohydrolase activity was determined by measuring the relative activity using sodium acetate (pH 5.0 - 5.5), sodium phosphate (pH 6.0 - 8.5) and sodium carbonate (pH 9.0 - 9.5) buffers. The maximum activity was considered as 100%, and used as reference in determining relative activities at different pH values. The effect of temperature on the reaction rate was determined by performing the standard reaction at different temperatures in the range of 30˚C - 90˚C.

The stability of the cellobiohydrolase as a function of pH was determined by measuring the residual cellobiohydrolase activity after incubation of the enzyme for 1 h at different pH at 30 ˚C. The relative activity was expressed considering the activity before incubation as 100%. Thermostability of cellobiohydrolase was determined by incubating the enzyme extract at different temperatures (30˚C - 90˚C) for 30 min. Protein was estimated according to the method of [12] using bovine serum albumin as the standard.

2.7. Sequence Analysis

Nucleotide and deduced amino acid sequences were analyzed with the sequence analysis tools. Signal peptide sequence was analyzed by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP). Related sequences were obtained from the databases using the software BLAST. Phylogenetic analyses were performed in MEGA 2.1, using the minimum evolution (ME) approach. GENSCAN online tool (www.genes.mit.edu/GENSCAN.html) was used for identification of gene features such as exons and splice sites in genomic DNA. Bio Edit (version

7.0.4.1) was used for sequence editing and analysis.

3. Results

Majority of cellobiohydrolase belong to the two families, GH6 and GH7 glycosyl hydrolases. To clone *cbhII* cellobiohydrolase, degenerate primers were designed (**Table 1**) based on conserved catalytic domain regions of known GH6 cellobiohydrolases of *Penicillium* species. Using *P. funiculosum* NCL1 genomic DNA as template, PCR was performed with degenerate primers Cbh6F & Cbh6R and to amplify *cbhII* cellobiohydrolase gene. The resulted amplicon of length 900 bp was cloned and sequenced. The partial cellobiohydrolase gene sequences were BLAST with the sequences of other fungal cellobiohydrolase in the GenBank and EMBL databases sequences. From the retrieved sequences, the ORF for family GH6 (*cbhII*) cellobiohydrolases were predicted and amplified. Sequencing of cellobiohydrolase *cbhII* gene from *P. funiculosum* NCL1 showed 100% identity towards sequences of the *A. cellulolyticus* Y-94 Acc2 gene. Based on comparison of this sequence with *A. cellulolyticus* Y-94 Acc2 gene *cbh*6 of *P. funiculosum* was found to have 5 introns.

Further, to amplify the full length cDNA of *cbhII* gene sequence, RT-PCR was done. First strand cDNA was synthesized using total RNA isolated from avicel grown culture. Using this first strand cDNA as template, PCR was performed with primers (ORFCBH6F & ORFCBH6R) specific for *cbh6* gene. An expected amplicon of 1.4 kb cDNA was obtained. The product was cloned and sequenced. The cDNA sequence showed identity towards cellobiohydrolase (Acc2) of *A. cellulolyticus* Y-94. The alignment of cDNA and genomic DNA sequence of *P. funiculosum cbh6* showed that the second intron was not spliced. As the cbh6 cDNA of *P. funiculosum* has one intron, attempt was made to fuse the exons of *cbh*6 gene. Primers were designed to amplify the two exons separately. Fusion primers were designed to fuse the exons. First round of PCR were performed with overlapping primer sets [Cbh6e345F-ORFCBH6R] and [ORFCBH6F-Cbh6E12R] which resulted in amplicons of 1.2 kb and 200 bp respectively. The second round of overlap extentsion PCR was performed without exon-primers. Final round of PCR was made with ORF specific primers (ORFCBH6F and ORFCBH6R) specific for *cbh6* gene which resulted in an expected amplicon of 1.4 kb. The *cbhII* gene sequence was submitted to GenBank accession number (FJ000002).

3.1. Nucleotide Sequence Analysis of the *cbhII* Gene

The *cbhII* gene consisted of an ORF 1700 bp. Five introns with an average length of 55 bp were identified in the *cbhII* gene based on alignment with other cellobiohydrolase genes belonging to family GH6 and also with predicted cDNA obtained using GENSCAN software. The size of introns resembled those of other filamentous fungal introns and varied between 55 and 76 bp. Conserved domain search (RPSBLAST) analysis confirmed the presence of catalytic domain of GH6 cellobiohydrolase. Deduced aminoacid sequence of *cbhII* was compared to other characterized GH6 cellobiohydrolases. The sequence of GH6 exhibited maximum identity with *A. cellulolytics* Y-94, 100% and followed by *A. terreus* 46%, *N. fischeri* NRRL 18.156%, *A. fumigatus* Af293 cellobiohydrolase 51%, *S. sclerotiorum* −33%, *A. niger* −39%, *T. ressei* (cbhII) 43%, *T. viride* cellobiohydrolase (cbhII) −43%, *Hyocrea koningi* −38%. The protein comprises of 529 residues including the signal peptide (**Figure 1**). The putative signal peptide was predicted with SignalP 3.0 Server and the most likely cleavage site is between position 20^{th} and 21^{st} aminoacid. The mature protein comprises of 422 residues, with a calculated molecular mass of 47.7 kDa and a theoretical pI of 4.62. Phylogenetic analysis revealed that *cbhII* from *P. funiculosum* formed a separate cluster and is closely related *A. cellulolyticus* Y-94, *P. marneffei* ATCC 18224, *Talaromyces stipitatus* ATCC 10500 (**Figure 2**).

3.2. Expression of *cbhII* of *P. funiculosum* NCL1 in *P. pastoris*

The *cbhII* gene without signal sequence was amplified using primers pPICbh6F and pPICbh6R from the genomic DNA of *P. funiculosum* NCL and cloned at *EcoRI* and *NotI* site of expression vector pPICZαA in order to make in frame fusion of the *cbhII* gene with the α-factor secretion signal in the vector. Thus generated recombinant plasmid pPICbh6 was confirmed with PCR and restriction analyses. The linearized recombinant plasmid pPICbh6 was transferred into *P. pastoris* X33 for integration by single crossover recombination. A total of 25 transformants were randomly picked and grown on YPD agar containing 1% avicel and zeocin (100 mg/ml). After a 72 h of growth, three recombinant colonies which produced large zone of hydrolysis were chosen for

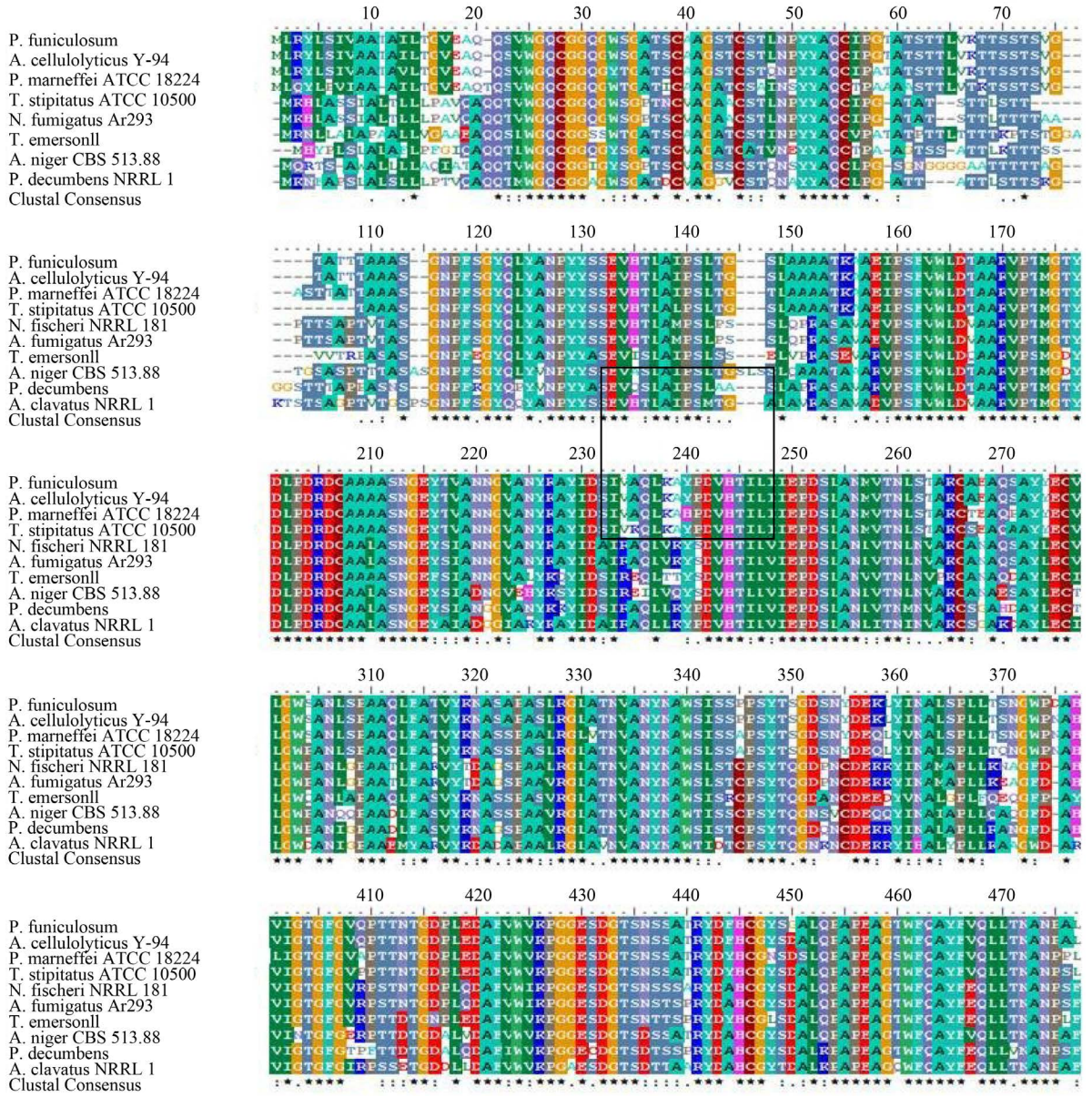

Figure 1. Comparison of GH family 6 cellobiohydrolase with cbh6 of *P.funiculosum*. Multiple alignments were obtained using ClustalX in Phylip format by the neighbor-joining method. The amino acid residues of are numbered; conserved regions are boxed; "*"—invariant residues; ":"—similar amino acids; "."—less similar amino acids. *A. cellulolytics* Y-94, *A. terreus*, *N. fischeri* NRRL 181, *A. fumigatus* Af293 cellobiohydrolase, *S. sclerotiorum*, *A. niger*, *T. ressei* (cbhII), *T. viride* cellobiohydrolase (cbhII), *Hyocrea koningi.*

further study. The selected clones were grown at 30˚C and induced with methanol in BMMY medium. The cellobiohydrolase production was determined every 24 h. The recombinant yeast strains produced 2 - 6 U/ml of cellobiohydrolase in the culture medium while one of the recombinant strains, PIC2 produced maximum cellobiohydrolase activity of 5.5 U/ml (**Figure 3**). The kinetics of cellobiohydrolase production by the transformant is shown in (**Figure 4**). The specific activity of the recombinant cellobiohydrolase was 0.8 U/mg. SDS PAGE analysis of culture supernatant showed expected protein band of 47.7 kDa (**Figure 5**).

3.3. Characterization of Recombinant Cellobiohydrolase

The activity profile of cellobiohydrolase in terms of pH was determined in different buffers (pH3.0 - 9.0). The

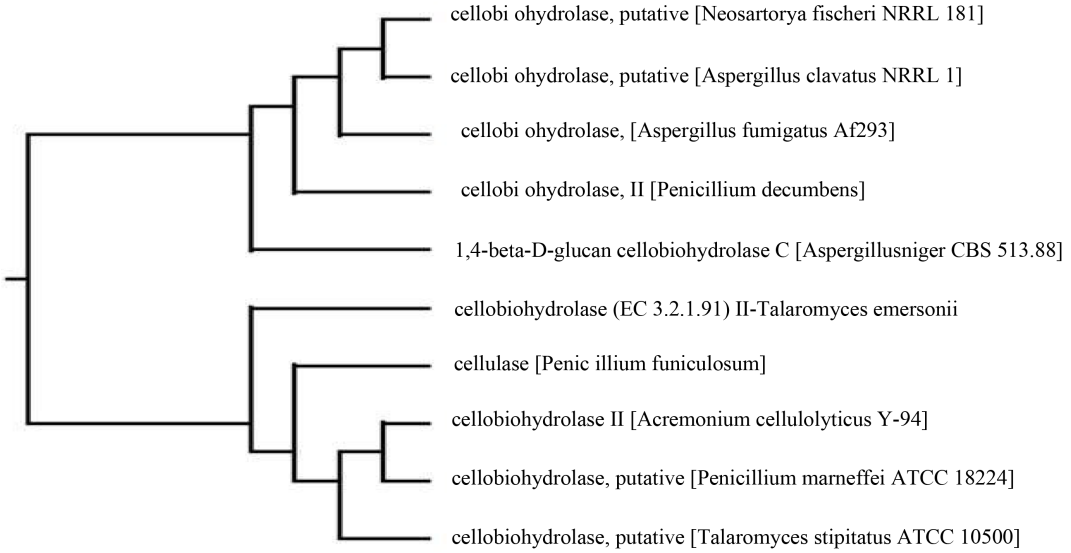

Figure 2. Phylogenetic analysis of cbh6 from *P. funiculosum* NCL1. The sequences of GH6 cellobiohydrolases were used to access their phylogenetic relationship. The phylogram was generated with ClustalW in FASTA format using the neighbor-joining method and displayed in tree view.

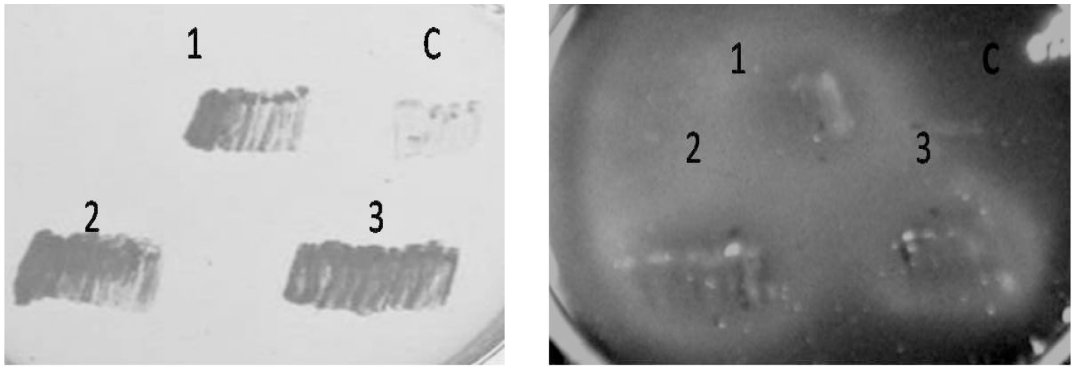

Figure 3. Expression of cellobiohydrolase in *P. pastoris* (pPICbh6). *P. pastoris* transformants were patched on minimal methanol agar medium with 0.5% Avicel. Methanol 100 ml was added to the lid of the inverted plate every 24 h. After 72 h, the plate was overlaid with 1% Congo red. Then destained with 1 M NaCl after 20 min and the zone of clearance was observed (**C**—control, **1**—pPIC1, **2**—pPIC2, **3**—pPIC3).

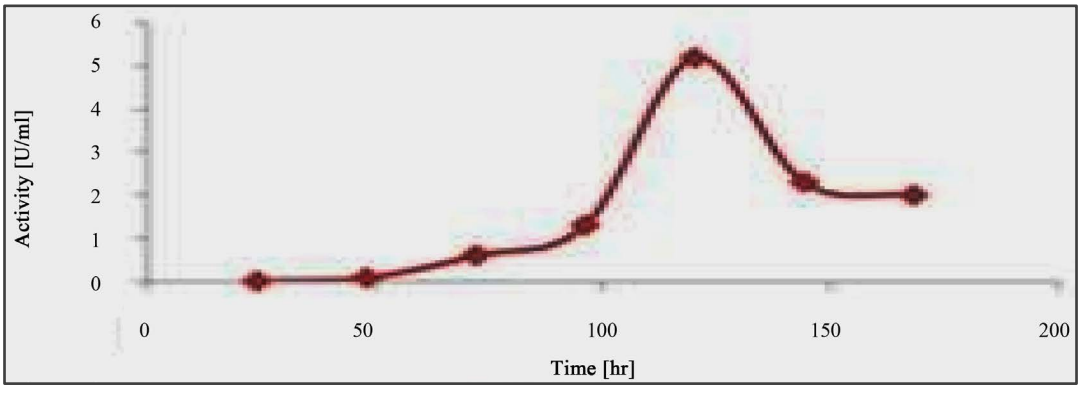

Figure 4. Kinetics of cellobiohydrolase production by recombinant *P. pastoris* (pPICbh6). *P. pastoris* (pPICbh6) was grown in minimal medium with 0.5% methanol. Samples were taken at 24 h intervals and assayed for cellobiohydrolase activity in the culture supernatant.

pH optimum for *P. pastoris* (pPICBH6) cellobiohydrolase was 5.6 and it showed 85% - 95% of relative activity at pH 3.0 to 5.2. When preincubated for 30 min in different buffers pH 3.0 to 5.0 at 30˚C. CBHII exhibited maximum activity at pH 5.0 (**Figure 6(a)**). When preincubated in different buffers for 1 h at 30˚C, cellobiohydrolase was 100% stable but the enzyme lost its activity at pH 8. Thus, the optimal pH for both cellobiohydrolases *P. pastoris* (pPICbh6) was 3.0 - 6.0 without much loss of activity. The *cbhII* higher specificity in hydrolysis of filter-paper.

4. Discussion

Partial cellobiohydrolase genes of *P. funiculosum* NCL1 900 bp for GH6 gene were amplified by PCR using degenerate primers. The partial cellobiohydrolase gene sequences were BLAST with the sequences of other fungal cellobiohydrolase in the GenBank and EMBL databases sequences. From the retrieved sequences, the ORF for family GH6 (*cbhII*) cellobiohydrolases were predicted and amplified. Sequenced cellobiohydrolase (*cbhII*) gene from *P. funiculosum* NCL1 showed 100% identity towards sequences of the *A. cellulolyticus* Y-94

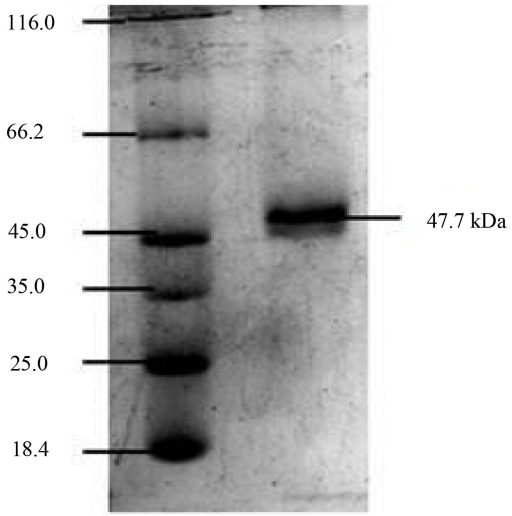

Figure 5. SDS-PAGE of the purified cellobiohydrolase of *P. pastoris* (pPICbh6). Purified cellobiohydrolase was resolved on a 12% SDS-PAGE and stained with coomassie brilliant blue R-250. Lane 1—purified cellobiohydrolase (coomassie blue stained), Lane M—Molecular weight marker (116.6 - 18.4 kDa).

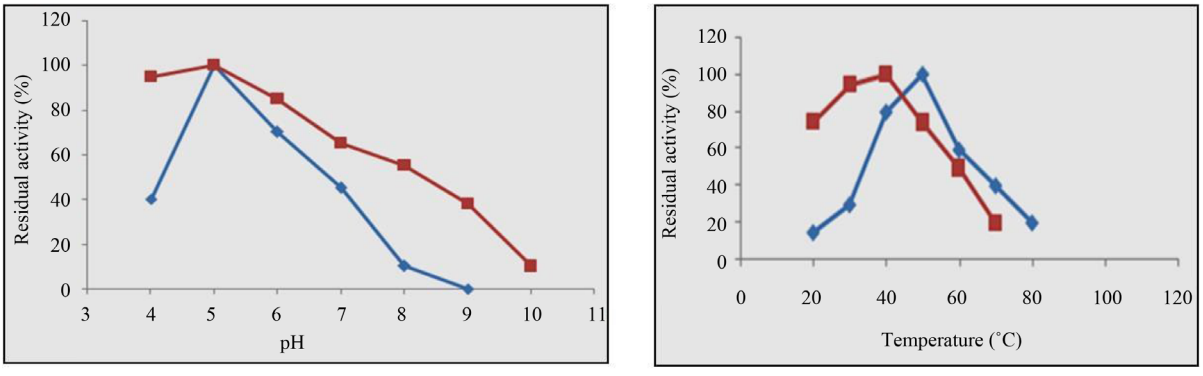

Figure 6. Effect of pH and temperature on the activity of purified cellobiohydrolase from recombinant *P. pastoris* (pPICbh6). (a) Cellobiohydrolase activity was determined in different buffers (pH 3.0 - 10.0) at 50˚C. The stability of the enzyme at different pH levels was determined by incubating the enzyme in different buffers for 1 h at 50˚C and the residual activity was measured at pH 4.8, 50˚C; (b) The cellobiohydrolase activity was measured at different temperatures (30˚C - 80˚C) at pH 4.8 the purified enzyme was preincubated at different temperatures for 30 min and the residual activity was measured at pH 4.8 and 50˚C. Relative activity was calculated by considering the maximum activity as 100%.

Acc2 gene. The *cbh6* gene consists of 1700 bp including five introns 71, 59, 71, 76 and 56 bp with consensus 5'and 3' intron splice sites. Toda *et al*. reported that cbhII from *Irpex lacteus* MC-2 is interrupted by eight small introns 53 - 59 bp in size [13]. The coding regions of both cbhI.2 of *P. chrysosporium* are interrupted by two introns [14]. Though a *cbhII* gene (FJ000002) showed about 100% identity to *A. cellulolyticus* Y-94 Acc2 gene, there is no study on the functional characterization of this cellobiohydrolase. Therefore, attempt was made to express this gene in *P. pastoris* and characterize the recombinant cellobiohydrolase.

4.1. Conserved Catalytic Residues Are in *cbhII*

CbhII multi domain structure composed of cellulose binding domain (CBD), a Ser/Thr/Pro-rich linker, and a family cellobiohydrolase II catalytic domain from the N-terminus. The cellulose binding domain contained four aromatic amino acid residues (Trp25, Phe33, Tyr51, and Tyr52) and four cysteines (Cys28, Cys39, Cys45, Cys55), which are highly conserved among carbohydrate binding modules classified into family 1 type CBM [14]. Prosite pattern search performed on the deduced *T. emersonii* Cel6A protein sequence indicated a GH family 6 signature 1 pattern V-x-Y-x (2)-P-x-R-D-C-[GSAF]-x (2)-[GSA](2)-x-G between amino acid 181 and 197 (VVYDLPDRDCAAAASNG) and a GH family 6 signature 2 pattern [LIVM YA]-[LIVA]-[LIVT]-[LIV]-E-P-D-[SAL]-[LI]-[PSAG] between amino acid 229 and 238 (ILVIEPDSLA). The putative catalytic residues in *cbhII* from *T. reesei* [15] are conserved in the *T. emersonii* cbhII gene at AA 235 (aspartate) and AA 413 (aspartate) in the deduced polypeptide sequence.

4.2. Expression of cbh6 Gene in *P. pastoris*

S. cerevisiae and the methylotrophic yeast, *P. pastoris* are the two most frequently used yeasts for expression of recombinant proteins [16] [17]. A great advantage of *P. pastoris* is that a low number of endogenous proteins that are secreted into the culture media. However, in most cases, greater success has been obtained with other signal peptides, such as the secretion signal sequence from the *S. cerevisiae* α-factor prepro peptide [18]. Family GH6 cellobiohydrolase genes from various fungi *Irpex lacteus* MC 2 [19], *Trichoderma parceramosum* [20] were cloned and expressed in *P. pastoris*. To express the *cbh6* gene of *Penicillium funiculosum* NCL 1 in *Pichia pastoris*, the exons were fused using PCR based method. The fused product was successfully expressed extracellularly with its own signal sequence. In similar manner a family GH6 cellobiohydrolase from *Irpex lacteus* MC-2 was expressed extracellular with *P. pastoris* system but with a leader peptide of the a-mating factor from *Saccharomyces cerevisiae* fused to the N-terminus of the translated product makes it possible to secrete recombinant proteins into the medium. Cellobiohydrolase gene (*cbhII*) from was successfully expressed in the *Saccharomyces cerevisiae* α-factor secretion signal [21]. *cbhI* and *cbhII* were isolated from *Trichoderma viride* AS3.3711 and *T. viride* CICC 13038 were successfully expressed in *Saccharomyces cerevisiae* H158 and secreted in yeast *S. cerevisiae* and they were secreted in an active form. The recombinant cellobiohydrolase produced by *P. pastoris* is active at pH 4.0 like other family GH6 cellobiohydrolases [22]. The recombinant enzyme exhibited optimum catalytic activity at pH 4.0 and 50 degrees C respectively. It was thermostable at 50˚C and retained 50% of its original activity after 30 min at 70˚C. The stability of the *cbhII* was observed till pH 6. The *cbhII* showed maximum activity at 50˚C and stable up to 60˚C. As the family GH6 cellobiohydrolases are true cellobiohydrolase, the recombinant cellobiohydrolase also showed highest specificity towards filter paper and not on other substrates, CMC and pNPG.

The importance of lignocellulose biotechnology and the many potential applications of lignocellulose enzymes in various industries such as chemicals, fuel, food, brewery and wine, animal-feed, pulp-and-paper, textile and agriculture are well documented [23]-[25]. Since the recombinant cbhII produced by *P. pastoris* found greater stability through wide range of pH. This property makes it useful for bios toning in the textile and detergent industries.

5. Conclusion

A gene encoding CBHII was successfully isolated from *Penicillium funiculosum* using RT-PCR. This gene contains the conserved catalytic amino acid residues that have been reported in other fungal cellulases. However, it does not contain a CBD. The enzyme was successfully expressed and secreted in yeast *P. pastoris* and it was secreted in an active form. Enzymatic properties of CBHII were also determined. Due to its temperature stability

and pH stabilities, our cellobiohydrolase might be useful for textiles, pulp and paper industries.

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