J Ind Microbiol Biotechnol (2015) 42:553–565
DOI 10.1007/s10295-014-1549-6

BIOENERGY/BIOFUELS/BIOCHEMICALS

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# Molecular cloning and expression of thermostable glucose-tolerant β-glucosidase of *Penicillium funiculosum* NCL1 in *Pichia pastoris* and its characterization

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Received: 5 June 2014 / Accepted: 14 November 2014 / Published online: 28 January 2015


**Abstract** A partial peptide sequence of β-glucosidase isoform (Bgl4) of *Penicillium funiculosum* NCL1 was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The cDNA (*bgl*4) encoding Bgl4 protein was cloned from *P. funiculosum* NCL1 RNA by consensus RT-PCR. The *bgl*4 gene encoded 857 amino acids that contained catalytic domains specific for glycoside hydrolase family 3. The cDNA was over-expressed in *Pichia pastoris* KM71H and the recombinant protein (rBgl4) was purified with the specific activity of 1,354.3 U/mg. The rBgl4 was a glycoprotein with the molecular weight of ~130 kDa and showed optimal activity at pH 5.0 and 60 °C. The enzyme was thermo-tolerant up to 60 °C for 60 min. The rBgl4 was highly active on aryl substrates with β-glucosidic, β-xylosidic linkages and moderately active on cellobiose and salicin. It showed remarkably high substrate conversion rate of 3,332 and 2,083 μmol/min/mg with the substrates *p*-nitrophenyl β-glucoside and cellobiose respectively. In addition, the rBgl4 showed tolerance to glucose concentration up to 400 mM. It exhibited twofold increase in glucose yield when supplemented with crude cellulase of *Trichoderma reesei* Rut-C30 in cellulose hydrolysis. These results suggested that rBgl4 is a thermo- and glucose-tolerant β-glucosidase and is a potential supplement for commercial cellulase in cellulose hydrolysis and thereby assures profitability in bioethanol production.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10295-014-1549-6) contains supplementary material, which is available to authorized users.

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**Keywords** *Penicillium funiculosum* NCL1 · β-Glucosidase · *Pichia pastoris* · Thermo-tolerant · Glucose-tolerant

## Introduction

Increasing demand for energy and continuous depletion of fossil fuel has raised interest on cellulosic ethanol production from cellulosic biomass. Cellulose is the most abundant biopolymer on earth and is composed of glucose monomers linked by β-1,4 glucosidic bonds [33]. Conversion of cellulose to glucose is involved synergistic action of cellulolytic enzymes namely endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) [16]. Endoglucanase randomly cuts the cellulose chains to yield glucose and cello-oligosaccharides, cellobiohydrolase attacks the polysaccharides from reducing and non-reducing ends to give cellobiose and cello-oligosaccharides, while β-glucosidase hydrolyzes these cello-oligosaccharides and cellobiose into glucose monomers [22].

*Trichoderma reesei* is the most widely used organism for cellulase production. However, lack of β-glucosidase secretion by this strain results in cellobiose and cello-oligosaccharides accumulation in the medium that generates catabolite repression on endoglucanase and cellobiohydrolase [30]. This limitation is considered as the major disadvantage of *T. reesei* and reduces the potential application of the same in bioethanol industries [14]. Though, exogenous supplementation of β-glucosidase alleviates the inhibition, the high cost and low availability of β-glucosidase turns the ethanol production very expensive. Therefore, there is always a search for potential β-glucosidase for successful bioconversion of cellulose.

β-Glucosidases are being produced by most of the cellulolytic microorganisms. Predominantly, β-glucosidases produced by filamentous fungi are preferred for industrial usage, due to their easy recovery and high titer of activity. Filamentous fungi like *Aspergillus* sp., *Penicillium* sp., *Thermoascus* sp., *Trichoderma* sp., *Talaromyces* sp. are reported for β-glucosidase production [16, 22, 29]. Particularly, *Penicillium* sp. is known for high β-glucosidase production [2, 4, 15, 21]. *Penicillium funiculosum* NCL1 has been reported to be an efficient cellulolytic fungus and it was found to bring about 97 % of cotton hydrolysis that was attributable to its high β-glucosidase production [3, 6, 25]. In our previous study, we found that *P. funiculosum* NCL1 was able to secret at least five different β-glucosidase isoforms and the major β-glucosidase isoform Bgl3 was purified and characterized [24]. The other isoform Bgl4 was markedly secreted by the culture grown with most of the cellulosic substrates that assured the broad substrate specificity of the isoform. In this study, we have identified a partial peptide sequence of the gene encoding Bgl4 by MALDI-TOF analysis and complete ORF of the corresponding gene was cloned by Consensus PCR and over-expressed in *P. pastoris* KM71H. In addition, the recombinant protein was purified, characterized and the synergistic action of recombinant enzyme with *T. reesei* cellulase on avicel hydrolysis was also evaluated.

## Materials and methods

### Microorganisms

*Penicillium funiculosum* NCL1 was obtained from Dr. Mala Rao, Division of Biochemical Sciences, National Chemical Laboratory, Pune [25]. *Trichoderma reesei* Rut-C30 was a kind gift from Dr. Ashok Pandey, National Institute of Interdisciplinary Science and Technology, Trivandrum [23]. Fungal strains were grown in potato dextrose agar (PDA) slants at 30 °C for 96 h and stored at 4 °C. *Escherichia coli* DH5α (Invitrogen, USA) was used for plasmid construction. *Pichia pastoris* KM71H (Invitrogen, USA) was used as heterologous host for gene expression.

### MALDI-ToF and peptide mass fingerprinting (PMF)

In-gel tryptic digestion and MALDI-ToF mass spectrometry of Bgl4 was performed at Department of Molecular Biophysics, Indian Institute of Science, Bangalore. Peptide mass spectrum was acquired using Axima CFR plus (KRATOS Shimadzu, USA) MALDI-ToF mass spectrometer in the reflectron mode. Data analysis was done with MS data analysis program MASCOT (http://www.matrixscience.com). Mass peaks with the signal to noise ratio of 20 and above was used for the databases search. The search parameters were as follows: enzyme digestion with trypsin, $[M+1]^+$ peptide charge state, monoisotopic mass values, mass tolerance −0.05 to 0.5 Da and maximum 1 missed cleavages per peptide, fixed modification—carbamidomethyl (for cysteine modification by Iodoacetamide), variable modifications—oxidation of methionine and propionamide (for cysteine modification by acrylamide). Obtained peptide sequences were further examined for number of matching peptides, the mass and pI accuracy of the matching peptides, the sequence coverage and distribution.

### Genomic DNA and RNA isolation

*Penicillium funiculosum* NCL1 was grown in Reese and Mandel medium [18] containing (g/l): $KH_2PO_4$ 3; $(NH_4)_2SO_4$ 1.5; $MgSO_4$ 0.3; urea 0.3; $CaCl_2$ 0.3; peptone 2.5; yeast extract 2.0; $FeSO_4{\cdot}7H_2O$ 0.005; $MnSO_4{\cdot}H_2O$ 0.0016; $ZnSO_4{\cdot}7H_2O$ 0.0014 and $CoCl_2$ 0.0012, pH 5.0 supplemented with appropriate carbon source (glucose for DNA isolation and avicel for RNA isolation) and incubated at 30 °C for 96 h [14]. Mycelium was collected by filtration using muslin cloth. Genomic DNA was isolated using Qiagen fungal DNA isolation kit and total RNA was isolated using Qiagen total RNA isolation kit.

### Polymerase chain reaction (PCR)

The PCR reaction mix consisted of genomic DNA/cDNA (5–10 ng/μl), forward and reverse primers (10 pmol each), dNTPs mix (0.2 mM), $MgCl_2$ (1.5 μM) and 0.5 units of *Taq* DNA polymerase in a 10 μl volume with reaction buffer adjusted to 1× concentration. PCR amplification was carried out for 30 cycles under the following conditions: initial denaturation at 94 °C for 5 min, denaturation for 94 °C for 30 s, annealing at 50–60 °C for 30 s and extension at 72 °C for 1.5 min. Final extension was at 72 °C for 5 min. PCR was carried out in an Eppendorf mastercycler (Eppendorf AG, Hamburg, Germany).

### Cloning and sequence analysis

The degenerate primers bgl4pf (5′-TGGGHAGYGGTACT GCCAACTTCCCT-3′) and bgl4pr (5′-TTCATTTACC CMTGGCTCAAYWCYACT-3′ where Y-C/T, H-A/C/T, M-A/C and W-A/T) were designed based on partial peptide sequence obtained from MALDI-ToF analysis and the C-terminal conserved domain of GH3 β-glucosidases. The partial *bgl*4 DNA fragment, that was amplified by consensus PCR from genomic DNA of *P. funiculosum* NCL1 was cloned into pTZ57R vector (MBI Fermentas, Opelstrasse, Germany) and sequenced. In order to amplify the

complete gene, primers bgl4f (5′-ATGCGGAACAGCTG GTTAATTTC-3′) and bgl4r (5′-CTACTTTCTAATGTC GAGAGCCAAC-3′) were designed according to GH3 β-glucosidase protein sequences that showed more than 95 % of similarity with partial *bgl*4 sequence. The amplified 2.7 kb fragment of *bgl*4 DNA was cloned into pTZ57R vector and sequenced.

To clone corresponding cDNA of *bgl*4, RT-PCR was performed with primers bgl4pf and bgl4pr from total RNA isolated from glucose, lactose and avicel-grown *P. funiculosum* NCL1 using Titanium One-step RT-PCR kit (Clontech, Badford, CA). The partial *bgl*4 cDNA was amplified from avicel-induced *P. funiculosum* NCL1 RNA. Similarly, the complete ORF of *bgl*4 cDNA was also cloned from avicel-induced RNA using the primers bgl4f and bgl4r and the amplicon was sequenced.

## Sequence analysis

Sequence similarity searches were performed using BLAST algorithm at the National Center for Biotechnology Information Server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment of DNA and protein sequences were performed using ClustalW version 2.0 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Signal sequence prediction was carried out using SignalP 3.0 online software (http://www.cbs.dtu.dk/services/SignalP). The molecular weight and the isoelectric point pI were calculated with ExPASy Proteomics server (http://www.expasy.org). Based on amino acid sequence homologies, a protein model was built using SWISS-MODEL and the structure was viewed by Pymol software package. The predicted structure was validated by Procheck and PSI-Pred.

## Heterologous expression and purification

The *bgl*4 cDNA was amplified using the primers bgl4fkpn (5′-TCGTCCGGTACCATGCGGAACAGCTGGTT AATTTCTCT-3′) and bgl4rxba (5′-GATCGCTCTAGACTT TCTAATGTCGAGAGCCAAC-3′) which are tagged with *Kpn*I and *Xba*I restriction sites, respectively. After the double restriction digestion with *Kpn*I and *Xba*I, the PCR product was ligated into the vector pPICZαA (Invitrogen, USA) that was digested with same restriction enzymes. This construct pPICZ*bgl*4 was propagated by transforming into *E. coli* DH5α. The pPICZ*bgl*4 plasmid was linearized with *Pme*I and transformed into *P. pastoris* KM71H by electroporation using Gene Pulser Xcell™ Electroporation System (Bio-Rad, USA). The transformants were screened by selection on YPDS (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol, 2 % agar) supplemented with zeocin (100 μg/ml).

The scored transformants were screened for resistance to higher concentration of zeocin (10×) according to Liu et al. [17]. Clones were grown on YPM solid medium (1 % yeast extract, 1 % peptone, 1.5 % methanol) to induce the expression of recombinant β-glucosidase as described by Hong et al. [9]. Thus grown clones were overlaid with 0.8 % agar containing 4 mM MUG (4-methyl umbelliferyl-β-D-glucopyranoside) (Sigma Aldrich, USA). After incubation for 10 min at 50 °C, β-glucosidase activity was detected by exposing the plate to UV light to detect luminescence. In order to evaluate the cellobiose utilization ability, the clones were grown on SCM solid and liquid medium (0.67 % yeast nitrogen base without amino acids, 2 % cellobiose, 0.5 % methanol, pH 5.0) and the growth of clones were compared with control.

Transformants with the strongest fluorescence on the YPM plate were inoculated into 1 l BMGY medium (1 % glycerol, 1 % yeast extract, 2 % peptone, 0.34 % yeast nitrogen base without amino acids and ammonium sulfate, 1 % $(NH_4)_2SO_4$ (w/v) and $4 \times 10^{-5}$ % biotin in 100 mM potassium phosphate buffer pH 6.0) for pre-culturing. After 48 h of incubation, the cells were transferred to BMMY medium (the same as BMGY, except that glycerol was replaced by methanol 5 ml/l). For continuous methanol induction, 1.5 % methanol was fed for every 24 h. Aliquots (1 ml) were collected every 24 h and the β-glucosidase activity was assessed. For purification of rBgl4, the supernatant was recovered and subjected to 80 % ammonium sulfate precipitation. The concentrated protein was purified with Ni–NTA Sepharose column.

## Enzyme assay and PAGE analysis

Total protein was estimated by Lowry's method using bovine serum albumin as standard. Total neutral carbohydrate content was calculated by phenol sulfuric acid method, using mannose as standard as per Masuko et al. [19]. β-Glucosidase assay was performed by incubating 1 ml of appropriately diluted enzyme with 4 mM *p*-nitrophenyl-β-glucoside in 50 mM sodium acetate buffer pH 5.0 at 50 °C (unless otherwise mentioned) for 10 min followed by addition of 2 ml of 1 M sodium bicarbonate. The yellow color developed was measured at 410 nm. One unit of β-glucosidase activity was defined as the amount of enzyme that hydrolyzes the substrate to release 1 μmol of glucose per min in the reaction mixture under assay conditions. Total cellulase activity was assayed by incubating avicel (2.5 % w/v) with appropriately diluted enzyme as described by Ghose [5]. The released glucose was estimated by HPLC (Shimadzu RID-10A, Kyoto, Japan) with a Luna amino column. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose per ml per min under the assay conditions.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the protein was performed for molecular weight analysis. Zymogram staining was done by resolving the protein on non-denaturing PAGE and incubating for 30 min at 50 °C in 50 mM sodium acetate buffer (pH 5.0) and then treating the gel with 4 mM (w/v) 4-MUG solution for 30 min at room temperature. The florescent band observed under UV light exposure was then documented using gel documentation system (UVP BioSpectrum, CA, US). Glycoproteins were visualized by the periodic acid–Schiff (PAS) method.

### Characterization of purified β-glucosidase

The influence of pH on rBgl4 activity and stability were analyzed by determining the β-glucosidase activity between pH 3.0 and pH 8.0 using acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–7.0), and Tris–HCl buffer (pH 7.0–8.0). Optimum temperature and thermostability were analyzed by assaying the purified enzyme at temperatures between 30 and 80 °C. The relative and residual activities were expressed in percentage with respect to the maximum activity obtained. Substrate specificity of purified enzyme was studied with various pNP substrates (pNβPG, pNP-α-D-glucoside (pNαPG), pNP-β-D-galactoside (pNβPGal), pNP-α-D-galactoside (pNαPGal), pNP-β-D-xyloside (pNαPX) (HiMedia, Mumbai, India), disaccharides (cellobiose, lactose, maltose, salicin, esculin, sucrose), and polysaccharides (cellulose powder (CP), carboxy methyl cellulose (CMC), avicel, xylan, starch) by determining the release of pNP and glucose, respectively. The effect of metal ions (10 mM) and chemical reagents (1 %) on purified enzyme was studied by incubating the purified enzyme with the corresponding additives for 30 min at 30 °C, and the residual activity was determined using pNβPG as substrate.

The effect of glucose on rBgl4 activity was examined with different concentrations of glucose ranging from 0 to 600 mM. The enzyme was incubated with varying concentration of glucose for 30 min at room temperature and the residual β-glucosidase activities were measured. A control reaction without glucose was set and the activity obtained was considered as 100 %.

### Catalytic properties of purified β-glucosidase

Michaelis–Menten constant ($K_m$) and maximum velocity ($V_{max}$) of purified β-glucosidase were determined with different concentrations (0.01–10 mM) of pNβPG and cellobiose.

### Effect of rBgl4 supplementation with *T. reesei* cellulase on avicel hydrolysis

*Trichoderma reesei* Rut-C30 was grown in basal medium supplemented with cellulose powder (1 %) for 96 h at 30 °C in shaking condition. The culture supernatant was collected by centrifugation at 9,000×*g* for 15 min, concentrated and 5 FPU/ml was used for saccharification. Avicel (2.5 % w/v) was incubated with 5 FPU/ml of *T. reesei* cellulase with rBgl4/commercial β-glucosidase (100 U/ml) at pH 5.0, 60 °C and 200 rpm. Control reaction was set without rBgl4. Samples (20 µl) were recovered from control and test reactions at every 6-h intervals, and glucose yield was analyzed by HPLC with Luna amino column using acetonitrile/degassed water (75:25) as the mobile phase at the flow rate of 1 ml/min.

## Results

### Identification and cloning of *bgl*4

The β-glucosidase isoform Bgl4 of *P. funiculosum* NCL1 was excised from non-denaturing PAGE gel, trypsin digested and MALDI-ToF analysis was performed. When the data was analyzed with MASCOT by searching the related sequences in Swiss-Prot database, the internal amino acid sequence (FIYPWLNSTD LEASSGDASY GQDSS DYLPE GATDGSAQPI LPAGGGPGGN PR) was obtained and it was matched with BGLA of *A. kawachi* with the molecular weight of 93 kDa (Supplemental File 1). Based on this partial peptide sequence and C-terminal-conserved motif (WGSGTAN) degenerate primers were designed. Subsequently, a DNA fragment of 850 bp (*bgl*4p) was amplified using these primers from genomic DNA of *P. funiculosum* NCL1 and the fragment was sequenced. BlastX analysis of *bgl*4p indicated the similarity of *bgl*4p with β-glucosidases belonging to GH3 family. Based on the homologous protein sequences, primers were designed to amplify the complete ORF and then 2852 bp fragment was amplified from genomic DNA of *P. funiculosum* NCL1 (Supplemental File 2). The corresponding complete cDNA of 2574 bp was also amplified from avicel-induced RNA of *P. funiculosum* NCL1 by Reverse transcription PCR (Supplemental File 2). The deduced amino acid sequence of Bgl4 was deposited in NCBI with the Accession No. AFU91382.

### Sequence analysis and homology modeling

Pairwise alignment of *bgl*4 DNA and cDNA sequences revealed that the coding sequence is made up of five exons which are interrupted by four introns located at 56–126, 260–314, 413–500 and at 1685–1748 bp (Supplemental File 3). The 5′ end of the deduced protein sequence of Bgl4 contained a putative signal sequence of 19 amino acids with the hydrophobic alanine-rich region (7-LAAAAV-12), preceding the mature protein of 838 amino acids. It shared 96, 90 and 70 % of sequence identity with β-glucosidase

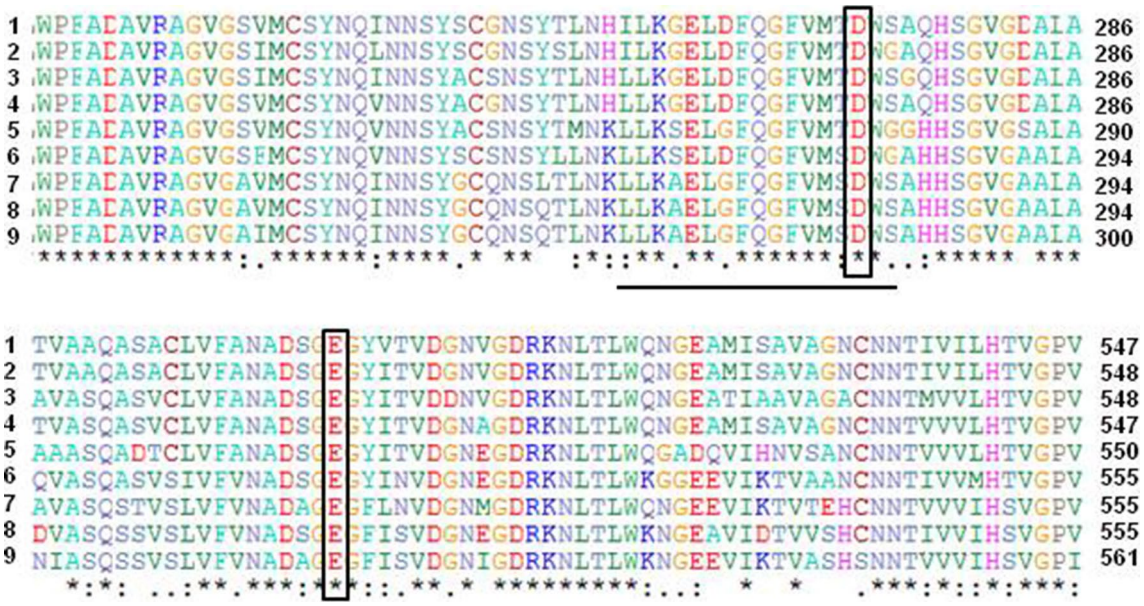

**Fig. 1** Multiple sequence alignment of Bgl4 with orthologs of *Penicillium* sp., *Talaromyces* sp., *Thermoascus* sp. and *Aspergillus* sp. Acid/base catalyst ($Glu^{273}$) and the catalytic nucleophile ($Glu^{502}$) of Bgl4 are shown in *boxes*. Signature sequence of GH3 β-glucosidases is *underlined*. The conserved residues are indicated with *asterisks*, conserved substitutions are shown as *semicolon*, semi-conserved substitutions are shown as *dot*. *1* Bgl4 of *P. funiculosum* NCL1, *2* β-glucosidase of *P. purpurogenum* (ACV87737.1l), *3* putative β-glucosidase of *T. stipitatus* (XP_002480480), *4* β-glucosidase of *P. marneffei* (XP_0021440891), *5* β-glucosidase of *T. emersonii* (AAL69548), *6* Thermostable β-glucosidase of *T. aurantiacus* (AAZ95587.1), *7* β-glucosidase precursor of *A. terreus* (XP_001212225), *8* extracellular β-glucosidase of *A. fumigatus* (ADX78143), *9* putative β-glucosidase of *A. clavatus* (XP_001269582)

of *P. purpurogenum* (ACV87737.1), putative β-glucosidase of *T. stipitatus* (XP_002480480.1) and thermostable β-glucosidase of *T. aurantiacus* (AAZ95587.1), respectively. Prosite scan revealed that Bgl4 possessed Glyco_hydro_3 super family domain (Pfam00933) and Glyco_hydro_C_3 domain (Pfam01915) while the domains are connected by ~63 amino acids long hinge region. Mature protein sequence contains 13 N-glycosylation sites and 9 cysteine residues.

Multiple sequence alignment of Bgl4 of *P. funiculosum* NCL1 with its closest homologs revealed the existence of signature sequence (259-ILKGELDFQG-FVMTDWSA-276) in Bgl4, which is specific for GH3 β-glucosidase (Fig. 1). Characteristic catalytic nucleophile Aspartic acid ($Asp^{273}$), and catalytic proton donor Glutamic acid ($Glu^{502}$) are conserved in Bgl4 that is the typical feature of GH3 β-glucosidases. Bgl4 was predicted to be a globular protein by Pred-class analysis with the calculated molecular mass of the mature protein of 90,631 Da. The 3-D structure of Bgl4 of *P. funiculosum* NCL1 was predicted by SWISS-MODEL, based on the structure of β-glucosidase of *Kluyveromyces marxianus* (pdb code: 3abz_A) [32] and visualized by PyMOL v0.98 (Fig. 2). The N-terminal domain (green) of Bgl4 consisted of residues 51–333 arranged in $(\alpha/\beta)_8$ TIM-barrel structure and C-terminal domain (blue) ranging from 392 to 677 arranged in six stranded β-sandwich structure. The catalytic residues $Asp^{273}$ and $Glu^{502}$ (red) most likely form the active site. Ramachandran plot suggested that 97.6 % of amino acid residues are in the favorable and allowed region.

## Over-expression and purification of recombinant enzyme (rBgl4)

The cDNA fragment encoding Bgl4 was cloned in frame with pPICZαA vector under AOX1 promoter, and the construct pPICZ*bgl*4 was generated via *E. coli* DH5α as cloning host. The pPICZ*bgl*4 plasmid was linearized with *Pme*I restriction enzyme and transformed into *P. pastoris* KM71H to facilitate the integration of *bgl*4 copies into its genome. Twenty-five randomly picked transformants were screened for multicopy integrants by 10× zeocin (1000 μg/ml) resistance (Fig. 3a). In order to confirm the expression of recombinant β-glucosidase, three transformants which showed high resistance to 10× zeocin, were grown on YPM solid medium and overlaid with soft agar containing 4 mM of 4-MUG. Transformants exhibited stronger fluorescence, while the control strain with pPICZα vector did not show fluorescence (Fig. 3b). The transformants were examined for their ability to utilize cellobiose by growing them on SCM solid and liquid medium supplemented with 2 % cellobiose.

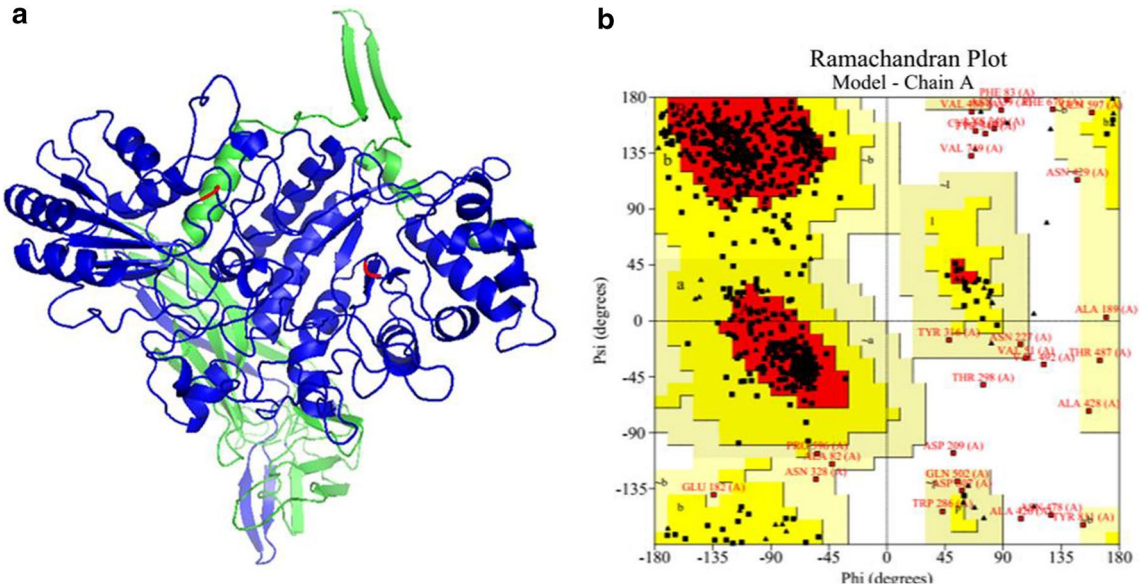


**Fig. 2** Predicted structure of Bgl4 of *P. funiculosum* NCL1. **a** The structural model of Bgl4 was constructed, based on structure of β-glucosidase of *Kluyveromyces marxianus* (3abz_A). The structure composed of C-terminal domain glyco_hydro_3C (*green*) followed by linker region and N-terminal conserved domain glyco_hydro_N (*blue*). The nucleophile $Asp^{273}$ and general acid/base $Glu^{502}$ are shown in *red*. **b** Ramachandran plot of predicted structure of Bgl4 of *P. funiculosum* NCL1. The most favored regions (*A*, *B*, *L*) are *colored red*, additional allowed (*a*, *b*, *l*, *p*) generously allowed (*~a*, *~b*, *~l*, *~p*) are indicated as *yellow* and disallowed regions are indicated as *light yellow* and *white fields*, respectively (color figure online)

They showed various degrees of growth on cellobiose-supplemented SCM medium, while the control strain did not grow properly (Fig. 3c, d). Particularly, clone 21 reached an OD of 2.0 at 48 h in SCM liquid medium, indicated the ability to utilize cellobiose efficiently and grow.

For over-expression of recombinant protein, *P. pastoris* (pPICZ*bgl*4) clone 21 was grown in BMGY medium till it reaches the midlog phase. Then, the cells were transferred to BMMY medium and induced with 1.5 % of methanol for every 24 h. The β-glucosidase activity was observed from 24 h (4.7 U/ml) and continued to increase gradually till 96 h (52.8 U/ml). Further incubation did not increase the enzyme production substantially (Fig. 4). When the culture supernatant was assayed with cellobiose, it showed highest cellobiase activity of 41.2 U/ml at 84 h. The culture filtrate (1 l) was concentrated to 100 ml by passing through 50 kDa molecular weight cutoff membrane. The redundant exhibited 584.7 U/mg of specific β-glucosidase activity with the purification fold of 13. The redundant was then passed through nickel affinity column and the active fraction was eluted between 225 and 242 mM of imidazole with the specific activity of 1,354.3 U/mg. The purification yield of the purified rBgl4 was 30.3 and the recovery was 29.7 %.

## Characterization of rBgl4

SDS-PAGE analysis indicated that the molecular weight of the rBgl4 was about 130 kDa (Fig. 5a). The protein showed a single activity band in zymogram with the substrate 4-MUG, suggested that the purified protein exhibit β-glucosidase activity (Fig. 5b). The glycoprotein nature of rBgl4 was confirmed by glycoprotein PAGE-gel assay using Periodic acid-Schiff's stain (Fig. 5c). Total carbohydrate content of rBgl4 was estimated to be 32 % by phenol–sulfuric acid assay.

## Physico-chemical properties of rBgl4

Effect of pH and temperature on rBgl4 activity were determined at various pH (3.0–8.0) and temperature (30–80 °C) using pNβPG as substrate. The maximum activity of rBgl4 was observed at pH 5.0 and the enzyme showed more than 95 % of the activity in the range of pH 4.0–7.0. Stability of the enzyme was also maintained between pH 3.0–6.0 (Fig. 6a). rBgl4 exhibited maximum activity at 60° and activity decreased sharply when the temperature exceeded 60 °C. The enzyme was stable at broad range of temperature between 30 and 60 °C (Fig. 6b) and it retained 77 and 40 % of its initial activity after 1 h of incubation at 60 °C and 30 min of incubation at 70 °C, respectively. However, the enzyme activity was lost after the incubation of 30 min at 80 °C.

## Substrate specificity of rBgl4

The substrate specificity of the rBgl4 on various aryl glycosides, disaccharides and polysaccharides were examined.

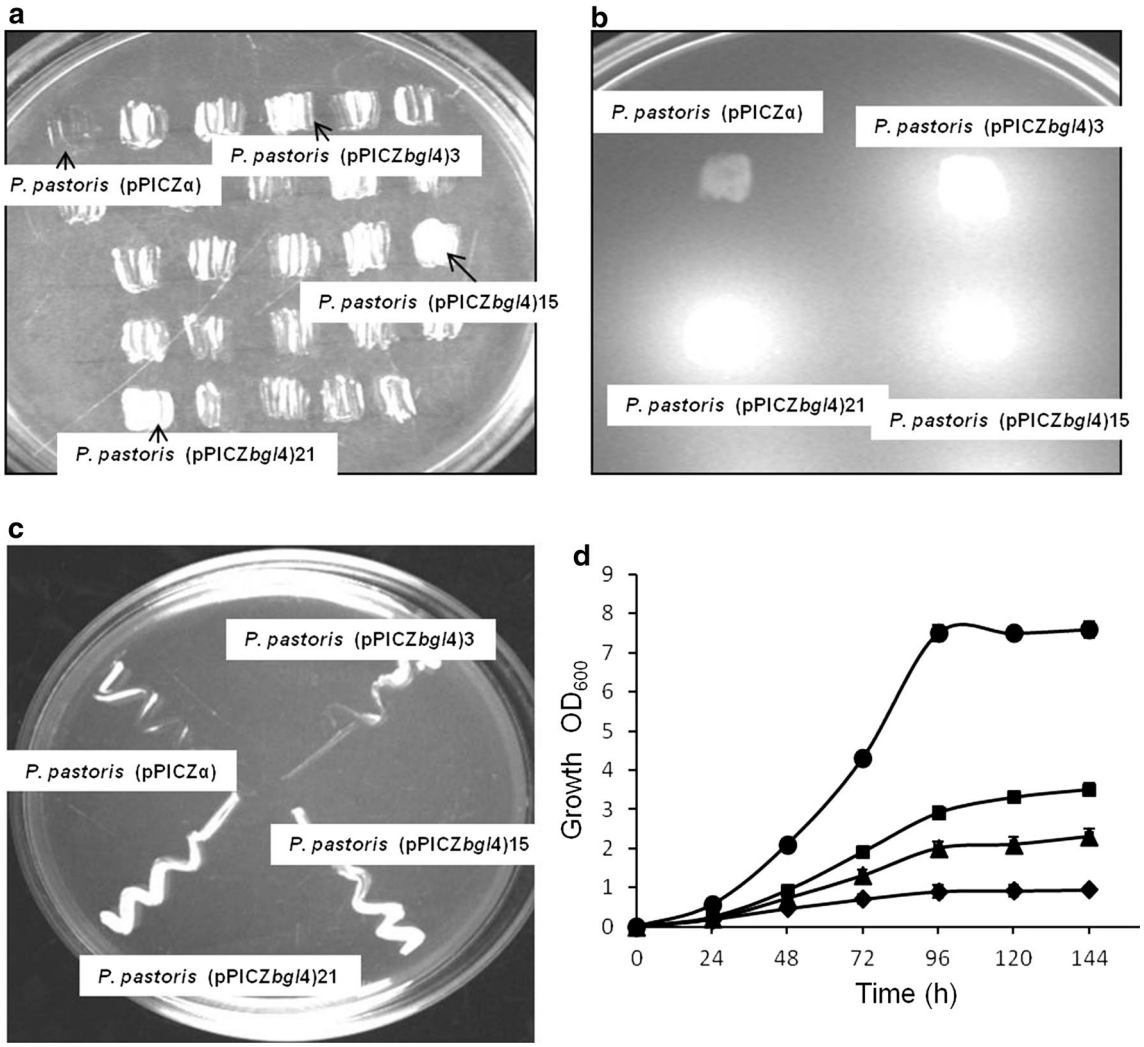


**Fig. 3** **a** Identification of *bgl*4 multiple integrants of *P. pastoris* through high zeocin resistant *P. pastoris* (pPICZ*bgl*4). Transformants 3, 15, 21 grew well in 10× zeocin plate, while *P. pastoris* clone with pPICZα-vector did not grow. **b** Identification of β-glucosidase over-expressing transformants by MUG-zymogram assay. Transformants 3, 15, 21 fluoresce under UV when overlaid with 4-MUG. **c** Cellobiose utilizing ability. *Pichia pastoris* (pPICZ*bgl*4) clone21 showed better growth in SCM solid medium. **d** Growth rate in cellobiose supplemented SCM medium. *Pichia pastoris* (pPICZ*bgl*4) clone21 reached optimal growth than other transformants. *Filled diamond* control *P. pastoris* (pPICZα); *filled triangle P. pastoris* (pPICZ*bgl*4)3; *filled square P. pastoris* (pPICZ*bgl*4)15; *filled circle P. pastoris* (pPICZ*bgl*4)21

It showed highest activity with pNβPG (100 %) and cellobiose (88 %), followed by pNPX (78 %). The enzyme could also hydrolyze salicin up to 70 % (Table 1). Fifty-one percentage of activity was observed on the esculin, which is natural coumaric substrate of β-glucosidase. The rBgl4 exhibited little or no activity on the substrates with α-1,4 linkages and polysaccharides.

## Effect of metal ions, enzyme inhibitors and reagents on rBgl4 activity

The effect of various metal ions (10 mM) or chemical reagents (1 %) on the rBgl4 activity were investigated and shown in Table 2. The $Zn^{2+}$ upgraded the activity by 142 %. In the presence of $Mn^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $K^{+}$ and $Na^{2+}$, 132–110 % of enhancement in the activity was observed. In contrast, the enzyme activity was inhibited by $Cu^{2+}$ and $Hg^{2+}$ with the retention of 32 and 23 % of activity, respectively. The $Ag^{2+}$ abolished rBgl4 activity completely. Significant enhancement in enzyme activity was observed with the addition of β-mercapto ethanol, while addition of $Ni^{2+}$, $Fe^{3+}$, DTT, EDTA, glycerol or SDS did not show any influence on rBgl4 activity.

Glucose tolerance of rBgl4 was investigated with the added concentration of glucose ranging from 0 to 600 mM (Fig. 7). Interestingly, addition of glucose, up to 150 mM resulted in positive influence on the rBgl4 activity. Highest activity of 143 % was observed with 150 mM of glucose.

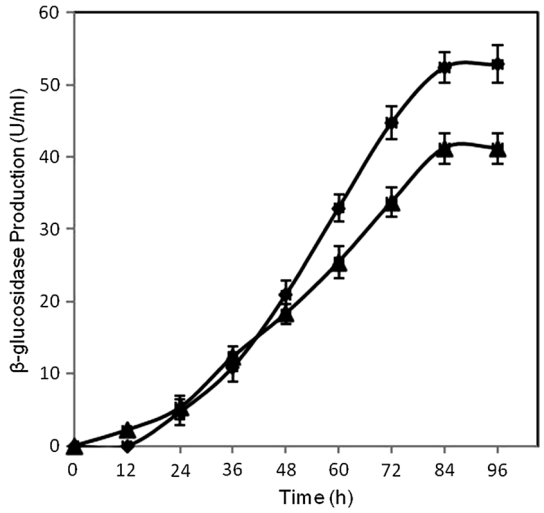


**Fig. 4** Kinetics of rBgl4 production. *P. pastoris* (pPICZ*bgl*4) clone21 was induced with 1.5 % (v/v) methanol in BMMY medium. Maximum of 52.8 U/ml of β-glucosidase (*filled square*) at 84 h (41.2 U/ml of cellobiase (*filled triangle*)) was obtained. Values are means of triplicates and error bars show the standard deviation

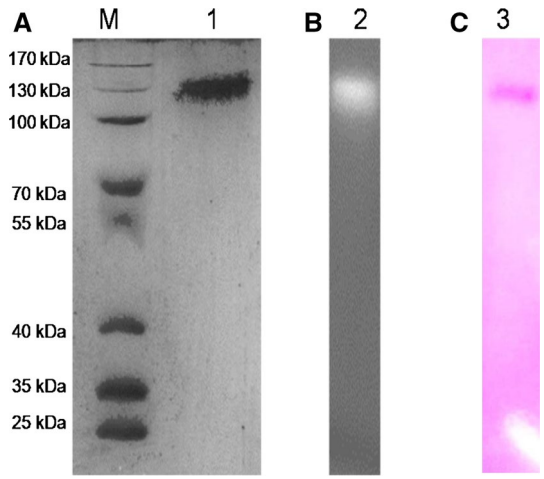


**Fig. 5** PAGE analysis of rBgl4. **a** SDS-PAGE analysis of purified rBgl4. *Lane M* Molecular weight marker, *Lane 1* Purified rBgl4. **b** *Lane 2* Activity staining of rBgl4 with 4-MUG. **c** Glycoprotein analysis *Lane 3* Schiff stained rBgl4

The rBgl4 exhibited tolerance to glucose till 400 mM and further increase in the glucose concentration resulted in inhibition of enzyme activity.

### Kinetic characterization of rBgl4

Based on Lineweaver–Burk plot, affinity constant and maximum velocity of the rBgl4 were determined at optimal conditions using pNβPG and cellobiose as substrates, respectively. The rBgl4 activity with increasing concentration of cellobiose and pNβPG followed typical Michaelis–Menton type kinetics. When pNβPG was used as substrate, rBgl4 exhibited $K_m$ and $V_{max}$ of 2.5 mM and 3,332 μmol/min/mg, respectively (Fig. 8a). When cellobiose was used as the substrate, the calculated $K_m$ of rBgl4 was 1.25 mM and the $V_{max}$ was 2,083 μmol/min/mg (Fig. 8b).

### Synergistic activity of rBgl4

The effect of supplementation of rBgl4 (100 U/ml) to the *T. reesei* total cellulase (5 FPU/ml) on avicel hydrolysis (2.5 % w/v) was compared with that of commercial β-glucosidase at optimal conditions (Fig. 9). Glucose yield was estimated for every 24 h by HPLC. When individually used rBgl4 and *T. reesei* cellulase produced 0.07 and 9.3 mg/ml of glucose, respectively, from avicel. Addition of commercial β-glucosidase to *T. reesei* cellulase yielded a maximum of 14.1 mg/ml glucose at 72 h. However, the rBgl4 supplementation resulted in improved glucose production with a maximum of 19.4 mg/ml at 72 h.

## Discussion

Identification of β-glucosidases with broad substrate specificity, improved thermostability and glucose tolerance is always being a thrust area of research, since β-glucosidase surmounts the feedback inhibition by hydrolyzing the accumulated cellobiose into glucose. β-Glucosidases from filamentous fungi are highly preferred for industrial applications due to their stability and broad spectrum of substrate specificity. In the present study, the gene encoding a β-glucosidase isoform (Bgl4) of *P. funiculosum* NCL1 was identified and expressed in *P. pastoris* KM71H. This isoform was selected based on its production by *P. funiculosum* NCL1 on media supplemented with sugar cane bagasse, avicel, wheat bran and rice straw, which authenticated the broad substrate specificity [24].

Based on the internal amino acid sequence that was obtained from MALDI-ToF analysis, the complete ORF of *bgl*4 gene was amplified by consensus RT-PCR using degenerate primers and the gene *bgl*4 was sequenced. Blast analysis of the deduced protein sequence of *bgl*4 revealed the highest sequence similarity with the thermostable β-glucosidase of *T. aurantiacus*, which indicated the possibility of Bgl4 being a thermostable β-glucosidase [8]. Alanine-rich signal peptide sequence in the N-terminal region of the protein endorsed that Bgl4 is a secretary protein. The presence of Glyco_hydro_3 domain in N-terminal region, Glyco_hydro_3_C domain in C-terminal region suggested that this enzyme belongs to the GH3 family. The fibronectin domain found at the C-terminal end is believed

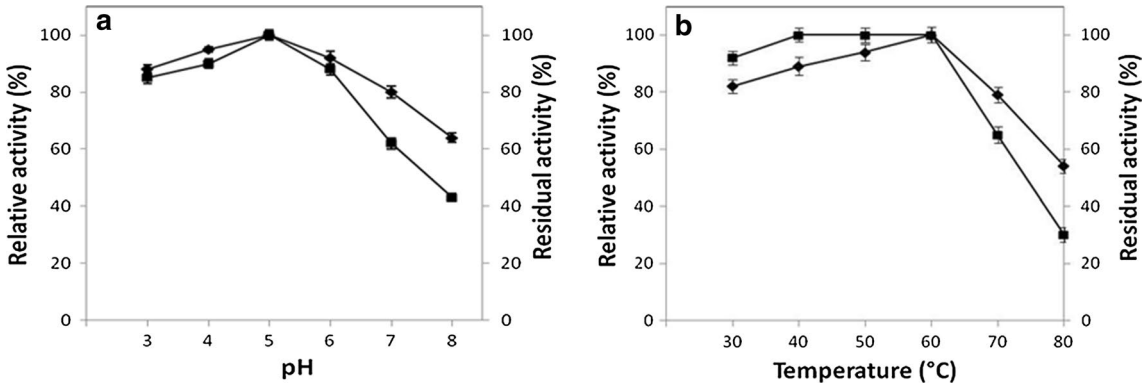


**Fig. 6** Effect of pH and temperature on β-glucosidase activity of rBgl4. **a** Optimum pH (*filled square*) of rBgl4 was determined by performing β-glucosidase assay at various pH (pH 3.0–8.0) Maximum activity was obtained at pH 5.0 and was taken to be 100 %. pH stability (*filled triangle*) was determined by pre-incubating the enzyme with different pH buffers for 30 min and assaying at standard conditions. rBgl4 was stable between pH 3.0 and 6.0. **b** Temperature profile was determined by performing β-glucosidase assay at temperatures ranging from 30 to 80 °C. Optimal enzyme activity (*filled triangle*) obtained at 60 °C was set as 100 % and the relative stability (*filled square*) was expressed in relative percentage. rBgl4 was more stable at the range of 30–60 °C. The activities are the mean values of three experiment values and the *bars* denotes the standard deviation

**Table 1** Substrate specificity of rBgl4

| Substrate | Relative activity (%)[a] |
|---|---|
| *Aryl glycosides* | |
| *p*-Nitrophenyl-β-D-glucopyranoside | 100 |
| p-Nitrophenyl-α-D-glucopyranoside | 0 |
| p-Nitrophenyl-β-D-galactopyranoside | 0 |
| p-Nitrophenyl-α-D-galactopyranoside | 0 |
| p-Nitrophenyl-β-D-xylopyranoside | 78 |
| *Oligosaccharides* | |
| Cellobiose | 88 |
| Lactose | 0 |
| Maltose | 0 |
| Salicin | 70 |
| Sucrose | 0 |
| Esculin | 51 |
| *Polysaccharides* | |
| Cellulose | N |
| CMC | N |
| Avicel | N |
| Xylan | N |
| Starch | N |

*N* negligible

[a] Depending on the substrates, the relative hydrolytic rate was determined under the optimal conditions (60 °C, pH 5.0) as percentage of that obtained with pNPG for pNP substrates and percentage of that obtained with cellobiose for di and poly saccharides

**Table 2** Effect of cations and chemical reagents on rBgl4 activity

| Metal ions/chemical reagents | Residual activity (%)[a] |
|---|---|
| Control | 100 |
| $Mg^{2+}$ | 124 |
| $Mn^{2+}$ | 132 |
| $Ca^{2+}$ | 122 |
| $K^{+}$ | 111 |
| $Zn^{2+}$ | 142 |
| $Na^{+}$ | 110 |
| $Hg^{2+}$ | 23 |
| $Cu^{2+}$ | 32 |
| $Ag^{+}$ | 0 |
| $Ni^{2+}$ | 109 |
| $Fe^{3+}$ | 103 |
| DTT | 102 |
| DMSO | 109 |
| Glycerol | 107 |
| EDTA | 105 |
| β-Mercaptoethanol | 121 |

Assay was carried out by incubating the enzyme in 10 mM of each metal ion and 1 % of chemical reagents and determining the residual β-glucosidase activity at standard conditions

[a] Values are mean value of three individual reaction results

to provide thermostability to the protein by managing the relative movements of C and N-terminal domains [11]. The signature sequence with the potential catalytic nucleophile ($Asp^{273}$) and a potential acid/base catalyst ($Glu^{502}$) are well conserved in Bgl4 of *P. funiculosum* NCL1. The characteristic hydrophobic cluster of GH3 β-glucosidase, 221-LYLWPF-228 reinforced the predictions. In general, the protein stability and elevated optimum temperature is directly correlated with number of glycosylation sites, number of acidic and aromatic amino acids. The Bgl4 sequence possessed 13 glycosylation sites, which is higher than that of

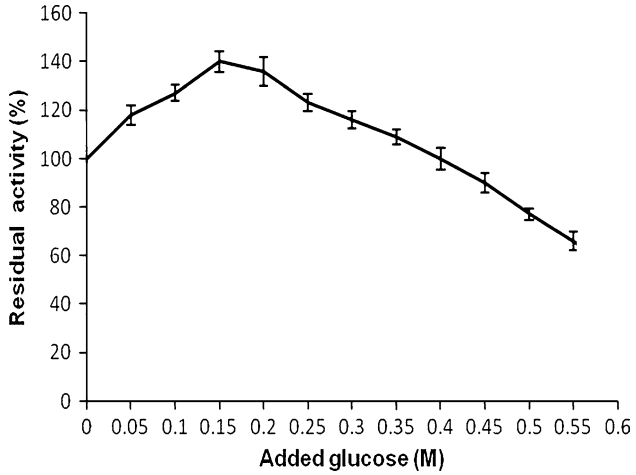


**Fig. 7** Influence of added glucose on the rBgl4 activity. Various concentrations (0–600 mM) of glucose was added to rBgl4 and incubated at room temperature. Residual activity was measured. Control was set with no added glucose and the activity was set as 100 %. Glucose concentration up to 150 mM has positive effect on rBgl4 activity. Results are average of three experiments and the *bars* denote the standard deviation of triplicates

earlier reported β-glucosidases, which implied the possible thermostability of Bgl4 [11]. The modeled structure of the Bgl4 consisted of $(\alpha/\beta)_8$ TIM barrel followed by six stranded β-sandwich, which is the characteristic feature of mesophilic and thermophilic β-glucosidase [7, 11]. The GH3-specific catalytic residues $Asp^{273}$ and $Glu^{502}$ most likely form the substrate-binding pocket [34]. Sixty-three amino acid-long hinge region that links the domains, probably play role in folding of the protein into the active form.

Though many genes belonging to the GH3 family were cloned from fungal origin, very few reports are available for their over-expression in heterologous host system such as *P. pastoris* (Table 3). The *bgl*4 gene of *P. funiculoum* NCL1 was successfully over-expressed in *P. pastoris* KM71H. The transformants grew well in the cellobiose-supplemented liquid and solid medium, implying that *P. pastoris* harboring pPICZ*bgl*4 construct is able to utilize cellobiose as sole carbon source. Moreover, when the transformants were overlaid with 4-MUG, they exhibited fluorescence under UV due to the release of 4-methyl umbelliferone, which indicated the secretion of recombinant β-glucosidase by *P. pastoris* KM71H (pPICZ*bgl*4). The recombinant protein rBgl4 was purified by metal affinity chromatography and the SDS-PAGE analysis showed that the purified rBgl4 has a molecular weight of ~130 kDa. N-Glycosylation of rBgl4 that was proved by Periodic acid-Schiff's staining can be corroborated to the increased molecular weight of rBgl4. Though the protein is hyperglycosylated, it did not affect the activity of the enzyme. This result is in concordance with hyperglycosylated recombinant β-glucosidase of *A. niger* that showed significant β-glucosidase activity [17]. Contrary to many of the β-glucosidases that exhibit dimeric or tertrameric conformation, Bgl4 is monomeric in nature [10, 28].

Since, substrate binding and catalytic mechanism of enzyme is based on the pH and temperature of the reaction, identifying the optimal conditions is essential for industrial applications [12]. When the rBgl4 activity was tested in various pH, it showed optimal activity at pH 5.0 and this range is similar to the reported fungal β-glucosidases [1]. Despite the mesophilic nature of *P. funiculosum*, purified rBgl4 exhibited optimal activity at 60 °C, which is higher than the reported optimal temperature of other β-glucosidases from mesophilic fungi [1]. Stability of β-glucosidase plays a vital role as they are involved in the cellulose hydrolysis at the elevated temperatures for long duration in order to increase solubility of reactants and to improve the reaction velocity of enzyme [20]. The rBgl4 displayed stability over

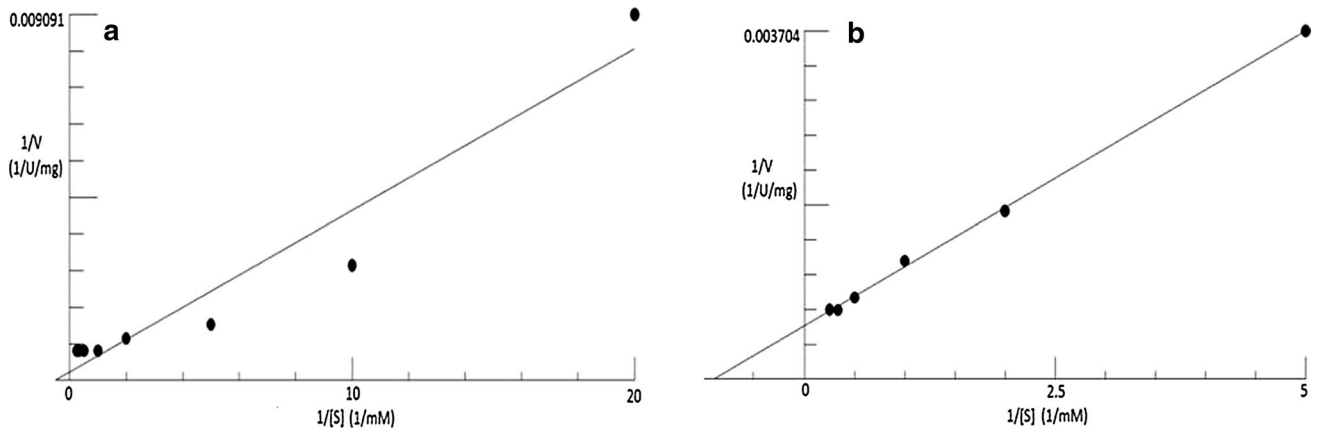


**Fig. 8** Double reciprocal plots stating enzyme kinetics of rBgl4. Enzyme kinetics analysis with substrate cellobiose (**a**) and pNβPG (**b**) as substrate. β-Glucosidase activity was calculated with 0.01 mM-10 mM cellobiose or pNβPG at 60 °C in 50 mM sodium acetate buffer pH 5.0

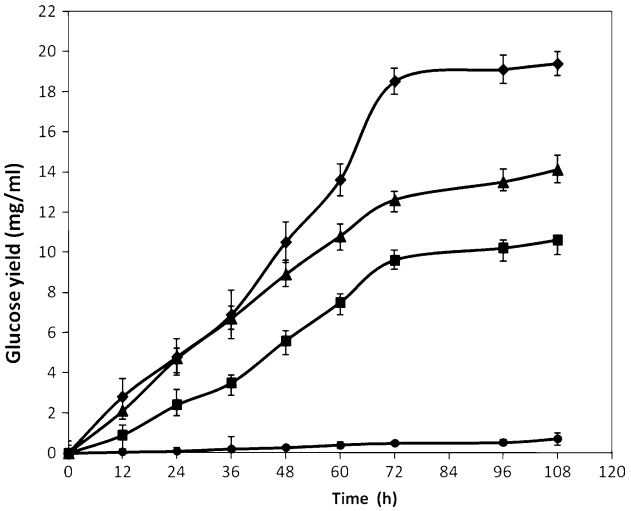


**Fig. 9** Synergistic activity of the rBgl4. Relative glucose production (mg/ml) from avicel (2.5 % w/v) by *T. reesei* extracellular cellulase system (*filled square*) (5 FPU/ml) in the presence of commercial β-glucosidase from sweet almond (*filled triangle*) and presence of purified rBgl4 (*filled diamond*) (100 U/ml) in 50 mM sodium acetate buffer pH 5.0 at 60 °C and 500 rpm at different time intervals. Experiment was carried out in triplicates and *error bars* show standard deviation between three observations. Results are the average of triplicates and the *bars* indicate the standard deviation of three experiments

a pH range of 3.0–7.0 and 40–60 °C and this is in contrast with other mesophilic fungal β-glucosidases that are stable only in the narrow range of temperature and pH [1, 27, 29]. The activity and stability of rBgl4 to broad range of pH and temperature of rBgl4 favors the industrial application.

The rBgl4 hydrolyzed a wide range of substrates such as cellobiose, pNβPG, pNβPX, salicin and esculin and this property can be correlated with the expression of native Bgl4 by *P. funiculosum* NCL1 grown with various carbon sources such as sugar cane bagasse, avicel, wheat bran and rice straw that ensures the ability of enzyme to hydrolyze various types of cellulosic substrates [24]. Xylan degrading activity of the rBgl4 is an added advantage for the biomass hydrolysis, which is made up of celluloses and xylans. Since the enzyme is specific for substrate with β-configuration, it did not show activity on the substrates with α-linkages. The rBgl4 activity was stimulated by $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Na^{2+}$ and $K^{+}$ implying that these cations could act as co-factor that facilitate the binding of catalytic amino acids with substrate thereby increasing the activity. $Ag^{2+}$ and $Hg^{2+}$ showed strong inhibition on rBgl4, suggesting the essential role of indole rings in maintaining proper tertiary structure of enzyme. Similarly, different levels of stimulatory and inhibitory effect by metal ions have been reported for fungal β-glucosidases [13, 26, 27]. A slight enhancement in the rBgl4 activity by β-mercapto ethanol suggested that the enzyme is a sulfhydryl enzyme and

**Table 3** Properties of recombinant β-glucosidases over-expressed in *P. pastoris*

| Source | Gene | Family | Vector | Host | Mr (kDa) | Quaternary structure | Optimal pH | Optimal Temp. (°C) | $K_m$ (mM) | | $V_{max}$ (μmol/min/mg) | | Glucose tolerance (mM) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | pNPG | Cellobiose | pNPG | Cellobiose | | |
| *Phanerochaete chrysosporium* | BGL | GH3 | pPIC9K | *P. pastoris* KM71 | 133 | NR | 4.5 | NR | 0.198 | 5.05 | NR | NR | 0.004[a] | [14] |
| *Thermoascus aurantiacus* | bgl1 | GH3 | pPICZα | *P. pastoris* KM71H | 115 | NR | 5 | 70 | 0.22 | 1.35 | 71.7 | NR | NR | [9] |
| *Aspergillus fumigatus Z5* | bgl3 | GH3 | pPICZα | *P. pastoris* X33 | 130 | Monomer | 6 | 60 | 1.76 | 2.2 | 131.4 | 52.9 | NR | [17] |
| *Neosartorya fisheri* P1 | NfBGL1 | GH3 | pPIC9 | *P. pastoris* GS115 | 80 | NR | 5 | 70 | 0.51 | NR | 2,172 | NR | 13.4 | [31] |
| *Penicillium funiculosum* NCL1 | bgl4 | GH3 | pPICZα | *P. pastoris* KM71H | 130 | Monomer | 5 | 60 | 2.5 | 1.25 | 3,332 | 2083 | 400[b] | This study |

[a] Ki calculated using glucanolactone

[b] The enzyme was incubated with various concentration of glucose for 30 min at room temperature and β-glucosidase activity was measured

the role of β-mercapto ethanol is in maintaining the enzyme in reduced state [26]. Glucose inhibition is generally found in fungal β-glucosidases with few exceptions such as β-glucosidases of *Aspergillus* sp. [23]. Interestingly, rBgl4 exhibited 37 % of enhancement in activity with 150 mM of glucose and showed tolerance up to 400 mM.

High conversion rate is the major phenomenon which evaluates the efficiency of enzymes, which is defined by the low $K_m$ and high $V_{max}$ with the specific substrate. rBgl4 exhibited $K_m$ value of 1.25 and 2.5 mM with cellobiose and pNβPG, respectively, which revealed that rBgl4 has higher affinity towards cellobiose than pNβPG. Moreover, this is the lowest value among the reported $K_m$ for fungal β-glucosidase for cellobiose (Table 3).

Since the commercial cellulolytic strain *T. reesei* is β-glucosidase deficient, administration of additional β-glucosidase from heterologous sources is mandatory to alleviate the cellobiose inhibition and to increase the conversion efficiency. Therefore, synergistic effect of rBgl4 with *T. reesei* cellulase on avicel hydrolysis was evaluated and enhancement in glucose yield was observed. Supplementation of the rBgl4 increased the glucose yield up to 37 % at 72 h. The higher glucose yield is probably attributed to the thermostability, high catalytic activity and glucose tolerance of rBgl4. The rBgl4 supplementation can be extended to hydrolysis of cost-effective cellulosic substrates by further optimizations of reaction conditions.

## Conclusion

In this study, the *bgl*4 gene encoding broad substrate-specific β-glucosidase isoform Bgl4 of *P. funiculosum* NCL1 was successfully over-expressed in *P. pastoris* expression system. Recombinant protein rBgl4 exhibits high-temperature optima, notable thermo- and pH-stability, high catalytic activity and glucose tolerance. The purified enzyme showed potential improvement in the saccharification yield by synergistic action with *T. reesei* cellulase, which favors the industrial application of rBgl4.

**Acknowledgments** This work was supported by the Department of Biotechnology (No. 5/258/51/2006-NMITLI). The UGC-CAS, CEGS, NRCBS, DBT-IPLS, DST-PURSE Programs of School of Biological Sciences, Madurai Kamaraj University, is gratefully acknowledged.

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