Characterization of a glucose tolerant β -glucosidase from *Aspergillus unguis* with high potential as a *blend-in* for biomass hydrolyzing enzyme cocktails

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Received: 9 May 2019 / Accepted: 17 August 2019 / Published online: 5 September 2019
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Abstract

Objectives Characterization of glucose tolerant beta glucosidase (GT-BGL) secreted by *Aspergillus unguis* NII 08123, determination of the gene and protein sequences of the enzyme and establishing its performance in blends for lignocellulose hydrolysis.

Results Supplementation of *A. unguis* beta glucosidase (BGL) to cellulase released 1.6 times more sugar within 12 h during the hydrolysis of lignocellulosic biomass. The enzyme was determined to be similar to BGL-F from *Emericella nidulans* by MALDI-TOF analysis, and was found to be a GH3 family protein. Molecular Docking simulation studies showed that the enzyme has lesser affinity for glucose (− 5.7 kcal/mol) compared to its substrate cellobiose (− 7.5 kcal/mol). The residues present in the N-

terminal domain are mostly involved in bond formation with both the substrate and the product, while the C-terminal domain contains the catalytic region. In-silico studies showed that its predicted structure is unlike that of previously reported BGLs, which might provide a clue to its exceptional catalytic activity.

Conclusion The GT-BGL from *A. unguis* NII 08123 was proven effective as a blend in for biomass hydrolyzing enzyme cocktails and the possible reasons for its glucose tolerance was determined through studies on its modeled structure.

Keywords β -glucosidase · Glucose tolerant · *Aspergillus unguis* · Biomass hydrolysis · Genome · Homology model

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10529-019-02724-z>) contains supplementary material, which is available to authorized users.

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Introduction

Conversion of the renewable lignocellulosic biomass to valuable products, including fuels, chemicals and chemical building blocks, is of strategic importance for sustainable development and energy security (Wu et al. 2016). Plant biomass is composed primarily of cellulose (35–50%), hemicellulose (20–35%) and lignin (15–30%), and its degradation is mediated by the hydrolytic enzymes cellulases and hemicellulases (Tabanag and Tsai 2018). Importantly, there are three classes of enzymes involved in cellulose breakdown—(1) endoglucanases which produce nicks in the cellulose polymer, thereby exposing reducing and non-reducing ends (2) exoglucanases, including d-cellobiohydrolases and cellobiohydrolases, which act on these reducing and non-reducing ends to liberate cellobiose units and (3) β -glucosidase (BGL) which cleave the cellobiose to liberate glucose. Majority of the microbes do not have a complete cellulolytic system capable of generating glucose from natural lignocellulosic material (Singh et al. 2017). On the other hand, fungi are known to secrete copious amounts of cellulases. The cellulolytic systems of most fungi are inducible and are tightly regulated by catabolite repression, which means that the organism produces cellulases only when cellulose or certain disaccharide hydrolysis products or their derivatives (e.g., sophorose, gentiobiose, cellobiose) are present in the medium, and the expression of cellulase genes is shut down in the presence of easily utilizable sugars like glucose (Amore et al. 2013). In several fungi, BGL is produced in lower quantities compared to the other two classes of enzymes, and it is slow acting, making BGL the rate limiting component in the hydrolysis of cellulose (Saloheimo et al. 2002). The BGL is also subject to inhibition by glucose and cellobiose.

This whole mechanism of induction and repression thus helps the fungus to save energy by eliminating the need to synthesize cellulases continuously when they are not required. However, this economy practiced by the fungus is not desirable when they are to be used as an industrial source of cellulases. From the foregoing discussions, the critical role of BGL in hydrolysis becomes apparent. As one of the major limitations in the saccharification of biomass is the product inhibition of BGL by glucose; and this key enzyme has to be present in sufficient quantities in the enzyme cocktail

used for biomass hydrolysis, for it to be efficient. Also, it is desirable for the enzyme to have better specific activity and to be tolerant to glucose.

The major sources of β -glucosidases are microorganisms like bacteria, filamentous fungus and yeast. Among these, filamentous fungi are considered as the most potent β -glucosidase producers (Tiwari et al. 2013). Preliminary studies in our lab showed that a fungal strain isolated from soil and identified as *Aspergillus unguis* produced a β -glucosidase with K_i of 0.8 M against glucose, which is the second highest glucose tolerance reported in the *Aspergillus nidulans* group (Rajasree et al. 2013). Among the several isoforms of BGL secreted by this fungus, a low molecular weight protein was identified as the highly glucose tolerant β -glucosidase (GT-BGL). This enzyme was produced in small quantities in response to induction by lignocellulosic substrates or known inducers like lactose.

In order to fully exploit the exceptional properties of this enzyme, it is necessary to understand the protein structure and sequence. The current study analyzed both genome-level and protein-specific data for elucidating the sequence of the *A. unguis* GT-BGL, and to use this, for gaining information on its structure and the molecular mechanisms behind its glucose tolerance. The performance of this enzyme in blends with different cellulases has also been evaluated. This information is expected to help understanding the regulation of BGL expression in this fungus, which in turn is needed for enhancing the production of this enzyme in commercially viable scales.

Materials and methods

Organism

Cultures of *A. unguis* NII 08123 were maintained on Potato Dextrose Agar (PDA) at 30 °C. Five days old sporulated PDA slants were used for enzyme production. The organism was sub-cultured at regular intervals and stored at 4 °C.

Production of β -glucosidase and zymogram and MALDI-TOF analyses for determination of the GT-BGL

β -Glucosidase from *A. unguis* was produced and assayed as described previously (Rajasree et al. 2013).

For zymogram analysis, 12% acrylamide gels without denaturing agents were used. Protein concentration was estimated by Bradford assay (Bradford 1976) and normalized before sample loading. For comparative analysis, two gels were cast, and both gels were loaded with replicates of the same samples. After electrophoresis, the gels were washed with distilled water and incubated in a 10 mM solution of 4-Methylumbelliferyl β -D-glucopyranoside (MUG) in citrate buffer (0.05 M, pH 4.8) for 10 min at 50 °C. Glucose tolerant proteins were detected by incubating one of the gels in a MUG solution containing 0.5 M glucose. The protein bands were visualized under UV. After separation by polyacrylamide gel electrophoresis and visualization using MUG, the band corresponding to BGL3 was excised from the gel and electro-eluted into sodium citrate buffer (0.05 M, pH 4.8). The protein was then digested with trypsin according to the protocol of (Trauger et al. 2002). The MS/MS spectra were acquired in the positive ion mode using a Axima-CFR Plus MALDI-TOF Mass Spectrometer from Shimadzu, Japan. Database search for protein identification was performed using the MASCOT search engine. The database query used was for microbial species against the SWISS-PROT and NCBI non-redundant databases, using only mono isotopic masses. MS-BLAST search was performed for sequence homology-based protein identity.

Cellobiase activity of *A. unguis* GT-BGL

Xylan-grown *A. unguis* cultures were filtered to remove the biomass. The filtrate was concentrated and then separated by 12% polyacrylamide gel electrophoresis. The gels were stained using MUG to visualize BGLs, and the band corresponding to GT-BGL was excised. The protein was electro-eluted from the gel and concentrated by lyophilization. The protein concentrate was then assayed for cellobiase activity by the protocol of Ghose (1987). Briefly, the protein was incubated for 30 min at 50 °C with 15 mM cellobiose, after which the mixture was analyzed by HPLC for the release of glucose. Appropriate substrate and enzyme blanks were included.

Hydrolysis of lignocellulosic biomass

Alkali pretreated rice straw (PT-RS) produced as per previously optimized protocols (Christopher et al.

2017) was used for analyzing the saccharification efficiency of enzyme blends containing *A. unguis* BGL. Cellulases from *Penicillium janthinellum* and BGL from *A. unguis* was produced according to protocols optimized in the laboratory and was assayed as per IUPAC methods (Ghose 1987). Hydrolysis was carried out in 150 ml screw capped conical flasks with a working volume of 30 ml. The biomass loading was 10% w/w, the cellulase loading was 20 FPU/g (0.606 ml) and BGL loading was 60 IU/g (3.75 ml). The reactants were incubated at 50 °C for 48 h, with sampling at every 12 h. Controls without BGL supplementation and with supplementation of 60 IU/g of commercial BGL preparations were run in parallel. Sugar release was monitored as total reducing sugar by the DNS method (Miller 1959).

Selection of the best inducer of BGL in *A. unguis*

A number of easily available carbon sources were checked for inducing the production of extracellular proteins and BGL by *A. unguis* NII 08123. The organism was grown in Mandels and Weber (MW) Medium (Mandels and Weber 1969) containing 1.0% glucose-as carbon source for 48 h to build up the biomass. The fungal biomass was then separated by filtration, washed with distilled H₂O to remove residual glucose, and then transferred to fresh MW medium (Mandels and Weber 1969) containing 1.0% (w/v) of one of the inducers—Glucose, Xylan, Cellulose, Cellobiose, Lactose, Wheat bran, Rice straw, Alkali-pretreated Rice Straw (PT-RS), Maltose or Fructose). Cultures were incubated at 30 $\pm$ 2 °C and 200 rpm for 120 h. Samples were collected at 24 h intervals for BGL assay and zymogram analysis.

In-silico characterization of BGL

The sequences encoding BGLs in *A. unguis* NII 08123 were obtained from the whole genome sequence data (generated through whole genome shotgun sequencing and submitted to GenBank with ID QLOF01000000 [unpublished results]) by using BLAST analysis to identify motifs conserved in β -glucosidases. The amino acid sequences of the proteins were obtained by translation using the EXPASY tool (Gasteiger et al. 2003).

Physicochemical characterization

Intrinsic properties like molecular weight, isoelectric point (pI), half-life, aliphatic index, instability index and Grand Average Hydropathy (GRAVY) were predicted using the EXPASY tool ProtParam (Gasteiger et al. 2003). The presence of signal peptides was checked using the SignalP program (Petersen et al. 2011).

Homology modelling of BGL structure

The three-dimensional structures of the proteins were homology modeled using the SWISS-MODEL workspace (Biasini et al. 2014), using appropriate templates. GT-BGL was modeled using the *Aspergillus oryzae* Family GH3 beta-D glucosidase structure (PDB ID-5FJJ) as template (Agirre et al. 2016). Visualization and analysis of the models were done using the Swiss PDB Viewer (Guex and Peitsch 1997) and PyMOL programs (DeLano 2002).

Docking studies

Computer-aided molecular docking of the substrates to the active site of modeled structures of the proteins was done using AutoDock (Goodsell and Olson 1990). Initial coordinates of the substrates (Cellobiose and Glucose) were obtained from the PDB database. The modeled structure of the protein was taken as the receptor protein. The binding energy of each complex was calculated and analyzed.

Screening and amplification of GT-BGL from the genome of *A. unguis*

The putative sequence of the highly glucose tolerant BGL (GT-BGL) of *A. unguis* was obtained from MALDI-MS analysis of the partially purified protein. The sequences of the peptide fragments were compared with those of the predicted secretory BGLs in order to identify the GT-BGL. PCR primers BGLF_F1 (TAGGTTCCGCCCTGAGGATCTTGG) and BGLF_R1 (CGTTTACGGGCTCACCGTCTTTC) were designed for amplification of the full coding sequence, and primer pairs BGLCAT_F1 (GCCAGAGATGCAATCACG) and BGLCAT_R1 (CCGCGTGATATCCATGGT) were used for amplification of the catalytic domain of the GT-BGL. The amplicons were then sequenced using standard protocols at the CSIR-NIIST DNA sequencing facility for confirmation.

Results

Hydrolysis of alkali pretreated rice straw using enzyme blends

Hydrolysis of the alkali pretreated biomass using *P. janthinellum* cellulase (at 20 FPU/g biomass enzyme loading) yielded 247 mg/g reducing sugar (25% of theoretical maximum efficiency) in 12 h. When supplemented with 60 IU of *A. unguis* BGL per gram biomass, a reducing sugar yield of 555 mg/g (55% of theoretical maximum) was obtained within 12 h (Table 1). Maximum hydrolytic efficiency (85% of theoretical maximum/858 mg/g) was attained in 36 h.

Table 1 Hydrolysis of alkali pretreated rice straw using *P. janthinellum* cellulase supplemented with *A. unguis* β -glucosidase or commercial BGL

Time (h)	<i>A. unguis</i>		Commercial BGL		Control—no additional BGL	
	60 IU/g		60 IU/g			
	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)
12	555 $\pm$ 1.9	55	351 $\pm$ 9.9	35	247 $\pm$ 12.7	25
24	834 $\pm$ 14.7	83	787 $\pm$ 14	78	696 $\pm$ 24.8	69
36	858 $\pm$ 15.6	85	701 $\pm$ 15.5	70	738 $\pm$ 18.4	73
48	819 $\pm$ 21.1	81	583 $\pm$ 33.9	58	708 $\pm$ 7.4	70

Fig. 1 Secreted BGL activity of *A. unguis* cultures grown with different inducers

Fig. 2 Zymogram analysis of secreted BGL isoforms from *A. unguis* grown with different inducers. Lane Information: 1-glucose, 2-xylan, 3-cellulose, 4-cellobiose, 5-lactose, 6-wheat

bran, 7-rice straw, 8-pretreated rice straw (PTRS), 9- maltose, 10-fructose. Arrows indicate the position of the BGL isoforms. *Image inverted for clarity

Sugar yields for experiments performed with commercial BGL supplementation to the cellulase, was lesser than the cellulase plus *A. unguis* BGL combination, at all the hydrolysis durations tried. This indicates the synergy between specific cellulases and BGLs, which is a key requirement for efficient biomass hydrolyzing cocktails. While the commercial BGL used here is one of the best currently available in the market, GT-BGL from *A. unguis* in blends with *P. janthinellum* cellulase, could perform better than this. Apparently, BGLs from *A. unguis* may have better

synergy with *P. janthinellum* cellulases than commercial BGLs, resulting in better hydrolytic performance.

Cellobiase activity of GT-BGL

By HPLC analysis of pure sugars, the retention times of glucose and cellobiose were determined to be 16.229 and 13.952 min, respectively. The chromatogram of the test sample showed a peak at 16.116 min indicating presence of glucose. No such peak was detected in the control samples, thereby

Fig. 3 Zymogram analysis of secreted BGL isoforms from xylan grown *A. unguis* in presence and absence of glucose. Zymogram performed in the absence (**a**) and presence (**b**) of 0.5 M glucose on *A. unguis* culture supernatants from 24, 48, 72 and 96 h xylan grown cultures (Lanes 1, 2, 3 and 4 respectively in both **a** and **b**). Arrows indicate the position of the BGL isoforms. *Image inverted for clarity

confirming that the glucose is released from cellobiose due to enzyme action, confirming that the enzyme is indeed a beta glucosidase.

Partial purification and identification of glucose tolerant β -glucosidase by zymogram and MALDI-TOF analysis

On comparing *A. unguis* cultures grown in media containing different inducers, the maximum BGL activity of 0.11 IU/ml was observed in the xylan induced culture (Fig. 1). The zymogram revealed that the organism secretes multiple β -glucosidases (Fig. 2) and five isoforms of the BGL were detected when the

fungus was grown in xylan, lactose, wheat bran or pretreated rice straw as carbon source. BGLs from xylan induced cultures were tested for activity in presence of 0.5 M glucose in a separate zymogram, which showed the predominant presence of three major bands which included the proposed GT BGL. Proteins corresponding to these activity bands were named as BGL1, BGL2 and BGL3 (Fig. 3). BGL1 is the high molecular weight isoform and with the highest expression among the three. On the other hand, BGL3 had the lowest molecular weight among these isoforms and it was identified as the GT-BGL as it persisted even on incubation with 0.5 M glucose (Fig. 3).

Based on its relative position on 12% Native poly acrylamide gel electrophoresis (Native PAGE), BGL3 was speculated to be a low molecular weight protein (Rajasree et al. 2013). MALDI-TOF-MS analyses revealed the presence of two peptide fragments, showing identity with the glycosyl hydrolase C-terminal domain of *Emericella nidulans* β -glucosidase F (Table 2 and Supplementary Fig. S1), a region common to all glycosyl hydrolase family 3 (GH3) members. The GH3 C-terminal domain (pfam00933) is involved in the catalysis and may also be involved in the binding of β -glucan.

Identification of putative glucose-tolerant β -glucosidase (GT-BGL) from the *A. unguis* genome

Whole genome shot gun sequencing of the *A. unguis* was performed using Next Generation Sequencing and the genome sequence was submitted to Genbank with Accession Number QLOF01000000 (unpublished results). Glycoside hydrolase (GH) family proteins were identified using the Multiple EM for Motif Elicitation (MEME) server (Bailey et al. 2006) from among the predicted proteins obtained from genome data. Motif families were identified by sequence

Table 2 Confirmation of GH3 family peptide matches from the BGL3 protein through MALDI-TOF-MS analysis

Peak name	Start	End	Observed	Mr (exp)	Mr (cal)	Peptide
P1	445	521	8179.66	8179.66	8179.89	K.GCNRGVLTMGWGSSTKLPYLITPQEAIANITPTA EFFITDSFPSSVDANDEDIAIVFINSDSGENYITVDGNPGDR.K
P2	730	777	5014.09	5014.09	5014.35	K.TYPYPDGYSTDPQPPPRAGGAEGGNPALWDVAFS VQVTVTNTGQHSGR.A

Table 3 Affinity of G2944 (GT-BGL) towards cellobiose and glucose determined through molecular docking studies

S. no.	Secretory BGL isoform	Affinity to cellobiose (kcal/mol)	Affinity to glucose (kcal/mol)
1.	G493	− 6.0	− 5.4
2.	G2944	− 7.5	− 5.7
3.	G3151	− 6.9	− 6.9
4.	G6460	− 7.1	− 5.7
5.	G9078	− 6.8	− 6
6.	G9081	− 6.8	− 5.5

searching in Pfam database. After analysis of the conserved motifs, 17 glycoside hydrolase genes were predicted to be β -glucosidases. Fourteen of these genes belonged to GH3 family while 3 belonged to the GH1 family. The SignalP program predicted six of these proteins to be secretory (Supplementary Table S1). These predicted secretory BGLs varied in their physicochemical properties (Supplementary Table S2). The molecular weights of the secretory BGLs ranged from 82 to 135 kDa. Among the 17 predicted BGLs, initial screening for the glucose tolerant β -glucosidase was done through peptide

similarity search, using the peptide sequences of GT-BGL obtained from MALDI-MS data. Translated protein sequence from the GH3 β -glucosidase gene with gene ID g2944 matched the GT-BGL peptide sequences obtained from MALDI-MS analysis. The peptide P1 from MALDI-MS data showed 100% query coverage with 91% sequence identity, while peptide P2 showed 85% sequence identity with 100% query coverage to g2944 (Supplementary Fig. S2). No significant matches were obtained with the other BGL sequences; therefore, it was hypothesized that g2944 is the coding sequence for the glucose tolerant BGL3.

Fig. 4 Molecular Docking Simulation of GT-BGL (G2944) with cellobiose and glucose. The sequence of GT-BGL was modelled using *Aspergillus oryzae* Family GH3 β -D-glucosidase (PDB ID: 5FJJ) as the template. Amino acid residues interacting

with cellobiose (blue) and glucose (red): Tyr 79 (blue), Arg 108 (Teal), Arg 210 (Teal), Asp 273 (Green), Ser 445 (Pink), and Glu 497 (Orange). Yellow lines show the hydrogen bonds. Groove where ligands bind is shown zoomed in inset for clarity)

Fig. 5 Confirmation of the presence of GT-BGL (g2944) in the *A. unguis* genome and its expression by PCR amplification of the gene using genomic and cDNA templates. PCR amplification of full length (~ 5000 bp) gene of GT-BGL (g2944) from *A. unguis* genomic DNA (a), amplification of catalytic domain of

G2944 from *A. unguis* genomic DNA (b), amplification of catalytic domain of G2944 from cDNA of Xylan grown cultures. M = O'GeneRuler 1 kb DNA ladder (Thermo Scientific), Lane 1 = Amplicons from respective experiments

Docking studies

3D models of the six secretory BGL proteins of *A. unguis* were generated through homology modeling at the Swiss Modeler Homology Modeling server using appropriate templates (Supplementary Table S3). *In-silico* docking studies with the substrate (cellobiose) and product (glucose) was performed to determine the affinity towards the substrate and the product, so as to identify the GT-BGL protein and to determine the amino acid residues interacting with the substrate and the product. The binding affinity to cellobiose was highest for the protein G2944 compared to the other secretory BGLs and this protein model showed a comparatively lower affinity for glucose confirming that G2944 could indeed be the GT-BGL (Table 3). As per the predicted model of G2944 (the speculated GT-BGL), cellobiose and glucose binds to Tyr 79, Arg 108, Arg 210, Asp 273, Ser 445, and Glu 497 (Fig. 4). All these residues except Tyr 79, Ser 445 and Glu 497 are part of the *N* terminal glycosyl hydrolase domain of BGL. The latter two amino acids among these belong to the GH3 *C* terminal (catalytic) domain.

Amplification of GT-BGL from genomic DNA and cDNA

The putative GT-BGL sequence g2944 (BGL3) is a 4309 bp long nucleotide sequence. The BLASTX analysis of the sequence revealed ~ 85% identity to β -1,4-glucosidase from *A. nidulans* FGSC A4, and other similar proteins. Conserved domains were detected by PROSITE analysis, including a β -D-glucoside glucosyl hydrolase domain (residues 3–815), the GH3 *N*-terminal domain (residues 92–332) and the GH3 *C*-terminal domain (residues 394–623), which is involved in catalysis and may be involved in binding β -glucan; and the DUF572 domain which is a eukaryotic protein domain present in proteins required under certain conditions (residues 873–1092).

The complete sequence was amplified from the genomic DNA of *A. unguis* using the primer pair BGLFF1-BGLFR1 which gave an ~ 5000 bp amplicon (Fig. 5a). The catalytic domain (Glycosyl hydrolase family 3 *C*-terminal domain) was also amplified using the primer pair BGLCAT_F1-BGLCAT_R1 and this gave an ~ 1200 bp amplicon as expected (Fig. 5b). Amplification of the catalytic domain was also performed using cDNA of the xylan-induced culture as template using the same primer pair, which gave the ~ 1200 bp amplicon, which was then

sequenced and confirmed as the GH3 family β -glucosidase (Fig. 5c). On alignment using CLUSTALW, it was observed that the peptide P1 is also a part of this domain, thus confirming our prediction that g2944 encodes BGL3 (Supplementary Fig. S3).

Discussion

The *Aspergillus* genus is well known for the secretion of different carbohydrate active enzymes (CAZymes) including BGLs. BGLs produced by *Aspergilli* have been widely studied and are used industrially. The BGL secreted by *A. niger* is considered as a standard in commercial β -glucosidase production (Sørensen et al. 2013). Since BGLs catalyze the rate-limiting step in the hydrolysis of cellulose and are susceptible to product inhibition, there is a lot of scope for utilizing β -glucosidases with glucose tolerance to develop highly potent cellulase enzyme cocktails.

There are several reports of other yeast and fungal strains producing glucose tolerant β -glucosidase (GT-BGL), notably the extracellular β -glucosidase from *Candida peltata* (NRRL Y-6888), which has a K_i of 1.4 M (252 mg/ml) (Saha and Bothast 1996), and the β -glucosidase from *Aspergillus niger* CCRC 31494 with a K_i of 0.543 M. The latter enzyme also has the added advantage of not being inhibited by other sugars like fructose, galactose, mannose, and xylose (Yan and Lin, 1997). In comparison, the strain used in this study—*A. unguis* NII 08123, produces a highly glucose tolerant β -glucosidase (BGL3) with a glucose inhibition constant (K_i) of 0.8 M, which is the second highest tolerance to glucose reported amongst BGLs from the *A. nidulans* family.

The BGL enzyme preparation from *A. unguis* also improved the rate of lignocellulose hydrolysis. The rate of glucose release was much higher, while using cellulase cocktails supplemented with *A. unguis* BGL as compared with commercial BGL supplementation. Another important observation was that on using the commercial enzyme mixture, there was a rapid drop in sugar concentration after 24 h, probably due to the conversion of glucose back to di- or oligosaccharides by the *trans*-glycosylation activity of BGLs in that preparation. Compared to this, the *A. unguis* enzyme supported peak yields for longer durations (up to 36 h) which is important for biomass conversion to fuels/chemicals, since the hydrolysate normally has

to undergo further unit operations before the final product recovery. On the whole, the *Aspergillus unguis* BGL preparation released approximately 1.6 times more sugar than the commercial BGLs within a 12 h period.

In addition to deconstruction of biomass, BGLs have other intracellular functions and several isoforms of this enzyme are normally secreted by fungi. The SDS-PAGE analysis of different strains of *A. niger* showed different BGLs ranging in molecular weight between 68 and 220 kDa (Narasimha et al. 2016), whereas in *Penicillium italicum* a 354 kDa β -glucosidase was identified (Park et al. 2012). β -Glucosidases predicted from the genome of *A. unguis* in this study, range in molecular weight from 82 to 135 kDa.

MALDI-TOF analysis of BGL3 indicated that it has a molecular weight of approximately 15 kDa. Almost all BGLs deposited so far in SWISS-PROT and NCBI non-redundant databases have a molecular weight above 20 kDa. BGL3 therefore may be considered as a rare example of a low molecular weight glucose tolerant β -glucosidase. Interestingly, comparison of the peptide sequences with the whole genome data showed that they are part of a predicted 135 kDa GH3 family β -glucosidase. We therefore speculate that this difference in the molecular weights of observed and predicted proteins may be because of a post translational modification.

The in silico characterization of the protein also confirmed that the protein has the ability to withstand high concentrations of its product glucose. Docking simulations indicated that the glucose tolerant β -glucosidase has lesser binding affinity for its product glucose (-5.7 kcal/mol) compared to the affinity for its substrate cellobiose (-7.5 kcal/mol). There are very few reports available regarding the mechanism of glucose tolerance. A novel β -glucosidase (Bgl6) with an IC-50 of 3.5 M against glucose was identified from a metagenomic library (Cao et al. 2015). Homology models of two GH1 β -glucosidases Bgl1A and Bgl1B, previously identified from bacteria were used to study the mechanism of glucose tolerance by Fang et al. (2010). The simulated models of Bgl1A and Bgl1B showed differences in eight sequences around the substrate channel. The two proteins were then mutated by interchanging these sequences between them. In Bgl1A there was no direct correlation between glucose tolerance and the eight amino acid residues. Surprisingly, in Bgl1B, mutations in two sequences—B4

(V₂₂₇H₂₂₈ mutated to F₂₂₇T₂₂₈) and B5 (N₃₀₁V₃₀₂ to Q₃₀₁F₃₀₂)-led to a surge in glucose tolerance. Further analysis using single-point interchange mutants showed that the change H₂₂₈T is accountable for the tolerance.

From the study of Bgl1A and Bgl1B mutants H₂₂₈T and N₃₀₁Q/V₃₀₂F, it was observed that in both cases, glucose binds outside of the active site, most preferably at the middle/entrance of the channel; reducing the chances of competitive inhibition between the substrate and the product probably accounting for the glucose tolerance. Binding of glucose to the middle of the channel can also stimulate substrate cleavage by trans glycosylation (Yang et al. 2015). However, these studies also revealed that glucose tolerance is influenced by other factors like folding because molecular docking of Bgl1A with glucose recommended several possible binding positions with different preferences.

In our docking analysis, it was found that the G2944-cellobiose complex was stabilized by means of hydrogen bonding with Tyr 79, Arg 108, Arg 210, Asp 273, Ser 445, and Glu 497, while all these residues except Tyr 79 were also involved in enzyme–substrate interactions in the G2944-Glucose complex (Fig. 4). All these residues except Tyr 79, Ser 445 and Glu 497 are part of the *N* terminal glycosyl hydrolase domain of BGL; Ser 445 and Glu 497 belong to the GH3 *C* terminal (catalytic) domain. Apparently, from the observation of glucose and cellobiose binding to the possible catalytic groove, it seems the amino acid residues of the enzyme interacting with these molecules are similar indicating the reasons for product inhibition. At the same time, the protein has a higher affinity for cellobiose compared to glucose, which could be the reason for its higher glucose tolerance. However, current results are insufficient to draw conclusions on the molecular reasons for higher glucose tolerance and further efforts are on to elucidate this.

Acknowledgements PKV wishes to thank CSIR for providing research fellowship to pursue his PhD studies at NIIST. We are thankful to Department of Biotechnology, Govt. of India for R&D funding to RKS under the project BT/PR20695/BBE/117/211/2016. The *P. janthinellum* strain is a kind gift from Dr. D. V. Gokhale, from the CSIR-National Chemical Laboratory, Pune, India. We thank Dr. N Ramesh Kumar, MPTD, CSIR-NIIST for sequencing services and Mass Spectrometry and Proteomic Core Facility, Rajiv Gandhi Centre for Biotechnology (RGCBC), Thiruvananthapuram for the MALDI-TOF analysis.

Compliance with ethical standards

Ethical approval This study was partially funded by Department of Biotechnology Govt. of India (BT/PR20695/BBE/117/211/2016). RKS, the corresponding author declare on behalf of all authors that we do not have any conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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