An Engineered *Aspergillus fumigatus* GH3 β-Glucosidase with Higher Glucose Tolerance

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β-glucosidases (3.2.1.21) are present in all domains of living organisms, and their importance in a number of essential biological processes and industrial applications has been highlighted. They are interesting for biomass conversion because b-glucans are the world's largest source of biomass. For this reason, several fungal β-glucosidases have been investigated. The β-glucosidase gene of *Aspergillus fumigatus*, as well as its mutants D262E and W263F, were cloned and expressed in *Pichia pastoris* in this study. Their optimum temperature, pH, glucose tolerance, metal ion effect, and V_{max}, k_m, and k_{cat} were determined. The optimal temperature for recombinant β-glucosidase was 65 °C. For mutant D262E, there is an improvement in pH stability ranging from 4 to 6. As compared to the D262E mutant and recombinant β-glucosidase, mutant W263F showed a higher glucose tolerance and k_{cat}.

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INTRODUCTION

Lignocellulosic material can be refined into commercially valuable products such as ethanol and glucose. Among the lignocellulosic materials, cellulose is the most plentiful and renewable organic molecule and carbohydrate on Earth (Zhang 2008). The rate of cellulose synthesis is estimated at 4×10^7 tonnes per year (Signh 1995). Cellulose is a biopolymer made up of hydro-glucopyranose molecules linked together by 1,4-glycosidic bonds (Pérez and Mazeau 2004). Cellulases comprise a complex enzyme system of β-1,4-endoglucanase, β-1,4-exoglucanase, and 1,4-β-cellobiosidase/β-glucosidase. These enzymes enable hydrolysis of β-1,4 linkages of glucose residues in cellulose (Xia *et al.* 2016). β-glucosidase hydrolyze glycosidic bonds to release non-reducing terminal glucosyl residues from glycosides and oligosaccharides. β-glucosidase releases glucose from cellobiose, and it also decreases cellobiose inhibition and improves the efficiency of the endoglucanase and exoglucanase enzymes (Liu *et al.* 2012). These enzymes are present in all realms of living organisms and perform a wide range of functions. D-glucosidases have

been isolated from a variety of fungal species (Lima *et al.* 2013). Novozymes is using *Aspergillus niger* for the production of β-glucosidase for commercial consumption (Novozymes SP 188; Novo Nordisk A/S, Bagsvaerd, Denmark). *Aspergillus fumigatus* is a thermophilic saprophytic fungus that secretes thermophilic cellulases. Based on their amino acid sequences, glucosidase genes belong to the glycoside hydrolase (GH) families (Dodda *et al.* 2018). GH1 and GH3 are the most common fungal β-glucosidase families (Suzuki *et al.* 2013). The modelled structure β-glucosidase displays the N-terminal (a/b)8 TIM barrel Domain A and C-terminal (a/b)6 sandwich structure Domain B with a shallow and open active site (Dodda *et al.* 2018). The predicted catalytic nucleophile is aspartate, which is present in the TIM barrel domain's conserved motif VMSDW, and glutamate acts as a proton donor in the catalytic site during catalysis (Da Costa *et al.* 2018)

Different important residues have been identified for their catalytic functions. Asp-247 had been identified as significant nucleophile by tandem mass spectrometry and site directed mutagenesis of D247E and D247G in *Flavobacterium meningosepticum* (Li *et al.* 2001). By using site-directed mutagenesis, the amino acids Glu-413, Gln-339, Ala-201, and Phe-269 were identified as important candidates in the acid/base catalytic reaction in the cassava plant (Keresztessy *et al.* 2001). Trp-49 and Trp-262 in the active site of β-glucosidase have been implicated in the transglycosylation reaction (Seidle *et al.* 2006). β-glucosidase has been recognized for their broad potential in the food, chemical, pharmaceutical, paper, textile, and biofuel industries (Kuhad *et al.* 2011; Molina *et al.* 2016). Many fungal β-glucosidases have been exploited for the production of better β-glucosidase. In this study, GH3 recombinant β-glucosidase and two of its catalytic site mutants from *Aspergillus fumigatus* were expressed in *Pichia pastoris.* All three β-glucosidases were purified and characterized to determine if the mutations enhance enzyme activity and glucose tolerance.

EXPERIMENTAL

Genomic Data Mining

The genome database of *Aspergillus fumigates* was examined for β-glucosidase genes (http://www.aspergillusgenome.org). The gene accession number HQ836475.1 for β- glucosidase was chosen for further research. The gene size is 2592bp, which produces protein of 863 amino acids.

Construction of Plasmids

The construct was designated as pPICZaA/β-glucosidase, corresponding to sequence coding for the full length β-glucosidase gene. The gene was codon-optimized for expression in *Pichia pastoris*, synthesized and inserted into the vector pPICZ α A (GenScript, Piscataway, NJ, USA) using the restriction sites Not I and Xba I.

Site-directed Mutagenesis

The β-glucosidase conserved domain region was selected for site-directed mutagenesis. Based on conserved domain functional importance, amino acid aspartic acid at nucleotide position (c.786C>A) was chosen to substitute the amino acid glutamic acid to study the glucose tolerance and functional effects of the enzyme. The site-directed mutagenesis was performed by PCR with the following conditions: denaturation at 97 °C for 2 min; followed by 30 cycles of 40 sec at 94 °C, 30 sec at 54 °C, and 40 sec at 72 °C;

and a final extension step for 7 min at 72 °C. The mutant p.Asp262Glu was generated using (β-glucosidase) with the forward primer D262E-F (5-aaggcttcgtcatgagtgaatggagc-gctcaccaca-3) and the reverse primer D262E-R (5-tgtggtgagcgctccattcactcatgacgaagcctt-3). The mutant p. W 263 F was generated from the β-glucosidase gene with primer F 5-AAGGCTTCGTCATGAGTGACTTCAAGCGCTCACCACA-3 and primer R 5-TGTGGTGAGCGCTCCATTCACTCATGACGAAGCCTT-3. The PCR product was purified with 1 µL of Dpn1.

Sequencing Analysis

Purified PCR product sequencing was performed by using the set of primers; forward primer: 5-aagaatgcggccgccaggaattggctttctctccac-3; reverse primer 5-agcgtctcta-gatagtagacagcgggcagagg-3; to validate the desired mutation in our desired position and the gene carrying mutation was introduced in *Pichia pastoris* by using vector pPICZαA for expression analysis (Liu *et al.* 2011).

Transformation and Expression

Constructs were first propagated in DH5α and selected on low salt LB plates supplemented with 25 µg/mL zeocin. The plasmid was extracted (GeneJet Miniprep Kit, Thermo Fisher Scientific, Waltham, MA, USA) and linearized using MssI (Thermo Fisher Scientific). Linearized plasmids were transformed into *Pichia pastoris* X-33 by electroporation using Gene Pulser Xcell™ Electroporation System (Bio- Rad, Hercules, CA, USA). Transformants were selected on YPD plates supplemented with 1000 µg/mL zeocin.

Expression of β-Glucosidase, D262E and W263F Mutants in *Pichia pastoris* X-33

Positive colonies were selected and inoculated into 25 mL of buffered glycerol-complex medium (BMGY) for 24 h at 28 °C and under agitation. Cells were then harvested by centrifugation at 2000 g for 5 min at room temperature and re-suspended to an $OD_{600\ nm}$ of 1 in buffered methanol-complex medium (BMMY). Cells were grown at 28 °C for 72 h. Methanol was added to the culture at a final concentration of 0.5% every 24 h to maintain induction. Cultures were then centrifuged, and the supernatant was ultrafiltered and concentrated on a 30 kDa crossflow membrane (Sartorius AG, Gottingen, Germany).

Purification

Crude extracts containing His-tagged proteins were purified using Ni Sepharose ® 6Fast Flow resin (GE Healthcare, Chicago, IL, USA). Expasy's ProtParam was used to calculate protein concentrations by calculating absorbance at 280 nm and using the molar extinction coefficients predicted by the tool (https://web.expasy.org/protparam/).

SDS-PAGE Analysis

SDS-PAGE was performed using a 12% (w/v) polyacrylamide gel and run at 200 V for 30 min, using Tris-glycine SDS buffer pH 8.3 (Bio-Rad) as running buffer. Proteins were stained with Coomassie Brilliant Blue R-250 (Laemmli 1970).

Enzymatic Assays

β-glucosidase activity was determined by the measure of the hydrolysis of p-nitrophenyl- b-D-glucopyranoside (pNPG) (Sigma, St. Louis, MO, USA). Different

concentration (1mM to 40mM) pNPG was used for analysis. The reaction was initiated by adding 10 µL of 0.02088 mg/mL purified enzyme to 20 µL of 50 mM sodium acetate buffer (pH 4.0) and 20 µL of 1 mM pNPG. The reaction mixture was pre-incubated at 65 °C for 10 min. The reaction was terminated by adding 100 µL of 1 M Na_2CO_3. The absorbance was read at 410 nm. A standard curve of pNP (from 0 to 0.5 mM) was used to determine the activity of the β-glucosidase. Lineweaver Burk plots were used to calculated k_m and V_{max} by plotting $1/S$ on the horizontal axis and $1/V$ on the vertical axis. Under the above assay conditions, one unit of β-glucosidase activity is described as the amount of enzyme needed to release 1 µmole of pNP per min.

pH and Temperature Optimum

The effect of pH on β-glucosidase activity was investigated by determining the activity on pNPG at 65 °C, as described above, in Britton Robinson buffers ranging from pH 2 to 9 (Reymond *et al.* 2009). The optimum temperature for β-glucosidase was investigated by measuring enzyme activity in a 50 mM sodium acetate buffer, pH 4.0, at different temperatures ranging from 20 to 90 °C.

Effect of Metal Ions

The effect of eight different cations (Cd^{+2}, Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2}, Ni^{+2}) on β-glucosidase activity was determined with 2 mM of each ion concentration in a standard enzyme assay. β-glucosidase enzyme activity without any cation was taken as control to measure relative activity. Initially ODs were calculated and then converted into enzyme activity according to pNPG standard curve readings. Each reaction was done in triplicate, and the average was taken for further analysis.

Glucose Tolerance

The effect of glucose on enzymatic activity was investigated as previously stated, by varying glucose concentration from 0 to 50 mM and using 1 mM pNPG. Initially ODs were calculated and then converted into enzyme activity according to pNPG standard curve readings. Each reaction was done in triplicate, and the average was taken for further analysis.

Determination of Kinetic Parameters

The kinetic parameters of purified β-glucosidase were determined at 65 °C and pH 4.0 using pNPG as substrates according to standard enzyme assay described above.

RESULTS AND DISCUSSION

Production and Purification

In this study *Aspergillus fumigatus* β-glucosidase and two of its mutant versions were expressed in *Pichia pastoris* X-33. The proteins were purified and characterized. Non-mutant β-glucosidase enzyme was produced at 0.75 mg per liter of *Pichia pastoris* X-33 culture. D262E and W263F mutant enzymes were produced at 0.70 and 0.71 mg, respectively, per liter of *Pichia pastoris* X-33. Size of β-glucosidase is 94 kDa but because of glycosylation bands of approximately 150 kDa β-glucosidase appeared on gel (Fig. 1).

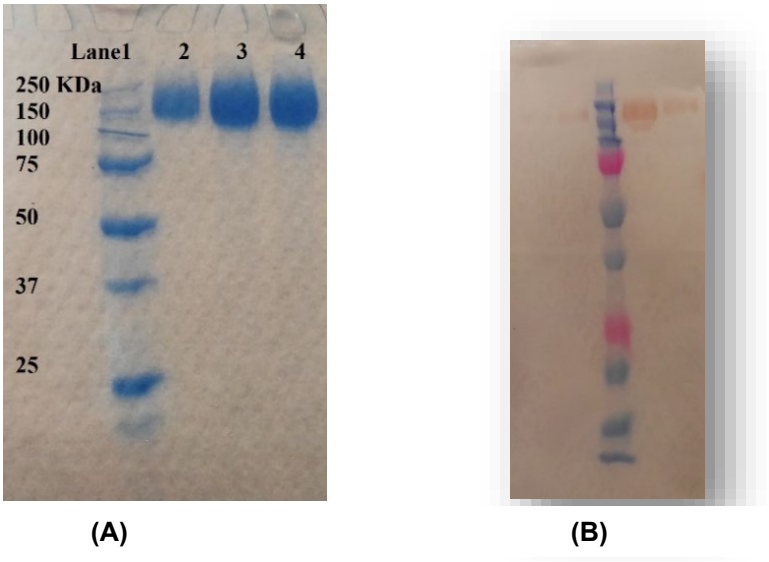

Fig. 1. Purification (A) SDS PAGE: Lane 1, Ladder; Lane 2, recombinant β-glucosidase; Lane 3, β-glucosidase D262E Mutant; Lane 4, β-glucosidase W263F mutant (B) Western Blot: Lane 1 is recombinant β-glucosidase, lane 2 is 250 KDa protein ladder, lanes 3 and 4 are mutant β-glucosidase

Optimum Temperature and pH

β-glucosidase enzyme and its mutants were optimized for their optimum temperature and pH (Fig. 2). Enzyme activities were calculated for the temperature range of 20 to 90 °C. The optimum temperature of β-glucosidase was 65 °C, which is higher than most other thermophilic fungal β-glucosidases reported as 40 to 70 °C (Molina *et al.* 2016). Both mutants showed an optimum temperature 60 °C, which is slightly less than the recombinant enzyme. β-glucosidase with optimum temperature of 65 °C is better than other fungal β-glucosidase. Enzyme activity was measured in pH 2 to 9 (Fig. 3). β-glucosidase had an optimum pH of 4. The D262E mutant showed stability in pH from 4 to 6, and W263F had an optimum pH of 5. These values were similar to many reported fungal β-glucosidase with an optimum pH of 4.0 (Krisch *et al.* 2010).

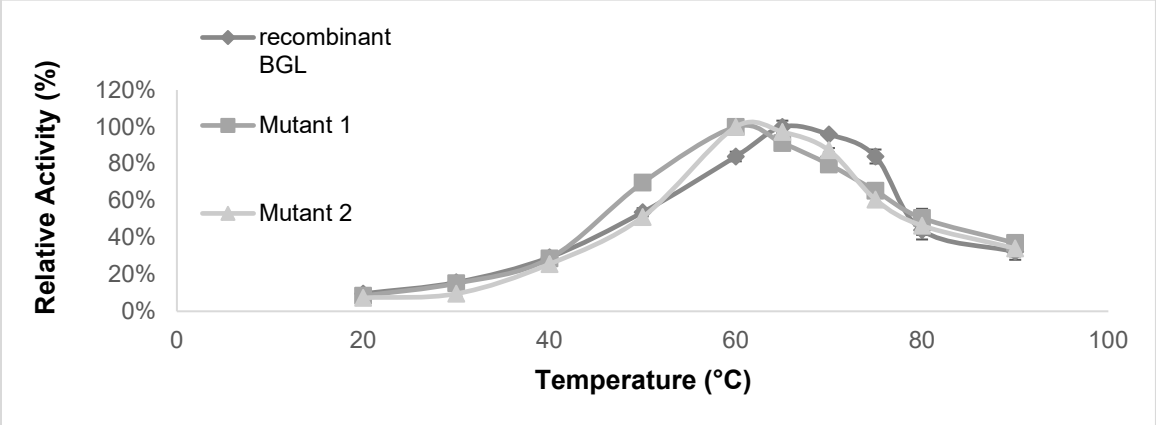

Fig. 2. Effect of temperature on activities of β-glucosidase, Mutant 1(D262E), and Mutant 2 (W263F). The standard deviation is represented by bars.

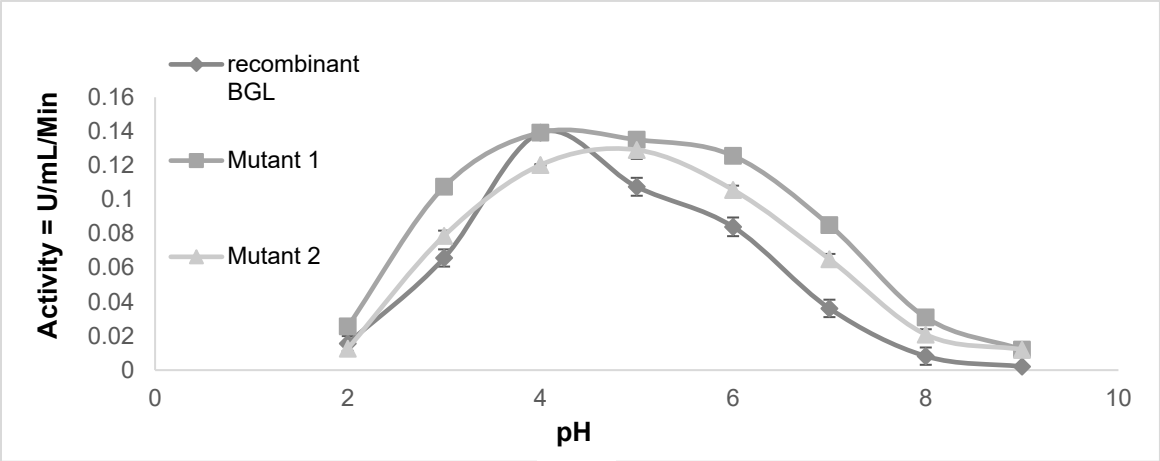

Fig. 3. Effect of pH on activities of β-glucosidase, Mutant 1(D262E), and Mutant 2 (W263F). The SD is represented by bars.

Effect of Cations

The effect of different cations on β-glucosidase activity was studied. The recombinant enzyme didn't work well with any of the eight cations, while the activity of D262E mutant was stimulated as increase in activity 112% with 2 mM calcium ion and 96% with 2 mM zinc ion. Meanwhile there was slight increase in activity in W263F mutant with 2 mM calcium ions. The other cations showed no significant effect on enzyme activities (Fig. 4). These findings were mostly in accordance with other studies of β-glucosidase of other fungal species that the divalent ions tested did not show significant effects on the activities at 2 to 5 mM concertation, whereas few studies revealed that β-glucosidase were strongly activated by Zn^{2+}, Ca^{2+}, Mn^{2+}, and Mg^{2+} (Bai *et al.* 2013; Oh *et al.* 2018).

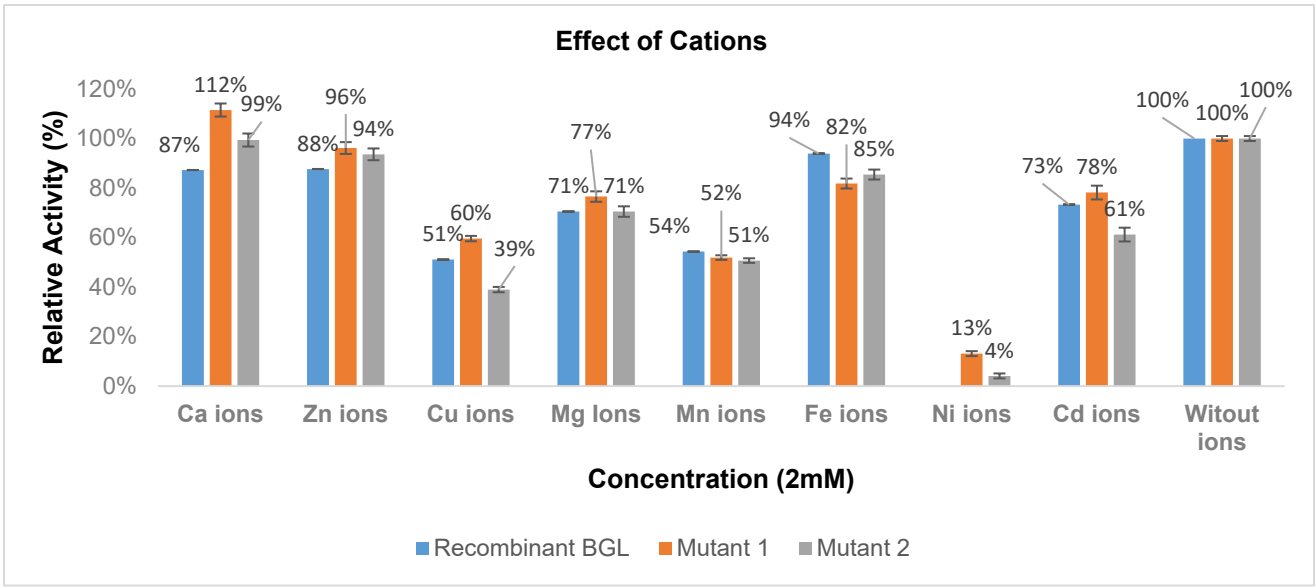

Fig. 4. Effect of cations on activities of β-glucosidase, Mutant 1(D262E), and Mutant 2 (W263F). The SD is represented by bars.

Glucose Tolerance

The effects of glucose on activity of β-glucosidase and its mutants were investigated. Glucose in concentrations of 0 to 50 mM was added to standard enzyme assay, and residual activities were measured (Fig. 5). At 50 mM glucose, mutants D262E and W263F resume 50% and 27% residual activities as compared to the recombinant β-glucosidase, which showed 16% residual activity in 50 mM glucose. Both mutants showed increase in glucose tolerance. Mutating Aspartate-262 residue into glutamate showed better glucose tolerance, which has not been reported previously. For the tryptophan to phenylalanine mutation, similar results have been reported for GH1 family β-glucosidase mutant W127F (Kuhad *et al.* 2011).

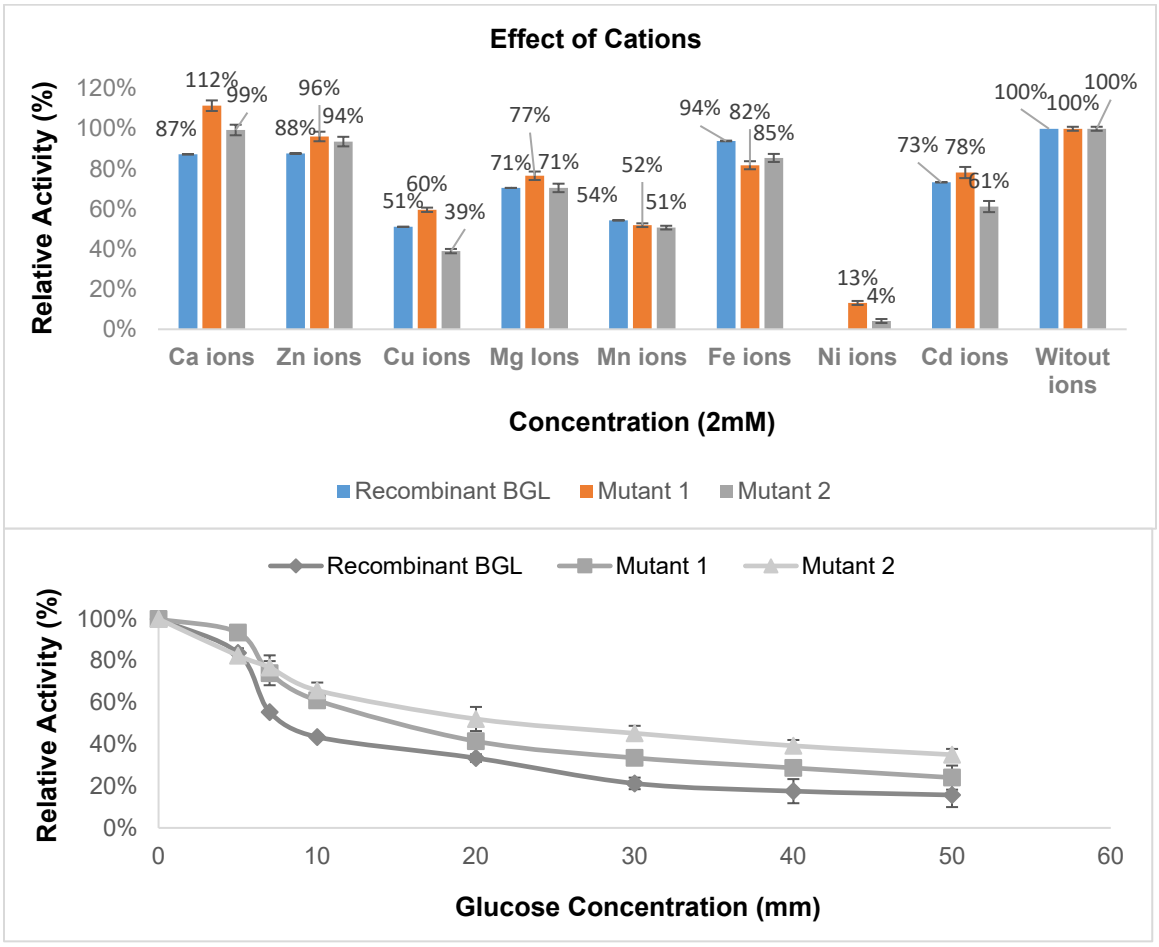

Fig. 5. Effect of glucose on activities of β-glucosidase, Mutant 1(D262E), and Mutant 2 (W263F). The SD is represented by bars.

Enzyme kinetics

Enzyme activities were analyzed for its K_m, V_{max}, and catalytic efficiency with standard enzyme assays using pNPG as substrate. The recombinant enzyme showed maximum catalytic efficiency as of 837.9 K_{cat}/s, which is better than many reported *Aspergillus* species β-glucosidase ranging from *Aspergillus fumigatus* (217/s) to (786/s) and *Aspergillus oryzae* (651/s) (Bohlin *et al.* 2010; Molina *et al.* 2016). The mutant D262E showed a slight decrease in K_{cat} 796.4/s as compared with recombinant *Kcat* 837.9/s. Mutant W263F showed the highest K_{cat} value of 1089.2/s (Table 1).

Table 1. V_{max}, K_m, and K_{cat} of Recombinant β-glucosidase and Mutant (D262E) and (W263F)

Substrate pNPG	Vmax (mM PNP/min)	K_m (μM)	K_{cat} (s^-)
Recombinant β-glucosidase	11.66 ± 0.26	14.36 ± 0.33	837.99
Mutant 1 D262E	10.88 ± 0.21	13.86 ± 0.29	796.44
Mutant 2 W263F	14.68 ± 0.30	9.180 ± 0.17	1089.24

In sum, these findings indicate that the recombinant *Aspergillus fumigatus* β-glucosidase and its W263F mutant produced in *Pichia pastoris* can be explored for further endeavors, as it has better catalytic activity. The D262E mutant can be studied further for its better glucose tolerance.

CONCLUSIONS

1. Recombinant β-glucosidase displayed maximum activities at 65 °C and retained most of its activity within the acidic pH range of 4, making it suitable thermophilic enzyme for industrial applications.
2. Mutational analysis showed increase in pH stability for D262E mutant form pH 4 to pH 6, which further enhance its applicability.
3. Both mutant (D262E and W263F) had increase in glucose tolerance as compared to recombinant and other fungal β-glucosidases. Mutants D262E resumed 50% and W263F resumed 27% residual activities as compared to the recombinant β- glucosidase which showed 16% residual activities.
4. Catalytic activity (K_{cat}) of W263F mutant was highest at 1089 K_{cat}/s, followed by recombinant 838 K_{cat}/s then 796 K_{cat}/s for D262E mutant. These results indicated the production of catalytically active β-glucosidase. W263F mutant showed good affinity towards its substrate as indicated by its low K_m value.

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