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Research Article

Biochemical and *in silico* evaluation of a recombinant, glucose tolerant, and highly thermostable β-glucosidase from *Thermococcus radiotolerans* DSM-15228

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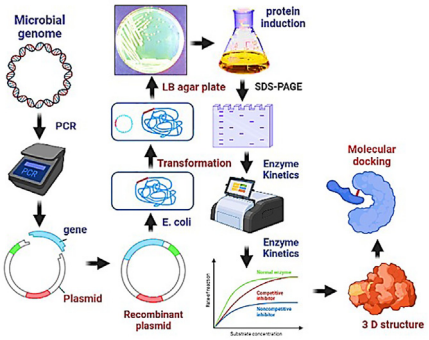
GRAPHICAL ABSTRACT



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ABSTRACT

Background: β-glucosidase (EC 3.2.1.21) catalyzing the β-glycosidic linkages in polysaccharides is a ubiquitous enzyme with great importance in biofuel and other industries. Enzyme inhibition by glucose has been considered as the major hurdle in the practical applications of this enzyme. Therefore, there has been a continuous search for novel β-glucosidase with high glucose tolerance and stability at industrial temperature. In the present study, recombinant of β-glucosidase from *Thermococcus radiotolerans* has been produced and characterized.

Results: The enzyme was overexpressed in *Escherichia coli* strain BL21 (DE3) codon plus RIPL and purified by selective heat denaturation, ethanol precipitation and anion exchange chromatography. Purified enzyme displayed a band on SDS-PAGE with a molecular weight of 50 kDa. Optimum enzyme activity was found at pH 5, and 85°C, it retained more than 46% activity when incubated at 100°C for 5 min and exhibited 80% activity in the presence of 800 mM glucose. Km and V_{max} values of the purified enzyme were found as 16.3 mM of pNPG and 25.8 μ moles per min. Molecular docking studies have shown a strong binding affinity of pNPG with the enzyme active site consisting of Glu^{365}, Asn^{266}, and Trp^{295} as major the active site amino acids. MD simulation analysis has shown a significantly high stability of enzyme active site and high potential of enzyme substrate complex formation.

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Conclusions: The novel characteristics such as relatively low Km value, extremely high-temperature stability and tolerance of high glucose concentration advocate the enzyme as a potential candidate for the industrial applications.

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1. Introduction

β-glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of glycosidic bond in order to free the non-reducing terminal residues of glucosyl from oligosaccharides and glycosides [1]. β-glucosidases are universally found in all domains of living organisms including eubacteria, archaea, and eukaryotes. Physiologically, these enzymes are responsible for the conversion of biomass in microorganisms, breakdown of exogenous glucosides and in some animals, lignification of oligosaccharides in cell wall, activation of the conjugate of phytohormone and plant defense mechanisms [2,3,4]. Microbial β-glucosidases are localized outside of or attached to cells, some of them are also found inside the cells. As for example, *Trichoderma reesei* are known to make β-glucosidase both inside and outside of their cells [5,6,7]. It has been shown that glycoceramides accumulation is a primary cause of Gaucher's disease, which is characterized by lysosomal glycoceramide accumulation [3]. Cellulases are the essential enzymes of the cellulose metabolizing system (cellulose hydrolysis) [8]. Among its many uses are the production of biofuels and ethanol from cellulosic agricultural waste, as well as the synthesis of useful β-glucosides [9,10]. In the process of extracting juice from wine grapes and liberating the aroma, these enzymes are utilized to hydrolyse bitter compounds [11]. An essential part of the flavour industry is the enzymatic release of aroma compounds from glucosidic precursors used in fruits and fermented products [5]. Food processing industries use enzymes to enhance the flavour of wine, tea, and fruit juices [12,13,14,15,16].

Modern genetic engineering tools and techniques have enabled the production of large quantities of beta-glucosidase recombinants for a variety of purposes [17]. Genetically modified strains of *Escherichia coli* have been extensively used as host organism for the production of recombinant enzymes and proteins on industrial scale [18]. The recombinants produced in *E. coli* have been approved by the food and drug administration authorities of USA; these proteins are easy to purify due to relatively less number of host proteins [19]. The recombinants of β-glucosidase have been produced from different sources including *Aspergillus saccharolyticus, Trichoderma harzianum*, *scleractinian corals*, and *Anoxybacillus ayderensis* and characterized for its properties and potential applications [20,21,22,23]. *Thermococcus radiotolarens* is a hyperthermophilic archaeon that has been isolated Mid-Atlantic Ridge (23°22′N, 44°57′W) at a depth of 3.5 km. The species grows at high salt concentration with an optimum growth as pH 6 and 88°C. The laboratory analysis of the *Thermococcus* genome sequence has shown the putative cellulase and translational genes for glucosidases present as constitutive enzyme [20,24]. Browsing microbial genomes at NCBI revealed that the genome sequence of some strains of *Thermococcus radiotolerans* harbor the genes encoding β-glucosidase. The objective of the current work is to clone, heterologously express, biochemically characterize, and *in silico* analyse the gene encoding β-glucosidase from *T. radiotolerans* for the first time ever.

2. Materials and methods

2.1. Materials

T. radiotolerans DSM-15228 genomic DNA was provided by DSMZ, Germany which was used as template for the amplification of β-glucosidase gene. DH5α cells (catalogue no. 18265017), TransformAid bacterial transformation kit (catalog no. k2710), DNA marker 1 kb DNA Ladder (catalogue no. SM0311), prestained protein marker PAGERulerTM, 10–180 kDa (26616). (USA), fast digest DNA restriction enzymes (*Nde*I and *Bam*HI), CloneJET PCR Cloning Kit (K1231), 2x PCR master mix (catalogue no. K0171) were purchased from Thermofisher Scientific. GeneEluteTM PCR Clean-Up Kit (Merck catalogue No. NA1020), GenElute™ Gel Extraction Kit (Merck catalogue No. NA1111), T7 promoter based expression vector pET21a (+) was obtained from Novagen, 4-Nitrophenyl-β-D-glu copyranoside (PNPG) was obtained from Calzyme laboratories Inc. California. Genetically, modified *E. coli* strain BL21 (DE3) codon plus RIPL (catalogue no. 230280) were obtained from Agilent Technologies.

2.2. PCR amplification and cloning of β-glucosidase gene

T. radiotolerans DSM-15228 has a 1254 bp gene (gene ID: WP_088866786.1) coding for β-glucosidase. The target gene was PCR amplified by using forward and reverse primer sequences 5'-catatgctcaggtttcggatggatt-3′ and 5′-ggatctcagccacctgacatcccatat-3′, respectively. In the reaction mixture 2x master mix (Thermofisher catalogue no. K0171), 40 picomole primers, and 10–15 ng of template DNA were used. The thermocycler was adjusted at 94°C for 40 s (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (extension), these conditions were repeated for 35 cycles. PCR amplified β-glucosidase gene was analyzed by agarose gel electrophoresis. GeneEluteTM PCR Clean-Up Kit (Merck catalog No. NA1020) was used to purify the PCR product stock. CloneJET PCR Cloning Kit (Thermo Fisher Scientific Catalogue No. K1231) supplied the pJET1.2 cloning vector for ligating the purified PCR product (Thermofisher catalogue No. K1231). Recombinant plasmids were synthesized according to the manufacturer's instructions and were used for the transformation of *E. coli* (DH5α) competent. TransformAid bacterial transformation kit (Thermofisher catalogue no. k2710) was used in the transformation process. Successfully transformed *E. coli* (DH5α) cells appeared as independent colonies on LB-agar plates containing 100 mg of ampicillin per ml of medium. Presence of target gene in the recombinant plasmid was verified by the restriction analysis of isolated plasmid with fast digest *Nde*I and *Bam*HI. The restriction reaction of 50 µl volume consisted of 100 ng of isolated plasmid DNA, 5U of each enzyme, 1x green buffer and nuclease-free water. The reaction mixture was incubated at 37°C for 10 min followed by analysis on agarose gel electrophoresis. Subcloning of the gene was performed by the elution of restricted gene from agarose gel using GenElute™ Gel Extraction Kit (Merck catalogue No. NA1111)

method as described by the suppliers, ligation of target gene and pET21a (+) plasmid restricted with the above-mentioned pair of enzymes. A recombinant plasmid pET21a-BG was generated, and BL21 (DE3) RIPL codon plus cells of *E. coli* were transformed using recombinant plasmid as described for first cloning step.

2.3. Expression induction of recombinant β-glucosidase

An LB broth flask with 30ml of LB broth containing 100 mg/ml of ampicillin per ml of medium was inoculated with a single colony of recombinant BL21 (DE3) RIPL codon plus cells, incubated overnight in an orbital shaker adjusted at 37°C and 200 rpm. One percent of the overnight culture was grown in the fresh flask under above conditions to acquire an optical density of 0.6 at 600 nm. A regular measurement of OD at 600 nm was performed to monitor the growth of the cells. Protein production was induced by incubation at 37°C and 150 rpm with 1 mM IPTG for 3 h. As a control experiment, the cells transformed with pET21a (+) minus β-glucosidase gene were also processed in parallel. Centrifugation at 10000 g for 5 min was used to harvest the cells, and the pellet was dissolved in distilled water and separated on SDS-PAGE [25].

2.4. Measurement of the β-glucosidase activity

A modified version of procedure described in the literature was used for the measurement of enzyme activity [26]. The activity of β-glucosidase was determined by preparing the experimental and control tubes in parallel. In the experimental test tube, 1000 µl acetate buffer pH 5 (buffer A), 500 µl of 50 mM 4-Nitrophenyl-β-D- glucopyranoside (PNPG) solution prepared in the buffer A, 100 µl of enzyme solution were carefully added. A negative control reaction (with 100 µl of water replacing the enzyme) was also prepared. Both tubes were incubated at 37°C for 15 min, and the reaction was stopped by the addition of 2.0 ml of Na_2CO_3 solution. The absorbance was measured by making the control as blank at 400nm, and enzyme activity was calculated by using **Equation 1** where ΔOD400nm indicated the change in optical density at 400 nm, ENZ.D.F stands for enzyme dilution factor.

$$Enzyme\ \ activity\ \ (U/mL) = \frac{\Delta OD400nm/\min \times Total\ \ reaction\ \ vol.(mL) \times Enz.\ \ D.\ \ F.}{(18.1) \times Enz.\ \ Vol.\ \ (mL)}$$

Equation 1

Total soluble protein content of the enzyme sample was determined by Bradford method [27], and specific activity of enzyme was calculated as units of enzyme per mg of solution.

2.5. Enzyme purification

After gene induction, bacterial cells were precipitated by centrifugation at 7000 × *g* for 10 min and suspended in buffer B (20 mM phosphate buffer pH 7.5, adjusted at 4°C). The suspended cells were sonicated [28,29]; clear supernatant was obtained by centrifugation at 12,000 × *g*. Enzyme activity was measured in the clear solution, which was purified by selective heat denaturation at 70°C for 10 min to denature the majority of proteins from *E. coli* origin followed by centrifugation as above. In the next step, enzyme from supernatant was precipitated by ice-chilled ethanol (35% of final sample solution) and dialysis was performed using buffer B. The clear supernatant was stored in an ice box after clarification of dialysate by centrifugation at 12000 × g for 15 min at 4°C. Equilibration of a 2.5 × 36 cm chromatography column with 30 mL of DEAE-Sephadex column was conducted by buffer B. A flow rate of 2 ml per minute was used to load the sample solution onto the column. An ice box was used to store the unbound proteins. A linear gradient of sodium chloride (0 to 400 mM) was used to elute the bound proteins. Fractions with higher protein concentrations are kept in an ice box and tested for activity and purity. Fractions with 0.5 to 2 mg/ml protein content were pooled and stored at −20°C.

2.6. Effect of temperature, pH, glucose, and substrate concentration on enzyme activity

The effect of temperature on the enzyme activity was determined by conducting the reaction at different temperatures

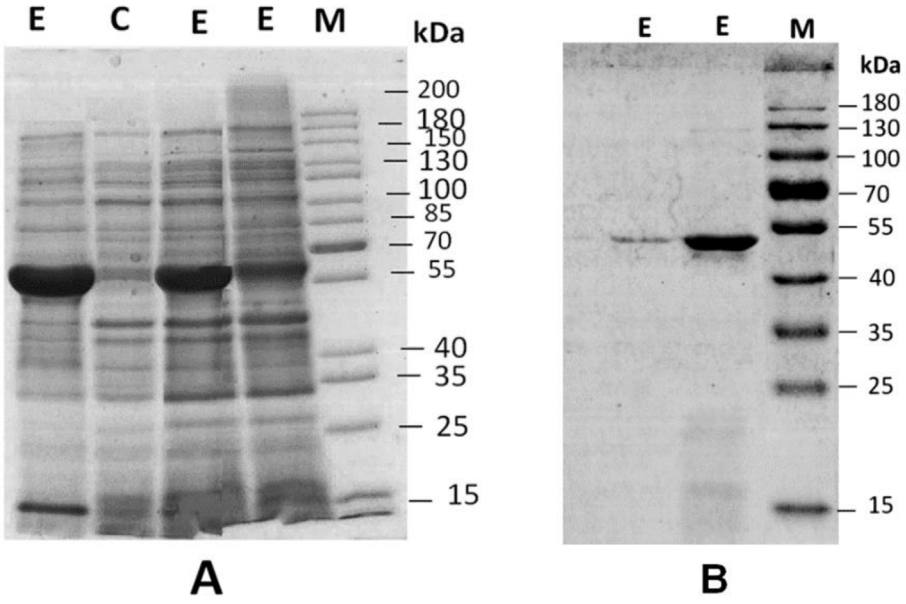

Fig. 1. SDS-PAGE photograph illustrating the expression and purification of β-glucosidase. A - Lane-M. Protein marker (Thermofisher - unstained protein ruler), Lane E - experimental with the expression of the gene, Lane C - a control experiment (without the gene), B - M represents protein marker (prestained protein marker PAGERulerTM, 10-180 kDa), Lane E - purified enzyme beta glucosidase. The molecular weight of the purified enzyme was found as 50 kDa.

Table 1
Activity of the enzyme, total protein content, specific activity, percentage yield, and fold purification of recombinant β-glucosidase. A total of 50 mL of crude extract was processed in three steps. One unit of enzyme is the amount of enzyme capable to convert 4-Nitrophenyl-β-D-glucopyranoside (pNPG) into its product per minute under our assay conditions.

Purification step	Activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Total units	Percentage recovery	Fold purification
Crude extract	19	6	3.16	1805	100	1
Dialysis of ethanol precipitate	98	18	5.4	1550	85.6	1.7
DEAE - Sephadex Colum	122	1.4	87.1	876	48	27.56

ranging from 35°C to 95°C; all other parameters were kept constant. To determine the effect of pH on the enzyme activity buffer solutions of different pH values, ranging from 4 to 7 were prepared and used as the medium for assay. For pH 4, 4.5, 5, and 5.5, sodium acetate buffer was prepared, for pH 6, 6.5, and 7, sodium phosphate buffer was prepared. These buffer solutions were selected on the basis of their pKa values and effective buffering range. Temperature stability of enzyme was determined by pre-reaction incubation of enzyme sample at temperatures from 40°C to 100°C for 5 min. To determine the glucose tolerance, activity of enzyme was measured in the presence of 25, 50, 100, 200, 400, and 800 mM of glucose. This was compared with its activity in the absence of glucose. Effect of linear increase on the enzyme activity was determined by increasing the substrate concentration in the reaction mixture. The values of 1/V and 1/[S] were subjected Lineweaver–Burk plot on Microsoft excel, Km and Vmax values were calculated.

2.7. In silico studies

AlphaFold server [30] was used to predict the 3D structure of beta-glucosidase from *Thermococcus radiotolerans*. This structure for pNPG was obtained from the PubChem database [31]. The Maestro interface [32] was used to prepare proteins and ligands, predict active sites, and perform extra precision docking [33]. Based on a molecular dynamics simulation of the Desmond package in the Schrödinger Maestro software, the stability of the beta-glucosidase and pNPG complex was estimated. A 50 ns run was performed, a TIP4P water model was applied, and the NPT temperature was set at 300 °K. In addition, in an effort to better estimate the binding affinity, the thermal_mmgbsa.py script of the Prime module is available in Schrodinger [34].

3. Results

3.1. Gene cloning and expression

An open reading frame of 1254 nucleotides representing β-glucosidase gene was PCR amplified, cloned into pJET1.2, and subcloned into pET21a (+) plasmids. Upon transformation with the recombinant pET-BG plasmid, the cells were subjected to gene expression, which resulted in the appearance of a prominent protein band at about 50 kDa on SDS-PAGE (Fig. 1A). All the recombinant enzyme was found in the soluble and active form which was purified, fractions with protein content ranging from 0.5 to 2 mg/ml were pooled together and analysed on SDS-PAGE (Fig. 1B).

3.2. Purification and kinetics of recombinant β-glucosidase

Recombinant β-glucosidase has been purified using DEAE-sephadex-based anion-exchange chromatography. A phosphate buffer (20 mM, pH 7.5) was used as the mobile phase, and a linear

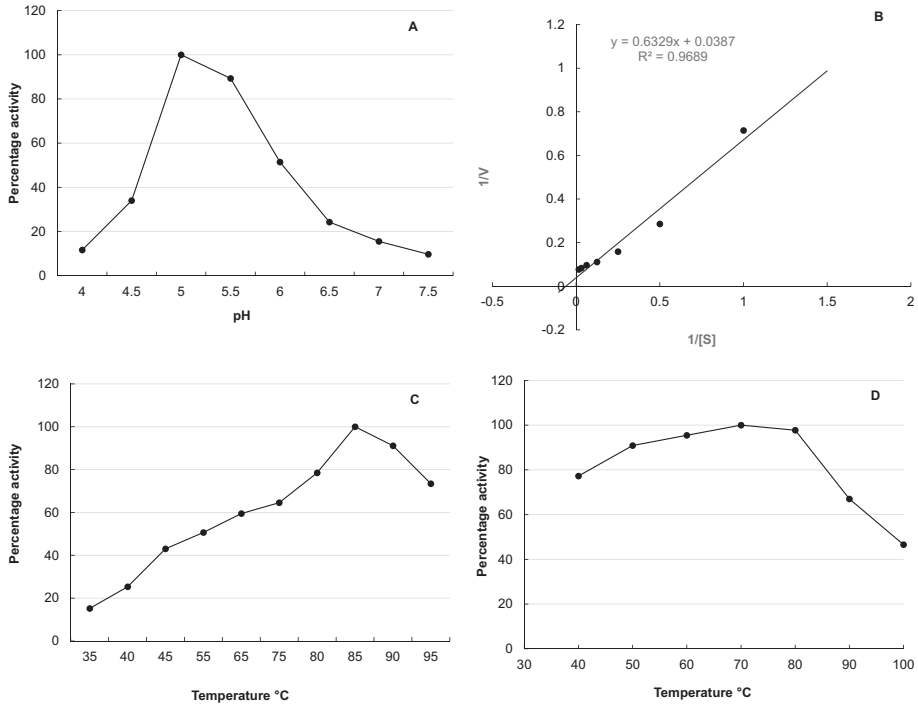

Fig. 2. A study of the kinetics of recombinant β-glucosidase. Effects of pH on enzyme activity (A) Lineweaver-Burk plot for Km and Vmax calculations (B) Effects of temperature on enzyme activity (C) Effects of temperature on enzyme activity, (D) Enzyme stability against temperature.

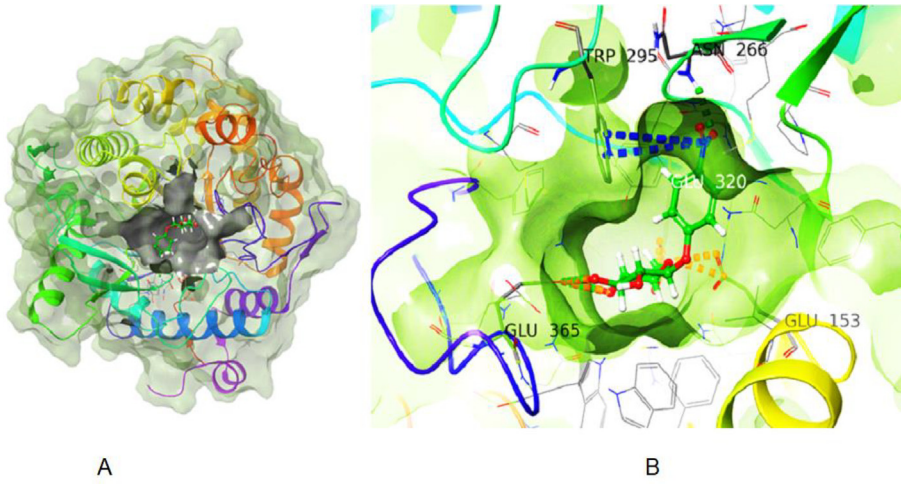

Fig. 3. 3D interaction of β-glucosidase and pNPG as enzyme-substrate complex, (A). Magnified model of the ES-complex showing the substrate in the active site. (B). The magnified view of ES-complex, the substrate is shown in the middle, the interacting residues and bonds are shown in different colours, H-bonds (green), (yellow), (blue).

NaCl gradient from 0 to 0.4 M was used to elute the protein. SDS-PAGE analysis was performed on fractions consisting of 2 mL of elution sample (Fig. 1). A pool of fractions containing purified enzyme was prepared. Purified enzyme had a specific activity of 87.1 U/mg, and a total of 876 units were obtained after 48% recovery and a 27.56-fold purification (Table 1). An optimal enzyme activity was measured at 85°C and pH 5; Km values for pNPG were 16.3 mM, and Vmax values for enzyme were 25.8 μmol per min (Fig. 2). When 25 mM, 50 mM, and 100 mM glucose were present, no significant change in enzyme activity occurred, however, enzyme activity gradually decreased at high glucose concentrations, and up to 20% decreased at 800 mM glucose.

3.3. In silico analysis of β-glucosidase

Molecular docking results showed a high binding affinity of beta-glucosidase to pNPG substrate in which ΔG = −7 kcal/mol were generated. Three H-bonds were generated with Glu365 (two bonds), one H-bond occurred with Asn266, and bi-cation bonds occurred with Trp295 as shown (Fig. 3).

4. Discussion

β-glucosidase (EC 3.2.1.21) is one of the three enzymes catalyzing the complete breakdown of cellulose to glucose which can be

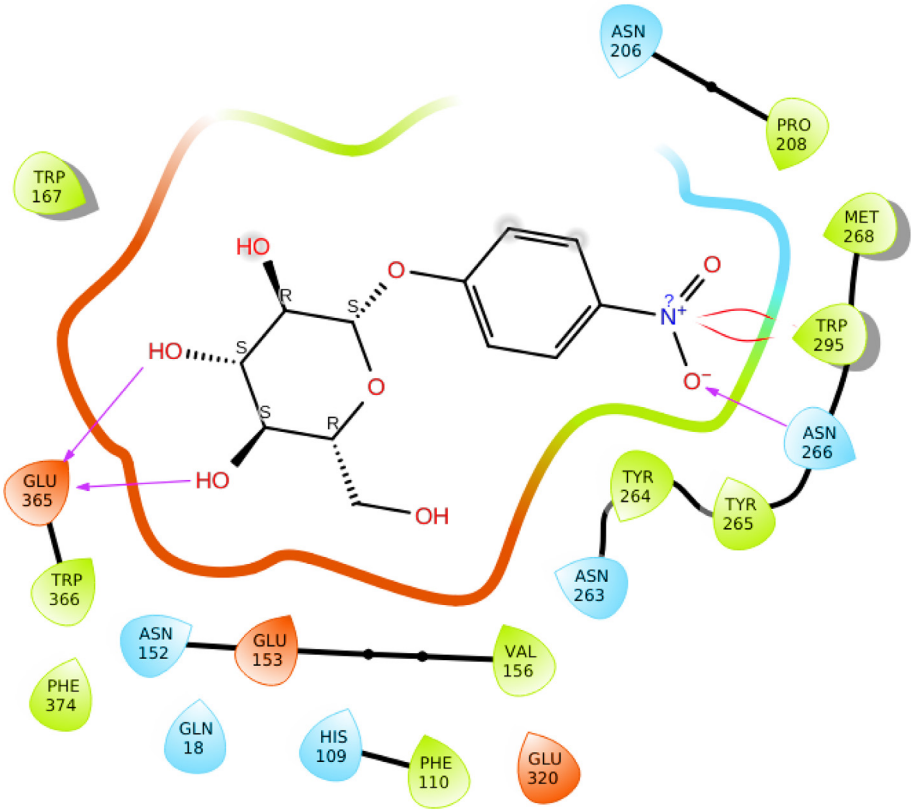

Fig. 4. 2D interaction of β-glucosidase and pNPG, showing the ligand in the center surrounded by active site amino acid residues. The H-bonds are shown in purple arrows, the bi-cation bonds are in red, and the amino acids are colored according to their charge.

further converted to ethanol - an environment friendly biofuel [35,36,37]. Several recent studies have reported the cloning, expression, and characterization of β-glucosidase from different sources in *E. coli* [38,39,40]. These investigations have generated a big pool of literature representing β-glucosidases. However, the end product (glucose) inhibition of enzyme remains a big issue in the practical applications of β-glucosidase in biofuel industry. This inhibition occurs due to better access of glucose product to the enzyme active site as compared to the real reactants [41,42]. Hence, there is a continuous research for the identification or engineering of novel β-glucosidase that can be useful at high glucose concentrations. On the basis of above information, the present study was aimed at the characterization of β-glucosidase from *T. radiotolerans*, a species unexplored for its enzymes and proteins. A 1254 bp gene coding β-glucosidase was PCR amplified, cloned, and subcloned by using pJET1.2 and pET21a (+) plasmid vectors in BL21 (DE3) codon plus RIPL strain of *E. coli*. The production of recombinant enzyme was induced in the presence of 0.5 to 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). A recombinant protein of molecular weight ranging from 45 to 50 kDa was detected on SDS-PAGE. The active enzyme was found in soluble form. β-glucosidases with molecular weight of 60 kDa [43], 330 kDa [44], 120 kDa [45], 135 kDa [46] have been reported in the literature indicating a diversity of molecular size of enzyme in different species. The enzyme was purified by anion exchange chromatography with 27.56 folds purity and final recovery of 48% (Table 1), it displayed a prominent protein band on SDS-PAGE (Fig. 1). The purified recombinant β-glucosidase has shown optimal activity at pH 5 and 85°C. The enzyme was highly thermostable, retained more than 46% activity at 100°C when incubated for 5 min. The enzyme with optimum pH 5 [47], 4 [48], and 3-9 [49] has been reported with optimum temperature of 30°C, 50°C, and 40-50°C, respectively. The optimum temperature and pH of enzyme may be associated with the growth conditions of source organisms rather than those of *E. coli*. The Km and V_{max} values were 16.3mM and 25.8 U/mg, respectively, when pNPG

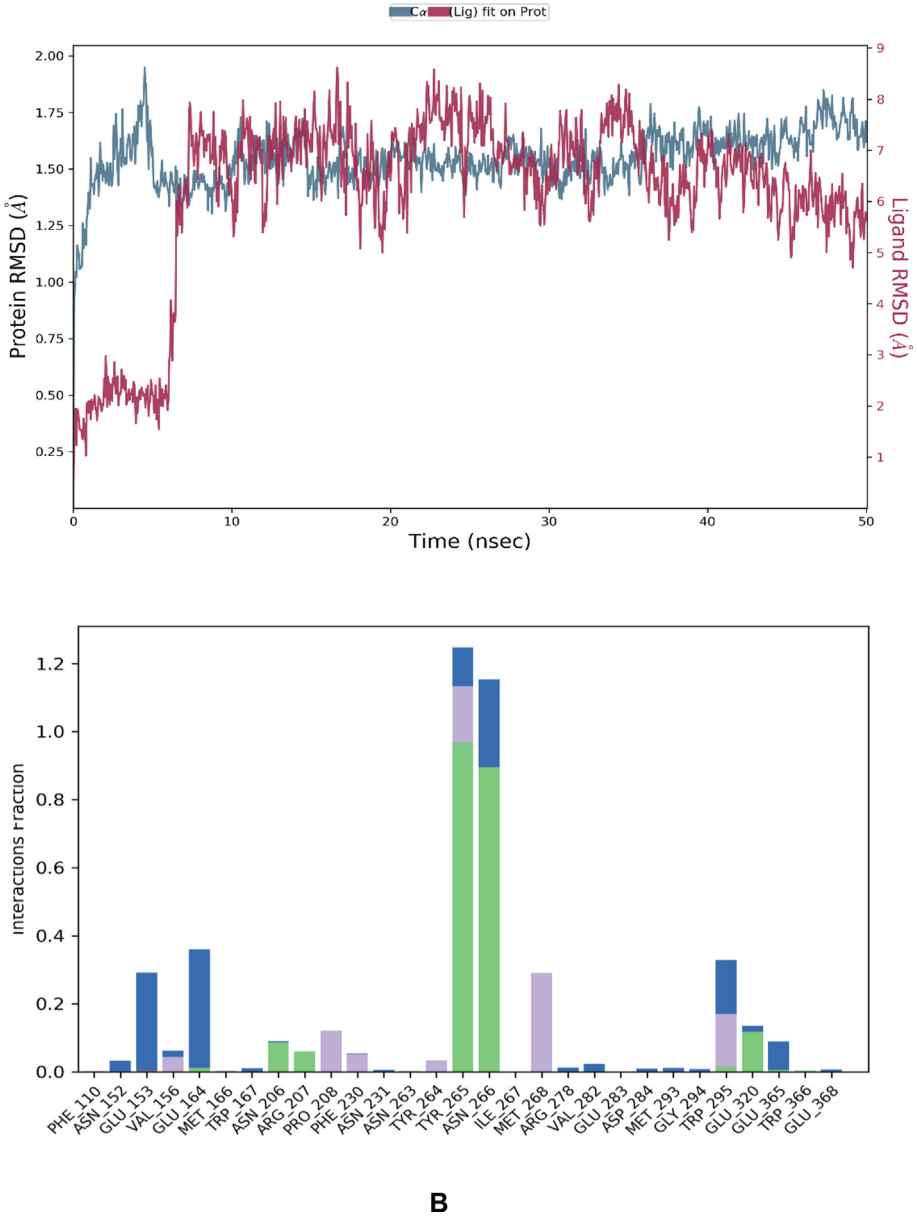

Fig. 5. MD simulation of the beta-glucosidase and pNPG complex during 50 ns run (A). Root mean square deviations (RMSDs) showing ligand in red and protein backbones in blue (B). Frequencies of the interactions between protein and substrate. Green blocks indicate H-bonds, blue blocks are water bridges, and violets indicate hydrophobic bonds.

was used as the substrate. A great inconsistency has been found in the Km and V_{max} values of β-glucosidases reported in the literature [50,51] (Fig. 2). There was no significant reduction in the activity of enzyme when measured in the presence of 25, 50, and 100 mM glucose in the reaction mixture. However, up to 20% decrease in enzyme activity was found when 800 mM glucose was present in the reaction mixture which indicates high level of glucose tolerance by the enzyme under our investigation. Similar information has been provided in a study conducted on engineered β-glucosidase [52].

In silico studies have evaluated 3D structure of enzyme that appears as a globular protein like majority of enzymes. The molecular docking between enzyme and substrate have shown a high negative energy change ΔG = −7 kJ/mol which indicates a strong affinity of pNPG with the enzyme active site (Fig. 3). The active site of enzyme consists of Glu365, Asn266, and Trp295 as the major components (Fig. 4). The MD simulation indicates the high stability of the ligand in protein backbones during the simulation time, as shown in (Fig. 5). Only a small fluctuation was observed from the beginning of the simulation until 7 ns of simulation, then the ligand showed a very small fluctuation of less than 2 Å, which remained in the acceptable range of fluctuation of 1–3 Å [53]. During MD simulation, two stable hydrogen bonds were observed with Tyr265, and Asn266, in which the H-bonds were observed in more than 80% as shown in Fig. 3B, stable bonds must exist in more than 70% of simulation time [54].

5. Conclusions

β-glucosidase from *T. radiotolerans* with novel properties like extremely high-temperature stability, low Km value, and high glucose tolerance has been produced and characterized. The calculation of the relative binding free energies (ΔG-bind) governed by the thermodynamic and strength of binding [30], usually more negative results indicate better stability [32]. The complex generated an average ΔG-bind in the range of −47.16 to −23.32 kcal/mol, with an average of −34.17 kcal/mol, and standard deviation (SD) 4.82. This low value of ΔG and small value of SD reflect the stability of the complex. The enzyme produced as a recombinant and evaluated by biochemical and *in silico* studies provides a potential candidate for industrial applications.

Author contribution

- Study conception and design: H Albalawi, MS Nadeem
- Data collection: H Albalawi, JA Khan, HN Altayeb
- Analysis and interpretation of results: HN Altayeb, S Iftikhar, MAA Al-Ghamdi
- Draft manuscript preparation: H Albalawi, MS
- Revision of the results and approval of the final version of the manuscript: H Albalawi, HN Altayeb, S Iftikhar, MAA Al-Ghamdi, JA Khan, MS Nadeem

Conflict of interest

None.

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