Abstract

With a paradigm shift in industry, moving from natural fuels to alternative renewable resource utilization, concisely the growing demands of bioenergy has led to the emphasis on novel cellulolytic enzymes to improve efficiency of bioconversion process of lignocellulosic plant biomass. Currently, biodegradation is an area of extensive research, the cynosure of biofuel industry is on the utilization of non-edible lignocellulosic biomass (feedstock, agricultural and municipal residues) as an exploitable, inexpensive and potential source of alternative renewable energy in the form of bioethanol, thus the need of efficient thermostable cellulases are expected to increase in the future. β-glucosidase is an essential member of cellulase enzyme system that plays a critical role in cellulosic substrate hydrolysis and in many other biological processes.

Therefore, the present study describes cloning of two novel highly thermostable cellulolytic enzymes β-1,4-glucosidases (*TnBgl*A and *TnBgl*B) from a bacterium *Thermotoga naphthophila* RKU-10^T and overexpressed in *Escherichia coli* BL21 CodonPlus (DE3)-RIPL. Purification and biochemical characterizations together with kinetic and thermodynamic analysis give insights about the thermostability of both enzymes. Various cultivation and induction strategies were applied to enhance the production of engineered host cells density and expression of highly efficient thermotolerant *TnBgl*A and *TnBgl*B, induced individually with IPTG and an alternative inducer lactose. Culture conditions and other parameters including media compositions, pre-induction optical density, agitation, inducer concentrations, temperature and time of induction were optimized to achieve maximum yield of heterologous proteins.

Genomic DNA of *T. naphthophila* was used as template to amplify two cellulolytic genes *Tnbgl*A and *Tnbgl*B (ADA66698.1 and ADA66752.1) of 1.341 and 2.166 kb, which encoded β-1,4-glucosidase proteins of 446 and 721 amino acid residues, respectively. Amplicons of genes were cloned initially in pTZ57R/T vector by employing dA×dT tailing technique and consequently subjected to sequence analysis. Sequence homology analysis demonstrated that *TnBgl*A and *TnBgl*B belong to glycoside hydrolase family 1 (GH1) and family 3 (GH3), respectively. Further, both genes were sub-cloned in pET-21a(+) vector

and over-expressed in *E. coli* BL21 under the control of inducible T7 *lac* promoter. Initially, LB medium was used for the production and optimization of various cultivation parameters to get the high level expression of both recombinant enzymes. However, optimal expression and activity of both enzymes were observed when culture induced with 0.5 mM IPTG after heat shock treatment (42°C, 1 h) at 0.6 pre-induction optical density (OD_{600nm}) followed by incubation at 22°C for 72 h in a shaking incubator at 200 rpm.

Both extracellular *TnBgl*A and *TnBgl*B with a molecular weight of 51.50 and 81.14 kDa, respectively were purified to homogeneity by ion-exchange and hydrophobic interaction chromatography after heat treatment at 70°C for 1 h. Purified enzymes *TnBgl*A and *TnBgl*B displayed optimal activity at pH 7.0 (95°C) and pH 5.0 (85°C temperature), respectively. Both enzymes were quite stable over a broad range of pH (6.0-8.5) and temperature (60-90°C), fairly stable up to 8 h at 80°C. GH1 *TnBgl*A activity was stimulated in the presence of glucose and xylose, with a K_i value of 1200 and 1300 mM, respectively. Whereas, GH3 *TnBgl*B revealed K_i value of 150 mM for glucose and 200 mM for xylose, both enzymes displayed affinity towards *p*-nitrophenyl substrates and cellobiose.

K_m, V_{max} and k_{cat} values of *TnBgl*A, using *p*NPG as substrate, were 1.5 mM, 297 mmol $mg^{-1}min^{-1}$, and 1527778 s^{-1}, respectively. Whereas, *TnBgl*B showed K_m, V_{max} and k_{cat} values of 0.45 mM, 153 mmol $mg^{-1}min^{-1}$ and k_{cat} 1214285.7 s^{-1}, respectively using *p*NPG as substrate. Thermodynamic parameters as ΔH^*, ΔG^* and ΔS^* for *p*NPG hydrolysis by *TnBgl*A were calculated at 95°C as 25.7 kJ mol^{-1}, 47.24 kJ mol^{-1} and -58.6 J mol^{-1} K^{-1}, respectively. Thermodynamic parameters for *p*NPG hydrolysis by *TnBgl*B like ΔH^*, ΔG^* and ΔS^* were calculated at 85°C as 24.1 kJ mol^{-1}, 46.55 kJ mol^{-1} and -62.74 J mol^{-1} K^{-1}, respectively. *TnBgl*A displayed a half-life ($t_{1/2}$) of 5.21 min at 97°C with denaturation parameters of enzyme including ΔH^*_D, ΔG^*_D and ΔS^*_D were 662.04 kJ mol^{-1}, 110.10 kJ mol^{-1} and 1.491 kJ mol^{-1} K^{-1}, respectively. *TnBgl*B showed a half-life ($t_{1/2}$) of 4.44 min at 94°C with denaturation parameters of enzyme including ΔH^*_D, ΔG^*_D and ΔS^*_D were 283.78 kJ mol^{-1}, 108.7 kJ mol^{-1} and 0.477 kJ mol^{-1} K^{-1}, respectively.

Generally, inadvertently preparing medium and unintentional induction of engineered *E. coli* BL21, give poor or variable yields of heterologous proteins. Therefore, to enhance the

activity and production of an industrially relevant cloned enzymes through various cultivation and induction strategies. High-cell-density and optimal heterologous proteins expression were obtained in 4×ZB medium after 72 h inducement at 22°C when culture gave heat shock (at 42°C for 1 h) at 0.6 OD_{600nm} and induced either with 0.5 mM IPTG/150 mM lactose. *TnBgl*A and *TnBgl*B activities were enhanced 3.8 and 0.096 fold in 4×ZB medium with 150 mM lactose, respectively under optimal conditions. However, considerably greater dry cell weight of *TnBgl*A and *TnBgl*B cultures were 11.30 g DCW L^{-1} and 11.08 g DCW L^{-1} in 4×ZB medium, respectively induced with 150 mM lactose. Use of the inexpensive and non-toxic inducer lactose, and an effective process strategy is essential to achieve a high level of enzyme expression.

The expression and purification scheme, presented here, has a potential of scaling up to obtain pure and active enzymes, relatively economical for further studies and other applications. Finally, this is a report on enhanced production of highly heat active cloned β-glucosidases from *T. naphthophila* (*TnBgl*A and *TnBgl*B) with high catalytic efficiency and low product inhibition, which have excellent tolerance against glucose and xylose, and also exhibit independence of detergents, chemical inhibitors and metal cations. All these significant features make both *TnBgl*A and *TnBgl*B appropriate candidates for biotechnological and industrial applications.

Keywords: thermostable; β-glucosidase; thermodynamics; glucose tolerance; heterologous protein expression; induction strategy; *Thermotoga naphthophila*.