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PURIFICATION AND CHARACTERIZATION OF CLONED ENDO-1, 4-β-GLUCANASE GENE OF *BACILLUS LICHENIFORMIS* FOR SAC CHARIFICATION OF PLANT BIOMASS

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Abstract

Endo-1, 4-β-glucanases are the hydrolytic enzymes which are commonly used for the saccharification of plant biomass. The endo-1, 4-β-glucanase (*bglC*) gene of moderately thermophilic bacterium *Bacillus licheniformis* ATCC 14580 was successfully cloned and expressed in *E. coli* BL21 (DE3) strain by means of pET-22b (+) vector. Purification of recombinant endo-1, 4-β-glucanase (*bglC*) enzyme was done by utilizing the ammonium sulphate method followed by immobilized metal affinity chromatography (IMAC) and gel filtration. Purification fold of 10.51 was achieved along with enzyme activity and specific activity of 18.25 U/ml/min and 75 U/mg, respectively. The molecular mass of purified recombinant enzyme was 56 kDas determined by SDS-PAGE analysis. The enzyme showed resistance to temperature up to 90ºC at pH 3 - 8. The activity of enzyme was improved in the presence of metal ions particularly Co^{2+} and Mg^{2+}. Organic solvents showed no significant effects on the enzyme activity and the purified enzyme also showed great stability against inhibitors such as Tween 80, β-marcaptoethanol and DTT upto 90%. Among different tested biomass, the endoglucanse enzyme showed highest saccharification (24.2%) with sugarcane bagass. These results showed that recombinant enzyme could be a useful candidate for bioconversion of natural biomass into simple sugars and eventually in biofuel production.

Key words: *Bacillus licheniformis*; Biomass; Characterization; Endo-1, 4-β-glucanase; Purification; Saccharification.

Introduction

Cellulose is a key polysaccharide present in the plants cell wall and is found to be the largest part of the earth biomass (Kipper *et al*., 2005). It is virtually a limitless source of bioenergy and the best source of carbon, which ultimately converted into glucose (Delmer & Haigler, 2002; Back & Kwon, 2007). Cellulose constitutes almost 40 – 60% lignocellulosic materials and remnants such as hemicellulose and lignin (Saha & Cotta 2008) The annual production of cellulose by land plants and algae is 8.5 x 10^{10} tons per year (Nowak *et al*., 2005). The utilization processes cause massive amounts of cellulosic wastes (municipal, agricultural and industrial), which accumulated because of inadequate use. Biofuel production from lignocellulosic biomass is an emerging area of research for sustainable development, but the cost of enzyme and the low productivity of fermentable sugars in enzymatic saccharificationare major limiting factors (Sukumaran *et al*., 2009).

Immense research efforts have been made due to the high requirement of production of bioenergy by bioconversion of cellulose into fermentable sugars (Kumar *et al*., 2008). The cellulose complex contains various enzymes (endoglucanase, exo-cellobiohydrolase, β-glucosidase) which act in a synergistic manner on cellulose for its degradation (Bhat & Bhat, 1997; Cai *et al*., 1999). Worldwide these enzymes constitute about 8% of industrial enzymes (Costa *et al*., 2008). Endo-1, 4-β-glucanases attack cellulose, which decreases the polymer chain length and increases the quantity of reducing sugars (Aygan *et al*., 2011; Onsori *et al*., 2005). Endoglucanases act on the amorphous region of cellulose and have been assembled into different families of glycoside hydrolase enzymes (Henrissat & Davies, 2000).

Cellulase complexes are produced by several microorganisms such as fungi and bacteria, which utilize cellulose as a carbon source (Kim *et al*., 2008). Amongst these are *Clostridium thermocellum* (Johnson *et al*., 1982), *Clostridium* strain C7 (Cavedon *et al*., 1990), *Clostridium cellulovorans* (Shoseyov and Doi1990), *Ruminococcusalbus* (Wood *et al*., 1982) as well as *Bacteroides cellulosolvens* (Lamed *et al*., 1991). The complex is hard to break without loss of its total activity and specific constituents (Gilbert & Hazlewood, 1993; Bayer *et al*., 1998a; Bayer *et al*., 1998b; Doi *et al*., 1998) but this complex is absent in *Bacillus* species (Mawadza *et al*., 2000) where individual genes for each cellulase enzyme are present. Numerous endoglucanases genes have been cloned from various *Bacillus* species (Baird *et al*., 1990; Gosalbes *et al*., 1991; Lloberas *et al*., 1991; Chen *et al*., 1992; Louw *et al*., 1993; Heng *et al*., 1997; Hakamada *et al*., 2002).

Cellulases are utilized in numerous industrial processes such as detergent industry as an additive (Shikata *et al*., 1990), manufacturing of paper in paper industry (Stork *et al*., 1995; Pastor *et al*., 2001), textile industry for manufacture of stone washed denim and to improve softness and brilliance of cotton (Tomme *et al*., 1995; Cavaco-Paulo *et al*., 1998). Few studies have been reported, where bioconversion of agricultural waste has been utilized by endoglucanases, and the search of new endoglucanases with more efficient and suitable properties for use in industrial applications is crucial (Okoshi *et al*., 1990; Lusterio *et al*., 1992; Han *et al*., 1995; Christakopoulos *et al*., 1999; Endo *et al*., 2001).Gene manipulation techniques have been utilized to generate favorable environment for production and application of cellulases at industrial scales (Zafar *et al*., 2014). Genetically modified *Escherichia coli* is very commonly used for the

production of cellulosic biofuels, in manufacturing of ethanol (Jarboe *et al*., 2007), butanol production (Atsumi *et al*., 2008), fatty acids production (Liu *et al*., 2012), biodiesel production (Steen *et al*., 2010) and alkane/alkenes production (Schirmer *et al*., 2010). *Bacillus licheniformis* is gram positive, spore producing slightly thermophilic bacterium which have shown to have a significant role in the production of numerous enzymes including endoglucanases, which have been shown to be proficient in using cellulose as carbon source (Rey *et al*., 2004).

In this research work purification and characterization of the cloned gene encoding endoglucanase from *Bacillus licheniformis* strain ATCC 14580 and its saccharification potential against various pretreated plant biomasses has been reported.

Methodology and Materials

Bacterial strains and plasmid: *Bacillus licheniformis* ATCC 14580 was purchased from USDA agriculture Research Service (ARS) Culture collection USA. Lyophilized culture was inoculated in recommended TGY (Tryptone, glucose, yeast extract) medium and further maintained in Luria Bertini medium. *Escherichia coli* BL21 (DE3) was obtained from the culture bank present at the Biotechnology department, GCU, Lahore, Pakistan. This strain was maintained in Luria Bertini medium and was further used for the expression of cloned gene. The pET 22b (+) was used as an expression vector which was obtained from the research laboratories, GCU, Lahore.

Cloning of *bglC*gene: Genomic DNA was isolated from *Bacillus licheniformis* by following the protocol of Kronstad *et al*., (1983) and used as template to amplify the endo-1, 4-β-glucanase (*bglC*) gene. Amplification was performed in a thermocycler using specific pair of primers, sequences of which are given below:

Forward primer: 5' GC**CATATG**CGTTCCATCTCTGTCTTCA 3'
Reverse primer: 5' GC**AAGCTT**ATTTAGGTTCAGTGCCC 3'

Primers were designed by using the software, Vector NTI against the available nucleotide sequence of endoglucanase gene of *Bacillus lichniformis* present on the NCBI database. Restriction sites for *Hind* III and *Nde*I were added at 5' of reverse and forward primers as shown in bold. Amplified product of *bglC* gene was purified by using Novagen Spin Prep™ gel extraction kit. Double restriction of both amplified product of *bglC* gene as well as pET 22b (+) vector was carried out by *Hind* III and *Nde*I and purification was carried out by using Novagen Spin Prep™ gel extraction kit. Double restricted fragment of *bglC* gene obtained after purification was ligated with expression vector pET 22b (+) with the help of T4 DNA ligase and further transformation was carried out into *E. coli* BL21 (DE3) competent cells (Sambrook *et al*., 2001) Method of Cohen *et al*., (1972) was used for competent cells preparation. Colony PCR was used for confirmation of cloning of *bglC* gene in addition to single as well as doubles restriction of recombinant expression vector cloned with *bglC* gene by using restriction enzymes i.e., *Nde*I and *Hind* III.

Expression study of cloned *bglC* gene: Submerged fermentation technique was used for the production of recombinant endoglucanase enzyme. Single hybrid colony was selected from LB agar plate containing ampicillin (100 µg/ml) and transferred into LB broth (50 ml) which also contained ampicillin (100 µg/ml) in flask. Overnight incubation was done at 37ºC in a shaking incubator at 150 rpm. The culture (inoculum) obtained was then diluted to 1% and incubated at 37ºC till its optical density approached to 0.4-0.6 at 600 nm. Culture was induced by adding isopropyl D-thiogalactopyranoside (0.2 mM) and incubated at 37ºC for 4 hrs. Centrifugation was carried out at 4ºC, 6000 rpm for 5 minutes to harvest the cells. After separation of supernatant, pellet was further re-suspended in Tris-HCl (50 mM, pH: 8.0). Cell lysis was carried out which taken 10 min in a heat sonicator. This process was repeated and the pellet obtained was re-suspended in 50 mMTris-HCl (pH 8.0). Analysis of *bglc* gene expression was performed with the help of SDS-PAGE in both intracellular as well as extracellular fractions by following the method of Laemmli (1970).

Enzyme assay: Enzyme assay was carried out by using DNS technique of reducing sugar estimation (Miller, 1959). One ml of enzyme was incubated with one ml of 1% CMC solution at 55°C for 20 minutes. Three ml of DNS was added to stop the reaction after incubation. A blank was run in parallel. After boiling for five minutes the samples were cooled at room temperature and absorbance was measured at 540nm in a spectrophotometer. Enzyme activity unit was demarcated as "Enzyme quantity that catalyzes conversion of one micromole of substrate per minute."

Total protein estimation: Bradford method (1976) was used for assessment of total proteins with help of bovine serum albumin as standard.

Purification of recombinant enzyme (*bglC*): Recombinant *bglC* enzyme from extracellular sample was partially purified using ammonium sulphate precipitation process (de-Moraes *et al*., 1999). Activity of enzyme as well as protein contents were resoluted for every fraction and fractions that exhibited high enzyme activity were combined and dialyzed by continuous stirring in the presence of citrate phosphate buffer (50mM, pH: 7.5).

Partially purified recombinant *bglC* enzyme was further subjected to IMAC Chromatography with the help of Protino® Ni-TED kit. 1 ml of partially purified recombinant *bglC* enzyme was loaded to the column and endorsed to drain by gravity. Elution was carried out by adding elution buffer and the column was permitted to drain by gravity. Elution fractions were collected after regular intervals and checked for its purity by SDS-PAGE analysis.

The concentrated *bglC* enzyme was finally purified by using Gel Filtration Chromatography (GFC). For this purpose, the enzyme sample was loaded to a Hi Load 16/60SuperDex 75 column (GE Health Care Life Sciences) with column volume of 120 ml and pre equilibrated with the help of gel filtration buffer in order to stabilize the bed. Elution fractions were collected and analyzed by SDS-PAGE.

Characterization of purified *bglC* enzyme

Molecular weight determination: The molecular weight of purified recombinant *bglC* was determined by SDS-PAGE (Laemmeli, 1970).

Temperature and pH stability: Stability at various temperatures was determined by incubation of the purified enzyme (1 ml). The range of temperatures and time were 40-90ºC for1-4 hours at neutral pH. Remaining enzyme activity was determined by using standard enzyme assay conditions. Likewise, the enzyme's pH stability was also determined at pH 3.0-8.0 for 1-4 hours at room temperature. Remaining activity of purified enzyme was determined by using standard conditions for assay.

Substrate specificity: Substrate specificity of purified recombinant *bglC* enzyme was determined by using 1% of various substrates like, CMC, PNPG, H_3PO_4 swollen cellulose, filter paper and avicel. Measurement of the activity of endoglucanase was carried out by means of standard assay conditions.

Effect of Inhibitors: The impact of different inhibitors on purified recombinant *bglC* enzyme was detected by incubation of purified enzyme with varying amounts (1–10%) of various inhibitors including sodium azide, SDS, dimethyl sulfoxide (DMSO), β-mercaptoethanol, DTT and Tween 80 for one hour at room temperature. Residual activity of the enzyme was determined under standard assay protocol.

Influence of organic solvents: Influence of different organic solvents i.e., methanol, iso-propanol, ethanol, acetone and n-butanol on enzyme activity was also studied by incubating enzyme with various concentrations (10–40%) of organic solvents for one hour at room temperature. Remaining activity of treated purified enzyme was determined under standard assay conditions.

Influence of EDTA and metal ions: Influence of different metals ions on the activity as well as stability of purified recombinant *bglC* enzyme was calculated by incubation of purified recombinant enzyme with varying concentrations (1–10 mM) of different metal ions (Co^{2+}, Na^{2+},Mn^{2+}, Ca^{2+}, Mg^{2+}, $Cu^{2+}$and Hg^{2+}). Residual activity of endoglucanase was determined under standard assay protocols.

Enzymatic saccharification of plant biomass: Saccharification potential of *bglC* enzyme was evaluated by using different plant biomass (sugarcane bagasse, rice straw and wheat straw). Pretreated cellulosic biomass (1%) was mixed with 1 ml of recombinant enzyme and incubated for 24 hrs at 50ºC. Primarily the saaccharification reaction was performed in Erlenmeyer flasks (100 ml) in a shaking incubator at 100 rpm. Saccharification reaction was also performed at large scale in a 1000 ml flask. For large scale saccharification study, 10 ml recombinant enzyme was used against 1% cellulosic plant biomass and incubated at 50°C in a shaking incubator at 100 rpm. Reducing sugars released were determined by the DNS method.Saccharification percentage was determined by using equation below (Pandiyan *et al*., 2014)

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar released (g/ml) x 0.9}}{\text{Biomass contents (g)}} \text{ x } 100$$

Results

Cloning of *bglC* gene: Amplification of *bglC* gene was achieved by using *Bacillus licheniformis* ATCC 14580 genomic DNA and specific pair of primers. Purification of amplified gene (1548 bp) was carried out by using Novagen Spin Prep™ extraction tool. Purified *bglC* gene as well as pET 22b (+) were double restricted by *Nde*I and *Hind*III and again purified with Novagen Spin Prep™ gel extraction kit. Double restricted purified *bglC* gene as well aspET 22b (+) were ligated with the help of T4 DNA ligase enzyme and transformed into *E. coli* BL21 (DE3).Cloning of *bglC* gene was analyzed by restriction analysis of plasmid containing *bglC* gene utilizing *Nde*I and *Hind*III restriction enzymes as well as by colony PCR. The products were observed on agarose gel and it was found that *bglC* gene (1548 bps) was detached from pET-22b (+) vector (5400 bps) (Fig. 1), which confirmed the successful cloning of *bglC* geneintopET-22b (+) expression vector.

Expression study of recombinant *bglC* gene: Cloned *bglC* gene was checked for its expression in both intracellular and extracellular samples. To determine the intracellular enzyme activity bacterial cells were sonicated. In extracellular samples *bglC* enzyme activity was 20U/ml/min, while in intracellular proteins the enzyme activity was negligible (0.87 U/ml/min). Cloned *bglC* gene expression was also analyzed with the help of SDS-PAGE as shown in Fig. 2.

Purification of recombinant *bglC* enzyme: Crude recombinant enzyme from extracellular sample was partially purified by using fractional ammonium sulphate purification technique with moderate stirring at 4ºC. The fractions with high activity of *bglC* enzyme were combined, dialyzed and subjected to Immobilized Metal Affinity Chromatography (IMAC) for further purification and concentration. Concentrated enzyme was loaded to Hi Load 16/60 SuperDex 75 column (GE Health Care Life Sciences) which was pre equilibrated using gel filtration buffer. Purified fraction specified as distinct band on SDS-PAGE exhibited 56 kDA molecular weight (Fig. 2). The result of all purification steps are shown in Table 1.

Characterization of purified recombinant *bglC* enzyme

Molecular mass determination: Fractions of purified endoglucanase enzyme were pooled and examined on SDS-PAGE. A discrete band (56 kDa) was detected in purified fraction but no specific protein band was observed in control as indicated in Fig. 2.

Table 1. Purification summary of recombinant endoglucanase enzyme produced from *E. coli*.

Purification step	Enzyme activity U/ml/min	Protein concentration mg/ml	Specific activity U/mg	% Age yield	Purification fold
Crude extract	58.5	8.2	7.13	100	1
$(NH_4)_2SO_4$ Precipitation	38	1.52	25	64.95	3.5
Affinity Chromatography (IMAC)	29	0.82	35.36	49.57	4.95
Gel filtration chromatography	18.25	0.24	75	31.19	10.51

Thermostability: Thermostability of purified recombinant endoglucanase enzyme was analyzed by examining the residual activity after pre-incubation of the enzyme for 1–4 hrs at varying temperature (40-90ºC) at neutral pH. The enzyme was found to be constant at elevated temperatures and reserved almost 100% residual activity after treatment for 4hrs at 40ºC and 50ºC.The enzyme showed very good stability and retained 82% residual activity at 60ºC after 4hrs of incubation (Fig. 3). Enzyme activity was hindered as the process of incubation progressed and was calculated to be73%, 64% and 59% at 70ºC, 80ºC and 90ºC, respectively after 4 hrs of incubation. After 3 hrs of incubation at 60ºC, 70ºC, 80ºC and 90ºC the residual activity of the enzyme was 86%, 80%, 72% and 68%, respectively. However, after 2 hrs of incubation enzyme was found to be very stable and showed up to 72% stability at 90ºC as shown in Fig. 3.

Effect of pH on purified *bglC* enzyme stability: The pH constancy of purified recombinant *bglC* enzyme was calculated by checking the residual activity of enzyme after pre-incubation of enzyme in buffers ranging from pH 3 - 8 for different time periods (1–4 hrs) using CMC as substrate at room temperature. The enzyme was found to be stable over a broad range of pH (4.0–7.0), retaining about 79% residual activity at pH 4.0, 88% at pH 5.0 and more than 96% at pH 6.0.The activity of the enzyme was decreased slightly at pH 7.0 and 58% residual activity was detected after4 hrs of incubation. After 4 hrs of incubation at pH 8.0 the remaining enzyme activity was 48% as shown in Fig. 4.

Substrate specificity: The substrate specificity of recombinant *bglC* enzyme was analyzed after incubation with 1% of various substrates including CMC, PNPG, H_3PO_4 swollen cellulose, avicel and filter paper. The enzyme was observed to be most active against CMC (20.024 U/ml/min) (Fig. 5).Low enzyme activity was found with PNPG, H_3PO_4 swollen cellulose, filter paper and avicel with activity of 2.25 U/ml/min, 1.08 U/ml/min, 0.277 U/ml/min and 0.52 U/ml/min, respectively.

Effect of Inhibitors on activity of *bglC* enzyme: The impact of variable amounts (1-10 %) of various inhibitors on activity of enzyme was also evaluated and residual activity was estimated. It was calculated that inhibition of 90% was observed by 10% SDS as shown in Fig. 6. However, considerable amount of enzyme inhibition (59%) was observed, when 10% NaN_3 was added in the enzyme solution. Interestingly, *bglC* enzyme was inhibited by 25%, 27% and 35% in the presence of 10% Tween 80, β-mercaptoethanol and DTT, respectively as shown in Fig. 6. In the presence of 10% DMSO, 82% inhibition in enzyme activity was observed. However, less inhibition of enzyme activity was observed when less concentration of these inhibitors were used.

Organic solvent's influence on endoglucanase stability: The influence of organic solvents i.e., isopropanol, methanol, n-butanol, ethanol and acetone on enzyme activity revealed that enzyme showed very good stability in presence of ethanol, methanol, isopropanol, acetone and n-butanol at lower concentration (10%). In the presence of 20% methanol, n-butanol, ethanol, isopropanol and acetone residual enzyme activity was found to be 74%, 71%, 80%, 74% and 65%, respectively as shown in Fig. 7. However, a slight decrease in enzyme activity was observed in the presence of 40% of these organic solvents.

Effect of EDTA and metal ions: Effect of EDTA as well as metal ions on activity and stability of recombinant *bglC* enzyme was determined after pre-incubation of enzyme with varying concentrations of metal ions (Co^{2+}, Na^{2+}, $Mg^{2+,}$ Mn^{2+}, Ca^{2+}, Cu^{2+} and Hg^{2+}) as well as EDTA. Residual activity was found to be improved by 20% and 35% in the presence of 1 mM Mg^{2+} and Co^{2+}, respectively. However, in the presence of other metal ions like, Na^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} and $Hg^{2+}$the enzyme activity was decreased by 43%, 68%, 60%, 15% and 7%, respectively (all metal ions concentration was 10 mM). The activity of enzyme was decreased in the presence of 1 mM and 5 mM EDTA and completely repressed when 10 mM EDTA was added as shown in Fig. 8.

Saccharification of pretreated biomass: In order to determine saccharification potential of purified recombinant *bglC* enzyme, 20 units of enzyme were incubated at 50ºC with different types of pretreated biomass (wheat straw, rice straw, and sugarcane bagass). Saccharification percentage and total reducing sugars produced under optimized conditions (pH, temperature, incubation time, enzyme concentration and substrate concentration) are tabulated in Table 2. Similar results of reducing sugars were obtained after saccharification of wheat straw and rice straw. Conversely somewhat improved effects (2.69 mg/ml) were achieved after saccharification was performed with sugarcane bagasse. Saccharification of 20.5%, 17.5% and 24.2% obtained when wheat straw, rice straw and sugarcane bagass were used as substrates, respectively. Saccharification potential of recombinant enzyme was also checked at large scale by using 1000 ml flask under the same conditions. For this purpose, 200 enzyme units were used to hydrolyze 1% plant biomass (Wheat straw, rice straw, sugarcane bagass). Reducing sugars releasing during hydrolysis of wheat straw and rice straw by endoglucanase enzyme, were 2.42 mg/ml and 2.13 mg/ml, respectively. While with sugarcane bagass slightly improved results (3.84mg/ml) were obtained same as in case of 100 ml flask. Saccharification percentage obtained during large scale experiment were 28.6%, 19.2% and 21.8% for sugarcane bagass, rice straw and wheat straw, respectively as shown in Table 2.

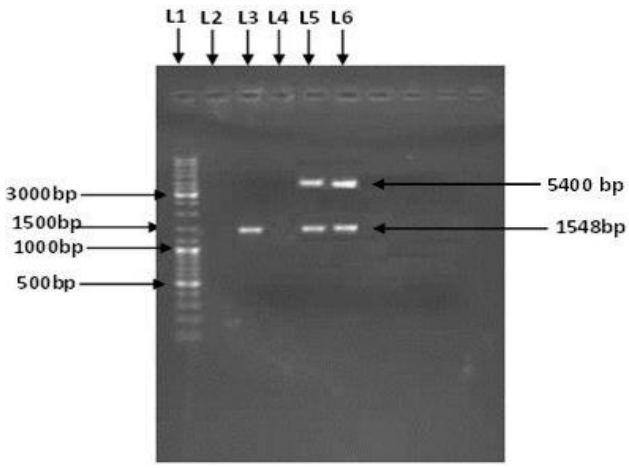

Fig. 1. Agarose gel showing amplified endo-1, 4-β-glucanase (*bglC*) gene and restriction analysis of recombinant pET-22b/*bglC* vector. Lane 1: DNA ladder (Fermentas#0341), Lane 3: amplified *bglC* gene (1548 bp) and lane 5 and 6: Double restricted recombinant plasmid pET-22b (+) (5400 bp) with *bglC*gene (1548 bp).

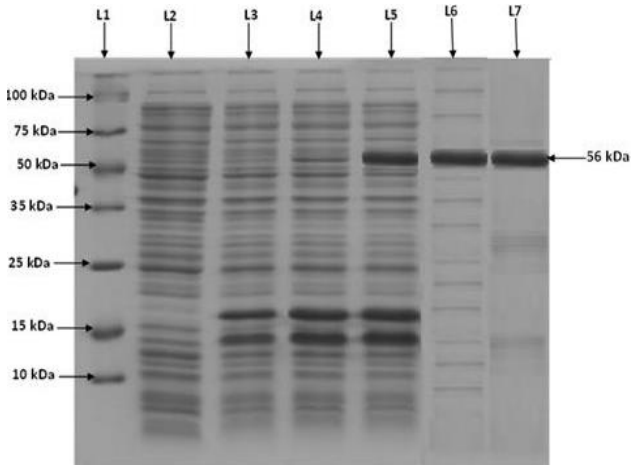

Fig. 2. SDS-PAGE analysis of cloned *bglC* gene expression. Lane 1 to 7 represents: protein marker, extract of wild *E. coli*, induced vector only, non-induced vector plus *bglC*gene, induced vector plus *bglC* gene, partially purified cloned *bglC* enzyme in *E. coli* and purified cloned *bglC* enzyme in *E. coli*.

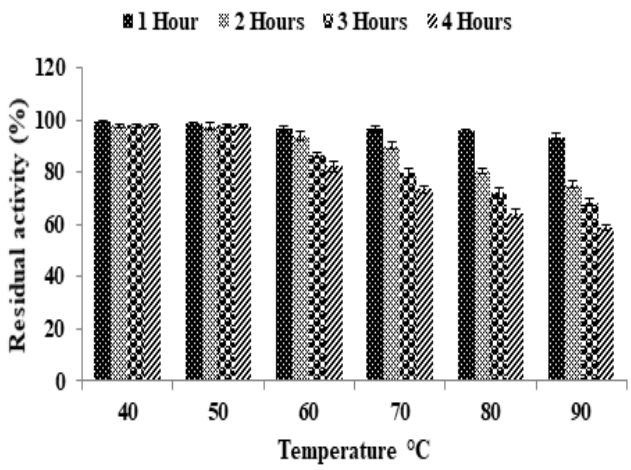

Fig. 3. Thermostability studies of recombinant *bglC* enzyme at various temperatures (40-90ºC) for different time intervals (1-4 hrs). Y error bars specify standard error among three parallel replicates.

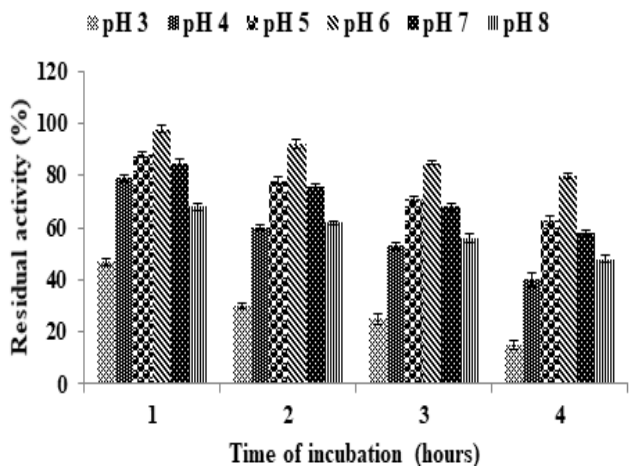

Fig. 4. pH effects on *bglC* enzyme stability. Enzyme was incubated at room temperature with buffers for various time intervals (1-4 hrs) and remaining activity was determined. Y error bars specify standard error among three parallel replicates.

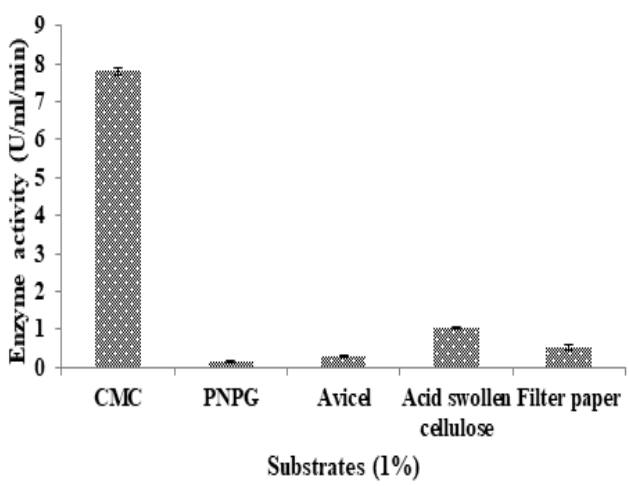

Fig. 5. Substrate specificity of purified *bglC* enzyme for different cellulose sources (CMC, PNPG, H_3PO_4 swollen cellulose, avicel and filter paper). Y error bars indicate standard error among three parallel replicates.

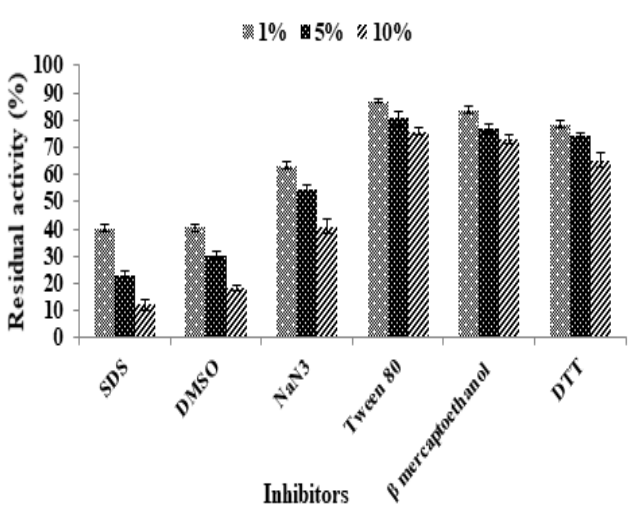

Fig. 6. Effect of various inhibitors with varying concentration (1–10 %) on *bglC* enzyme activity. Y error bars specify standard error among three parallel replicates.

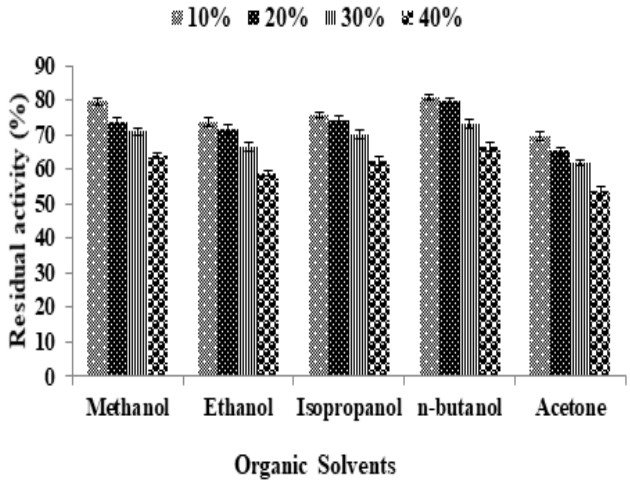

Fig. 7. Effect of various organic solvents with varying concentrations (10-40%) on *bglC* enzyme activity. Y error bars specify standard error among three parallel replicates.

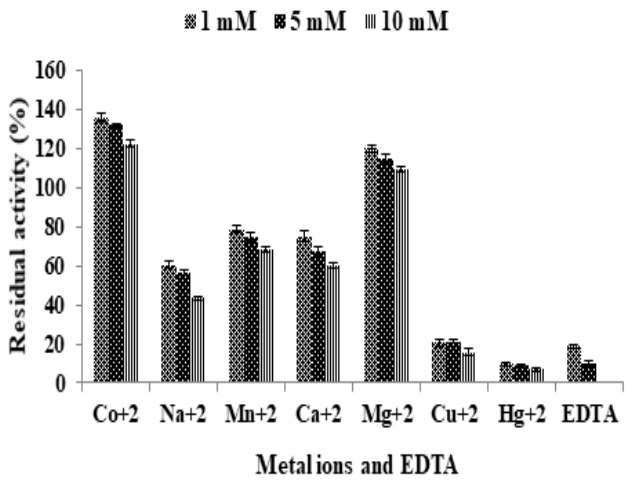

Fig. 8. Metal ions and EDTA effect with varying concentration (1-10 mM) on activity of purified *bglC* enzyme. Y error bars specify standard error among three parallel replicates.

Table 2. Percentage saccharification of various biomasses

Sr. No.	Substrates	In 100 ml flask		In 1000 ml flask	
		Sugar released (mg/ml)	% Saccharification	Sugar released (mg/ml)	% Saccharification
1	Wheat straw	2.277	20.5	2.42	21.8
2	Rice straw	1.95	17.5	2.13	19.2
3	Sugarcane Bagass	2.69	24.2	3.84	28.6

Discussion

Endo-1, 4-β-glucanase is an essential industrial enzyme and its manufacturing is considered crucial for the effective consumption of cellulosic constituents (Zhang and Lynd 2004). The aim of the current research was purification and characterization of cloned endoglucanase (*bglC*) enzyme and its application in conversion of cellulosic biomass into fermentable sugars for biofuel production. Partial purification of recombinant enzyme was carried out by the ammonium sulphate method, which has been extensively used for the purification of proteins of mesophiles (Bajaj *et al*., 2009). Endoglucanases from multiple sources has been purified using the ammonium sulphate purification technique (Shyamala *et al*., 2010). In this research work, 50 - 60% ammonium sulphate saturation showed high activity of endoglucanase (38.0 U/ml/min) having purification fold of 3.5. Sudan and Bajaj (2007) also reported elevated activity of cellulase at ammonium sulphate saturation level of 40-60%, while Sadhu *et al*., (2013) partially purified endoglucanase from *Bacillus* strain (40 – 80%). Further purification was achieved using immobilized metal affinity chromatography (IMAC) as well as gel filtration, with reduced the levels of endoglucanase activity, 29 U/ml/min and 18.25 U/ml/min, whereas purification fold was increased to 4.95 and 10.51, respectively. A distinct band on SDS-PAGE showed that the enzyme was purified to consistency having molecular mass 56 kDa (Fig. 2). The molecular weight of endoglucanses has been determined in other organisms e.g 55 kDa from *Penicillium notatum* NCIM No-923 (Das *et al*., 2012), 52 kDa from *Bacillus subtilis*(Yang *et al*., 2010) and 56 kDa from *Bacillus subtilis* (Wei *et al*., 2015).

Biological characterization of endoglucanase (*bglC*) showed that the enzyme was stable at high temperature and retained 93%, 96% and 97% of residual activity at 90ºC, 80ºC and 70ºC, respectively after one hour of incubation. The stability of endoglucanase from this study is higher than most of endoglucanase enzymes isolated from mesophiles reported earlier. Bischoff *et al*., (2007) demonstrated the endoglucanase enzyme from *Bacillus licheniformis* showed 10% activity after 1 hour of incubation at 65ºC. Similarly, Lima *et al*., (2005) and Zafar *et al*., (2014) reported stability of endoglucanases at 50ºC – 60ºC and 30ºC – 40ºC cloned from *Bacillus pumilus* and *Bacillus subtilis*, respectively. In the present study, the endoglucanase activity was reduced as the incubation temperature and time was increased. The activity of recombinant endoglucanase enzyme was reduced to 82%, 73%, 64% and 59% after 4 hours of incubation at 60ºC, 70ºC, 80ºC and 90ºC, respectively. A decrease in the catalytic action of the enzyme might be caused by its denaturation at elevated temperatures.

The pH stability of enzyme was determined over a wide range (3 - 8) of pH values. Maximum activity (80%) was retained at pH 6 after 4 hrs of incubation indicating that the endoglucanase enzyme of *B. licheniformis* could be used for industrial purpose employing wide range of pH values. Sadhu *et al*., (2013) also reported an endoglucanase from the *Bacillus* strain showing stability at a range of pH 6 – 9 reserving more than 80% enzyme activity after 1 hour, while Dhar *et al*., (2015) reported an endoglucanase from *Paenibacillus* spp, which was stable at pH ranges 4–12 with more than 50% residual activity. The influence of PNPG, CMC, H_3PO_4 swollen cellulose, filter paper as well as avicel on endoglucanase activity

showed that the enzyme was most active against CMC. Annamalai *et al*., (2013) reported 100% active against CMC and very low or negligible activity with other substrates of an endoglucanase enzyme from *Bacillus halodurans*. Similar findings were reported for endoglucanases from *Bacillus* species (Zhang *et al*., 2012; Li and Yu 2012). Results showed that purified enzyme possess no hydrolytic activity towards crystalline substrates (Ito, 1997; Hakamda *et al*., 2000).

The activity of the enzyme was improved by 35% and 20% in the presence of 1 mM $Co^{2+}$and Mg^{2+}, however $Hg^{2+}$and Cu^{2+}significantly decreased the activity (20% and 9%) in 3 mm. Changes in the structure and conformation of the enzyme may be the reason of its activity in the presence of metal ions. A reduction of the activity induced by $Cu^{2+}$and Hg^{2+}may imply that the active site of enzyme may contain histidine and thiol (Pol *et al*., 2012). The findings of this study are similar to those from Saha (2004), who stated that the presence of $Mg^{2+}$and Co^{2+}improvedthe synthesis of endoglucanase. The enzyme activity was reduced in the presence of 1 mm and 5 mm EDTA and completely inhibited in the presence of 10 mm EDTA, which suggested ametalo-enzyme nature of endoglucanase. The purified recombinant endoglucanase enzyme showed high stability in the presence of 10% organic solvents, however, at higher concentrations (40%) minor decrease in the enzyme activity and stability was observed. Deep *et al*., (2016) also reported an organic solvent tolerant endoglucanase enzyme from *Photobacteriumpanuliri* strain LBS5T, which showed stability using various organic solvents including ethanol, methanol, isopropanol and acetone etc.

Recombinant endoglucanase enzyme from *B. licheniformis* showed a high stability (75%, 73% and 65%) in the presence of Tween 80, DTT and β-mercaptoethanol. However, in the presence of NaN_3, the activity of enzyme was considerably decreased (41%). Similarly, enzyme activity was also reduced with the addition of 10% SDS and DMSO. Manavalan *et al*., (2015) reported an endoglucanase from *Ganodermalucidum*, which showed stability in the presence of 1% Tween 80, but this endoglucanase exhibited 82% stability, when 1% SDS was used. However, Bagewadi *et al*., (2016) also reported an endoglucanase enzyme from *Trichoderma harzianum* strain HZN11 that was stable against various inhibitors like Tween 80, DMSO, SDS, β-mercaptoethanol and DTT.

Endoglucanases are being used extensively in the process of plant biomass saccharification and are attracting considerable attention in biofuel production from plant biomass. For effective hydrolysis of plant biomass, endoglucanases are being used along with exoglucanases and β-glucosidases to increase the number of reducing ends of polysaccharide chains by random hydrolysis of the interior glycosidic bonds (Goryachkovskaya *et al*., 2015). Enzymatic saccharification potential of purified recombinant endoglucanase enzyme was measured by calculating percentage saccharification of three different kinds of pretreated plant biomass (wheat straw, rice straw, sugarcane bagasse). Almost similar amount (2.27 mg/ml, 1.95 mg/ml) of reducing sugars were released from wheat and rice straw by the action of purified recombinant endoglucanase enzyme, though slightly greater amount (2.69 mg/ml reducing sugar) was released from sugarcane bagass as a result of enzymatic saccharification. Saccharification percentage was calculated to be 20.5%, 17.5% and 24.2% for wheat, rice straw and sugarcane bagass, respectively. These initial results obtained in this study are much higher than previous studied employing mesophilic system. Alimon (2011) reported 9.03% and 5.92% saccharification of sugarcane bagass and sawdust, respectively, after 96 hours of incubation by using the culture filtrate of *Aspergillus oryzae*. While ulHaq *et al*., (2015) claimed 5.12% and 7.31% saccharification against wheat straw and bagass, respectively, at 45ºC and pH 7.0 by using endoglucanase from *Clostridium thermocellum*. A large scale (1L) saccharification experiment was also performed to scale up this process to achieve the ultimate goal, that is production of biofuel. Same conditions as in preliminary study (100 ml) were used in this experiment and 28.6%, 19.2% and 21.8% saccharification was obtained from sugarcane bagasse rice straw and wheat straw, respectively. These results are slightly better from the primary study which might be due to extensive area which provides efficient enzyme-substrate interaction. The proposed research work is also important with respect to the process economics. Various characteristics (pH stability, thermo stability, stability against various inhibitors and organic solvents) of the recombinant endoglucanase enzyme proved it an advantageous addition in the industrial sector usage. In the light of the present study, it is suggested that endo-1, 4-β-glucanase (*bglC*) cloned from a thermophilic strain of *B. licheniformis* ATCC 14580 might be a useful candidate for use in biofuel industry to produce the fermentable sugars from pretreated plant biomass.

Conclusion

Endoglucanase (*bglC*) of *B. licheniformis* ATCC 14580, which belong to glycosyl hydrolase family, was effectively cloned, expressed in *E. coli* BL21 (+) and characterized. A cost effective enzyme expression was achieved in this study. A detailed characterization of recombinant purified enzyme proved it as a valuable candidate for use in various industrial processes especially in biofuel industry for conversion of plant biomass into fermentable sugar.

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