African Journal of Biotechnology Vol. 11(12), pp. 2846-2854, 9 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2902
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Cloning and expression of endo-1,4–β-glucanase gene from *Bacillus licheniformis* ATCC 14580 into *Escherichia coli* BL21 (DE 3)

Saima Aftab, Muhammad Nauman Aftab*, Ikram-Ul-Haq, Muhammad Mohsin Javed, Asma Zafar and Irfana Iqbal

Government College University, Institute of Industrial Biotechnology, Lahore, Pakistan.

Accepted 4 November, 2011

An endo-1,4-β-glucanase gene from moderate thermophilic strain *Bacillus licheniformis* ATCC 14580 was cloned into *Escherichia coli* DH5α using pTZ57R/T as cloning vector. After confirmation of clone through polymerase chain reaction (PCR) and restriction analysis, endoglucanase gene (1.5 kb) was further expressed in *E. coli* BL21 (DE 3) using pET-22b (+) expression vector. The molecular weight of endoglucanase gene was found to be 52.2 kDa as determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The addition of isopropyl-β-D-thio-galactoside (IPTG) as an inducer in the fermentation medium, caused an increase in expression of recombinant endo-glucanase. The activity of enzyme in optimized condition was found to be 1.5 U/ml using carboxymethylcellulose as substrate. The optimum pH and the temperature of enzyme were also determined and found to be 6.0 and 60°C, respectively.

Key words: Endo-1,4-β-glucanase, *Bacillus licheniformis*, cloning, expression.

INTRODUCTION

Cellulose, a polymer of β-1,4-linked glucose units, is a major polysaccharide constituent of plant cell walls. Cellulases provide a key opportunity for achieving tremen-dous benefits of biomass utilization (Wen et al., 2005). Endo-1,4-β-glucanases (1,4-β-D-glucano-glucohydrolases E.C. 3.2.1.4) are wide spread group of cellulolytic enzymes that act on cellulose by randomly hydrolyzing internal β-1,4-D-glycosidic bonds resulting in decreased length of polymer and increased concentration of reducing sugar (Onsori et al., 2005). Endoglucanases show more affinity for amorphous zone of cellulose where hydrogen bonds between cellulose fibres have weak interactions (Dominguez et al., 1992). In comparison to fungal cellulases, bacterial cellulases are usually obtained easily. In addition, due to short generation time and ability to grow to very high cell density using inexpensive carbon and nitrogen sources, bacteria are good sources of secreted enzymes. Further utilization of the expression system and manipulation of bacteria is much more convenient than fungi; therefore, high-level expression of endogenous cellulase is more easily achieved in bacteria (Li et al., 2009).

Bacillus species produce several hydrolytic enzymes (Mawadza et al., 1996). Recently, extensive studies on proteins (such as cellulase, protease, and amylase) secreted by *Bacillus* species have shown that the following *Bacillus* species produce cellulases: *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus polymyxa* (Han et al., 1995). *B. licheniformis* is a gram-positive endospore forming organism that can be isolated from soils and plant material all over the world (Sneath et al., 1986). The organism was never reported to be pathogenic for either animals or plants and is used extensively for large-scale industrial production of exoenzymes as it can secrete large quantities of proteins (Veith et al., 2004).

Endo-β-1-4 glucanase has been exploited in a vast range of biotechnological applications like brewing industry (Celestino et al., 2006), vinification (Blattel et al., 2010), animal feed production (Walsh et al., 1995), and waste management (Liu et al., 2004). Bacterial β-1-4 glucanase belongs to the glycodyl hydrolase family 16

*Corresponding author. E-mail: nauman535@yahoo.com.

(GH16). Endo-β-1-4 glucanase has been studied and cloned from various organisms such as *B. subtilis* (Qiao et al., 2009), *Bacillus amyloliquefaciens* (Li et al., 2009), *Opuntia vulgaris* (Shyamala et al., 2011), *Trichoderma harzianum* (Noronha and Ulhoa, 2000), *Bacillus halodurans* (Akita et al., 2005), *Clostridium thermocellum* (Schimming, 1992), *Thermomonospora fusca* (Jung et al., 1993), *Oerskovia xanthineolytica* (Shen et al., 1991), *Ruminococcus flavefaciens* (Flint et al., 1993), *Streptococcus bovis* (Ekinci and Flint, 2001), *Phaffia rhodozyma* (Bang et al., 1999), *Rhodothermus marinus* (Spilliaert, 1994), *Pyrococcus furiosus* (Gueguen et al., 1997) and *Phanerochaete chrysosporium* (Ishida et al., 2009).

The genus *Bacillus* represents one of the most important groups of bacteria not only for the production of commercially valuable enzymes, such as proteolytic, amylolytic and cellulolytic enzymes, but also for the study of the secretion mechanism of extracellular enzymes (Khan and Husaini, 2006). Molecular cloning with plasmids has been well documented in some *Bacillus* species. *B. licheniformis* is a gram-positive, spore-forming soil bacterium that is used in the biotechnology industry to manufacture enzymes. *B. licheniformis* bears the generally regarded as safe (GRAS) status (Lam et al., 1998). The availability of the genome sequence from *B. licheniformis* ATCC 14580 facilitates direct genetic manipulations and thus maximum exploitation (Waldeck et al., 2007). *Escherichia coli* is one of the most widely used host for the production of recombinant proteins as it is the best characterized host with many available expression systems (Choi and Lee, 2004).

Due to wide scope of applications of endo-1,4-β-glucanases, this study was mainly concerned with cloning of endo-1,4-β-glucanases gene, *bgl C*, (1.54 Kb) of *B. licheniformis* ATCC 14580 and subsequent expression in E. coli BL21 (DE3).

MATERIALS AND METHODS

Bacterial strain and cultural conditions

B. licheniformis strain ATCC 14580 was obtained from the USDA ARS Culture Collection maintained at the USDA National Center for Agricultural Utilization Research, Peoria. The lyophilized culture was maintained in a medium of the following composition: tryptone (1%), yeast extract (0.5%), glucose (0.1%) and K2HPO4 (0.1%). Then, it was incubated overnight in shaking incubator (Ecocell, MMM Medcenter) at 37°C and maintained on agar slants. The glycerol stock of the culture was also maintained in eppendorf tubes at -80°C for long term storage.

Isolation of genomic DNA and PCR amplification

Genomic DNA from *B. licheniformis* ATCC 14580 was isolated by the protocol given by Kronstad et al. (1983). DNA was spooled out and resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA) buffer. DNA quantification was carried out by visualization and comparison of sample with bands of ladder on 1% agarose gel as well as by spectrophotometeric calculations. Endoglucanase gene (1.5 Kb) was amplified by polymerase chain reaction (PCR) using the following set of forward and reverse primers: forward primer- 5' GCCATATGCGTTCCATCTCTGTCTTCAT 3'; reverse primer- 5' TTATTTAGGTTCAGTGCCC 3'

In the forward primer, *Nde* I site was introduced at 5' end that will introduce ATG codon after restriction with *Nde I*. The 50 µl reaction mixture contained 5 µl of 10 × PCR buffer, 5 µl of 2.5 mM dNTP mixture, 5 µl of 25 mM magnesium chloride, l µl of 10 µM of each primer, l µl of 3 units of Taq DNA polymerase and 1 µl of genomic DNA. The reaction mixture was incubated in a thermal cycler at 94°C for 5 min and then underwent 35 amplification cycles of 30 s at 94°C, 30 s at 54°C and 90 s at 72°C, followed by a final incubation of 20 min at 72°C. The PCR product was analyzed on 1% agarose gel and purified by using Spin prep gel melt kit (Novagen).

Cloning of endo-glucanase gene in pTZ57R/T

The purified *B. licheniformis* endo-1,4-β-glucanase gene was ligated into cloning vector pTZ57R/T (2886 bp) by using T4 DNA ligase and incubated overnight in an incubator at 22°C. The ligated mixture was transformed into *E. coli* DH5α competent cells which were prepared according to calcium chloride method (Cohen et al., 1972). Transformed cells were selected on LB agar plates containing isopropyl-*β*-D-thiogalactopyranoside (IPTG), (130 ng/ml), X-Gal (270 µg/ml) and ampicillin, (100 µg/ml) by blue/white screening technique. Plates were then incubated at 37°C overnight. Positive clones were identified by colony PCR.

Plasmid isolation and restriction analysis

Plasmid was isolated by alkaline lysis method (Birnboim and Doly, 1979) and cloning of *B. licheniformis* endoglucanase gene in pTZ57R/T was further confirmed by single and double restriction of the recombinant plasmid with *Hind* III and *Nde* I.

Cloning of endo-glucanase gene in pET-22b (+)

The recombinant pTZ57R/T containing *B. licheniformis* endoglucanase gene was double digested with *Nde* I and *Hind* III. Expression vector pET-22b (+) was also digested with the same enzymes. The restricted fragment as well as the double digested pET-22b (+) vector were run on 1% agarose gel, purified and then ligated to the pET-22b (+) using T4 DNA ligase. The ligated product was then transformed into competent cells of *E. coli* BL 21(DE 3) which were prepared according to the method of Cohen et al. (1972). Confirmation of positive clones was performed by colony PCR as well as by double restriction of recombinant plasmid with *Hind* III and *Nde* I.

Expression of *B. licheniformis* endo-glucanase gene in *E. coli* BL21

Single transformed colony from LB agar plate containing 100 µg/ml ampicillin was picked up and inoculated into 50 ml of LB medium containing ampicillin (100 mg/ml) and incubated overnight at 37°C in shaking incubator. 1 ml of overnight grown culture was then inoculated to 100 ml of the above mentioned LB medium and incubated at 37°C in shaking incubator until an OD of 0.4 to 0.6 at 600 nm was obtained. Then, this culture was induced with IPTG and incubated for another 3 h at 37°C. After 3 h, this culture was centrifuged at 6000 *g* for 10 min. Supernatant was discarded and the pellet was resuspended in 5 ml of 50 mM Tris-HCl (pH 7.5 to 8). After resuspension, the mixture was lysed by sonication in a

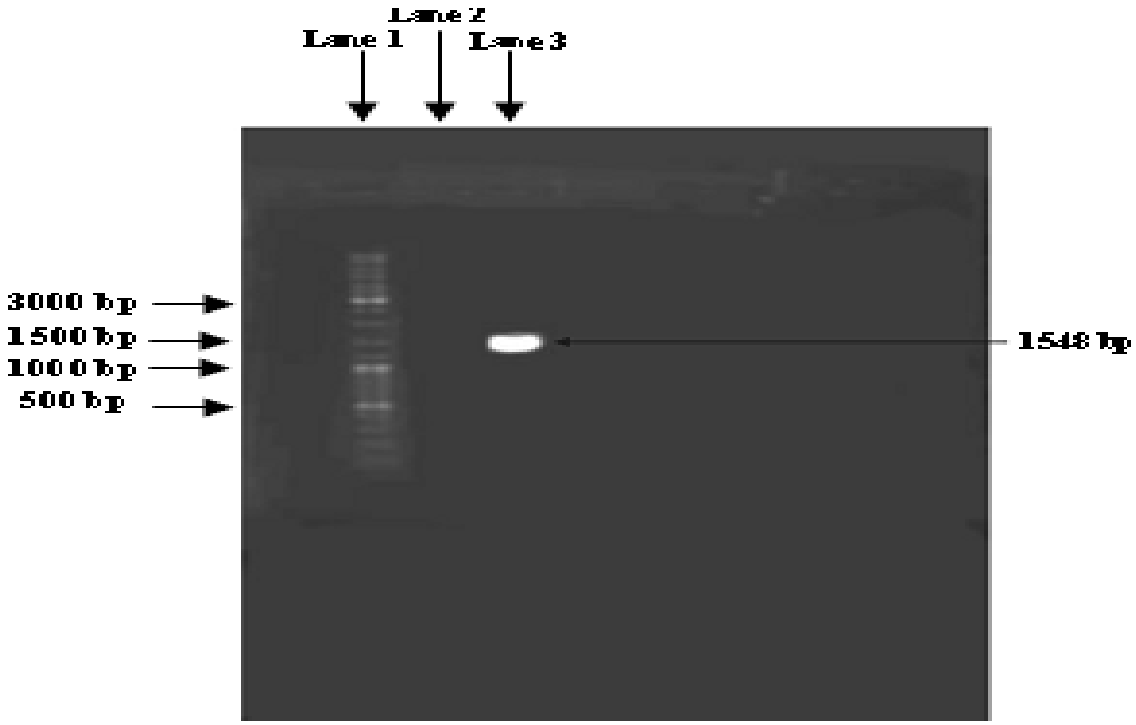

Figure 1. PCR amplification of endo-1,4-β-glucanase gene. Lane 1, Ladder; lane 3, endo-1,4-β-glucanase gene (1.5 kb).

sonication heat system (Hielsher, ultrasound technology) for 10 min and again centrifuged at 6000 *g* for 10 min. Pellet obtained was again resuspended in 5 ml of 50 mM Tris-HCl (pH 7.5). *B. licheniformis* endoglucanase protein was confirmed by using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970).

Electrophoretic analyses

DNA samples were analyzed by 0.8% agarose gel electrophoresis followed by staining with ethidium bromide and visualizing under the UV light. Protein samples were analyzed by 12% PAGE in the pre-zsence of 0.1% SDS followed by staining with Coomassie brilliant blue.

Fermentation technique

Shake flask

Submerged fermentation technique was employed for production of endo-1,4-β-glucanase. 50 ml of fermentation medium was transferred to each 250 ml cotton plugged conical flasks. The inoculum was transferred to each flask in laminar flow hood. The flasks were then placed in rotary incubator shaker at 200 rpm at 37°C. The fermented broth was used for estimation of endo-1,4-β-glucanase.

Enzyme assay

The fermented broth was centrifuged at 6000 rpm for 10 min. The supernatant was analyzed for estimation of endo-1,4-β-glucanase by using dinitrosalicylic acid (DNS) method (Miller, 1959). Carboxymethyl cellulose (CMC) was used as substrate for enzyme assay. The reducing sugar liberated was measured at 550 nm in spectrophotometer using glucose as standard. One unit of enzyme activity was defined as “the amount of enzyme that liberates one µmole of reducing sugar per ml per minute from appropriate substrate under assay conditions”.

Sequence comparison

Sequence database similarity searches were performed using BLASTP (Altschul et al., 1990). Sequence alignments were done using ClustalW (Thompson et al., 1994) and visualized by Jalview (Waterhouse et al., 2009).

Statistical analysis

Treatment effects were compared by the protected least significant difference method after Snedecor and Cochrane (1980) using computer software Costat by applying analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Cloning of β-glucosidase gene in pET-22b(+)

B. licheniformis strain ATCC 14580 was grown in LB medium and genomic DNA was isolated (Kronstad et al., 1983). Amplification of endoglucanase gene was performed by polymerase chain reaction using specific primers (Figure 1). After completion of PCR cycles, additional 20 min incubation of mixture was carried out at 72°C. This incubation will add adenine at the ends of PCR product

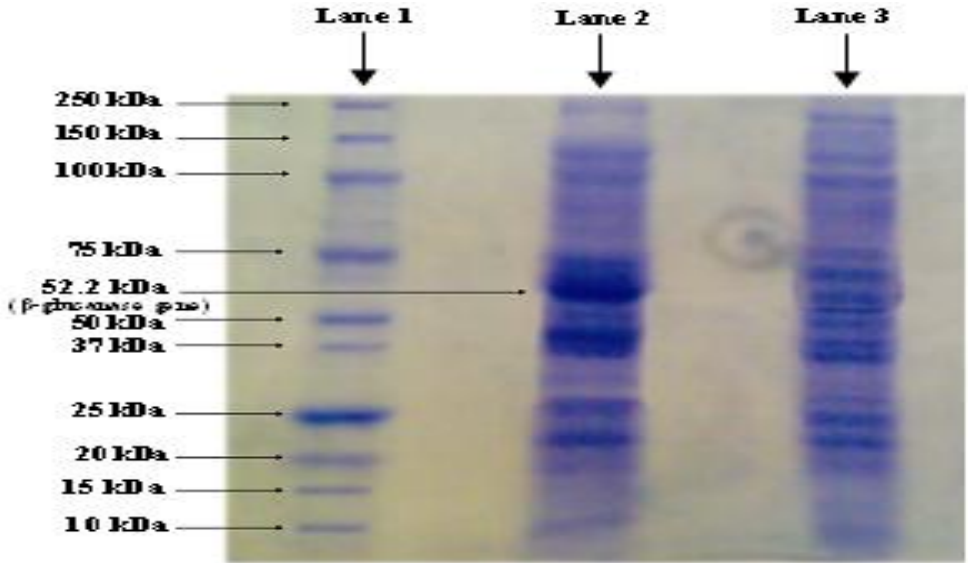

Figure 2. SDS-PAGE analysis of recombinant endo-1, 4-β-glucanase gene. Lane 1, protein marker; lane 2, extract of cloned *E. coli* (sample); lane 3, extract of wild *E. coli* (control).

as overhangs. A 1500 bp band of endoglucanase gene obtained after PCR was cut from the gel and purified by using Novagen kit. Purified PCR product obtained was ligated with cloning vector pTZ57R/T using T4 DNA ligase and recombinant plasmid was transformed into competent cells of *E. coli* DH5α.

Rabbani et al. (2009) also reported the use of pTZ 57 R/T vector for cloning procedures. Positive clones were identified using blue and white screening. Colony PCR was performed to confirm process of transformation. The transformed plasmid was isolated by alkaline lysis method (Birnboim and Doly, 1979). The pTZ57 R/T plasmid was double restricted with *Nde* I and *Hind* III to separate the ligated product from the plasmid. The restricted plasmid was run on the gel and appearance of 1.5 kb band confirmed the ligation of endoglucanase gene with plasmid. The gene was then cloned in expression vector pET22b (+). For this purpose, double digested endoglucanase gene was ligated with pET 22b (+) which has been double restricted with *Nde* I and *Hind* III. The ligated product was transformed in the freshly prepared competent cells of *E. coli* BL21.

Expression of recombinant β-glucosidase

Endoglucanase activity was investigated in both intracellular and extracellular samples. For the presence of endoglucanase enzyme in intracellular samples, the bacterial cells were broken by using the sonicator. Quantitative analysis was performed using DNS method (Miller, 1959). Higher activity of endoglucanase enzyme was observed in extracellular samples and was calculated to be 1.5 U/ml, respectively.

Reasonable amount of endoglucanase was also observed in intracellular samples, that is, 0.8 U/ml. To check expression of cloned gene in *E. coli* BL21, the samples along with marker and control (wild BL21) were run on SDS-PAGE.

The band at position 52.2 kDa was observed in the sample of *E .coli* cloned with pET 22b (+) containing endoglucanase gene (Figure 2). No such band was observed in the control sample obtained from wild BL21 strain that was cloned with pET 22b (+) indicating the absence of endoglucanase activity.

Similarity search for recombinant β- endoglucanase

Sequence similarity searches for amino acid sequence in *β*-glucanase of *B. licheniformis* were conducted using BLASTP tool. Results demonstrate that *β*-glucanase of *B. licheniformis* exhibited high similarity with β-glucanases of glycodyl hydrolase family 16 (GH16) of carbohydrate-active enzyme classification (Cantarel et al., 2009; http://www.cazy.org/). The *B. licheniformis* β-glucanase showed 73, 72, 71, 72, 51 and 50% identity (Figure 3) with β-glucanase from *B. subtilis* (Genebank accession No P1O45), *Geobacillus stearothermophilus* (E3V048), *Bacillus amyloliquefaciens* (A7Z597), *Paenibacillus campinasensis* (D8WN01), *Erwinia carotovora* (Q47096), and Pectobacterium wasabiae (D0KFU8).

Characterization of recombinant β-glucosidase

To increase the yield of endoglucanase, the induction parameters including optical density (OD) of the culture

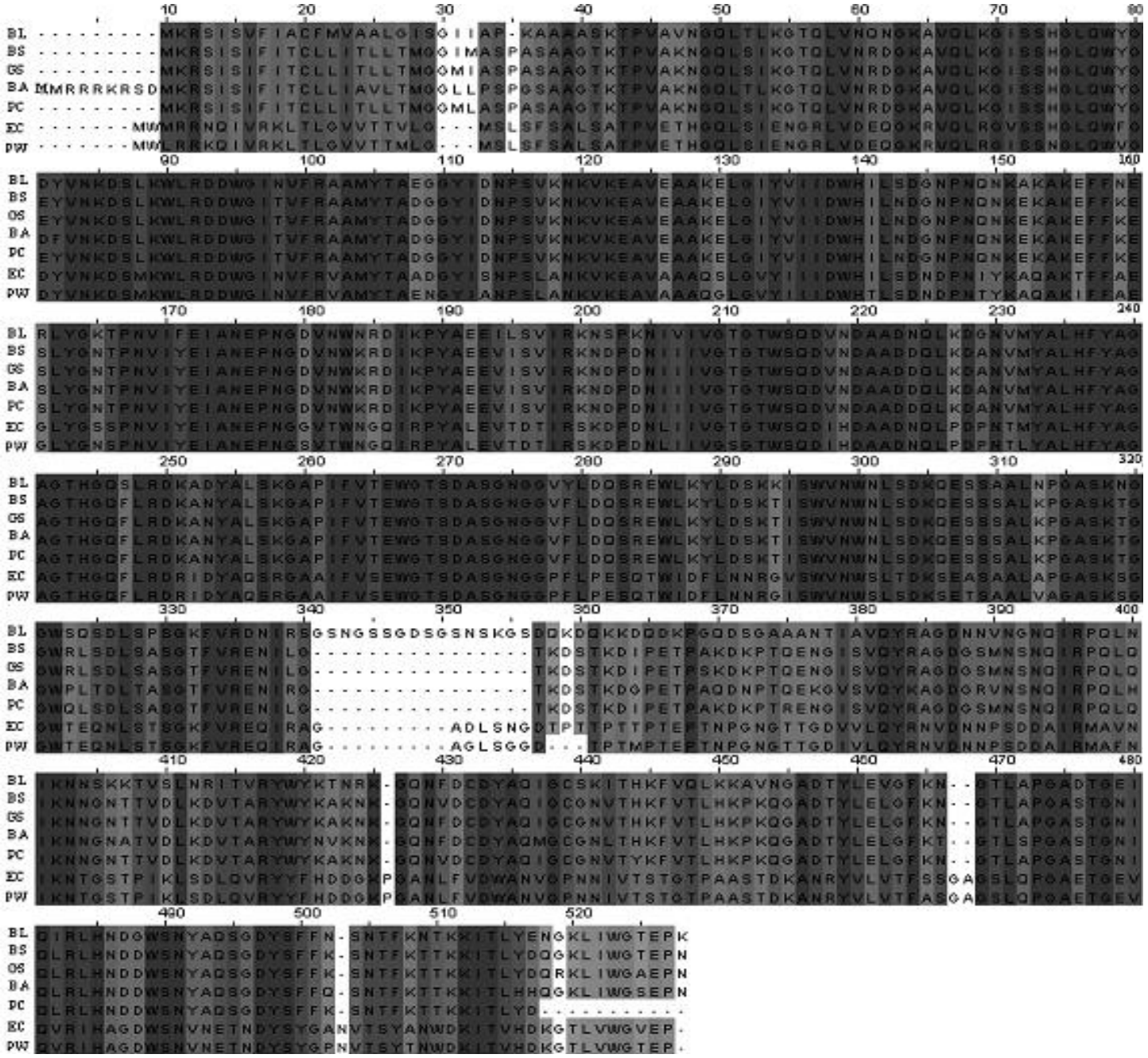

Figure 3. Jalview representation of multiple sequence alignment of *B. licheniformis* β-glucanase with other glycodyl hydrolase family 16 β-glucanase. BL, *B. licheniformis* ATCC 14580; BS, *B. subtilis*; GS, *Geobacillus stearothermophilus*; BA, *B. amyloliquefaciens*; PC, *Paenibacillus campinasensis*; EC, *Erwinia carotovora*; PW, *Pectobacterium wasabiae*. Identities in all sequences are represented as black shaded areas while other identities are shown as grey shaded areas.

was assessed to confirm maximum number of cells in the culture before induction with inducer. This was achieved by varying optical density of cells from 0.2 to 1.0 nm. However, an OD600 of 0.6 nm which represent the exponential phase gave maximum activity of 0.7 U/ml (Figure 4A). Further increase in O.D caused a decrease in enzyme activity. This might be due to the fact that the bacterial cells may enter the stationary phase of growth due to lack of nutrients in medium. The expression of endoglucanase gene was further enhanced by adding different inducers such as lactose, maltose and IPTG to the fermentation medium that induces the promoter region of pET 22b(+).

It was found that IPTG which is an analog of lactose gave maximum enzyme production. The concentration of inducer was then optimized by using IPTG in range of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM. However 0.3 mM IPTG

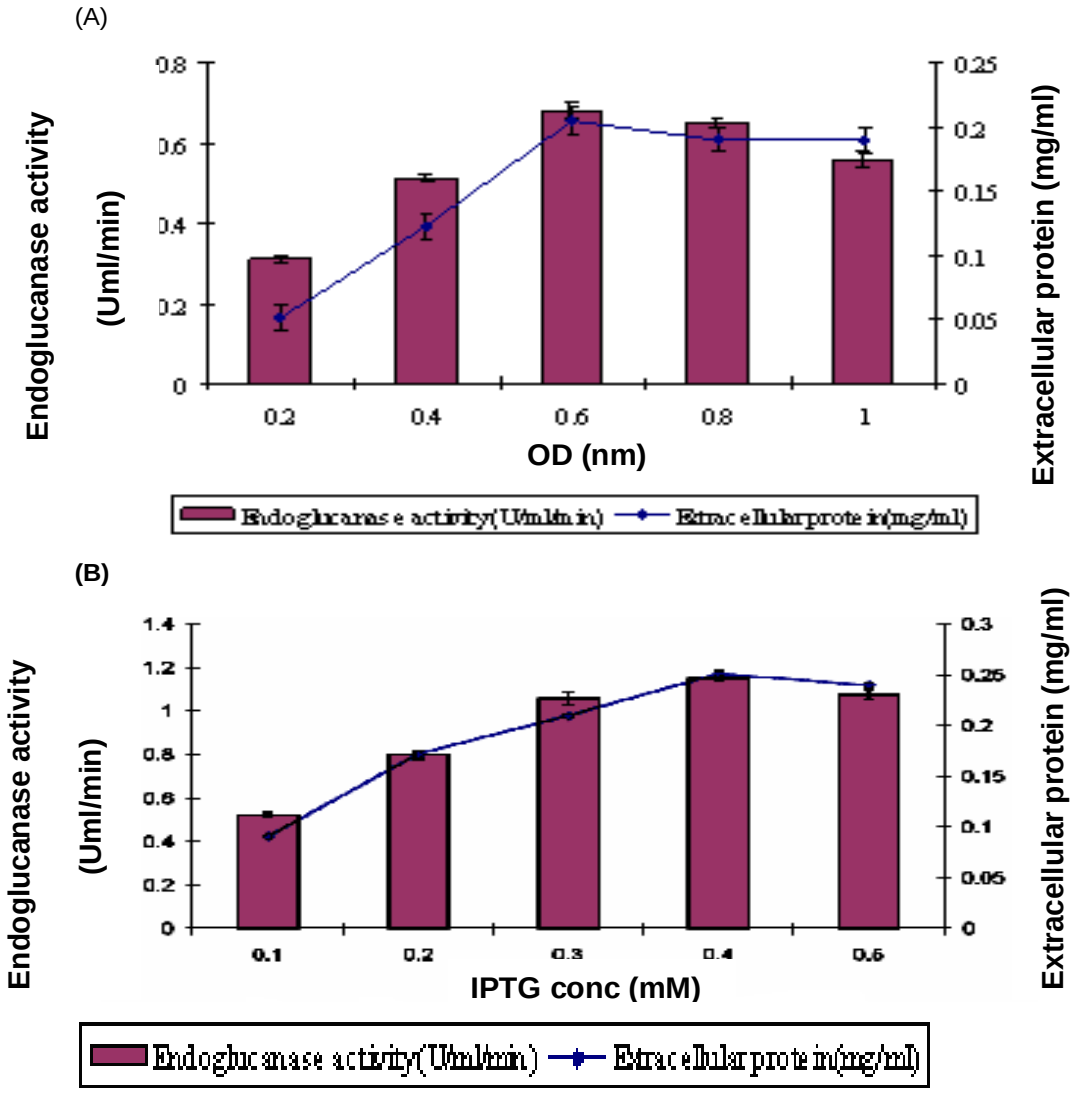

Figure 4. (A) Effect of O.D on production of endo-glucanase in shake flask. (B) Effect of IPTG concentration on production of endo-glucanase in shake flask. Y error bars indicates standard error of mean among three parallel replicates. These values are statistically significant at probability level of ≤ 0.05.

gave maximum enzyme activity of 1.09 U/ml (Figure 4B). Afzal et al. (2010) also reported that the presence of IPTG in the fermentation media increase the activity of recombinant enzyme. The effect of pH on endo-glucanase production was studied by incubating at different pH (4 to 7).

However, maximum activity was observed at pH 6.0 using citrate phosphate buffer (Figure 5 A). Further increase or decrease in pH resulted in a decrease in the enzyme activity. Our result is in accordance with the work of Bischoff et al. (2006) who also obtained maximum endo-glucanase production at pH 6.0. Nurachman et al. (2010) also found that pH 6.0 is optimum for maximum endo-glucanase production from *Bacillus amyloli-quefacians*. To determine the effect of temperature on assay conditions, the enzyme was assayed at various temperature ranges (30 to 70°C). Maximum activity of endo-glucanase (1.5 U/ml) was obtained at 60°C (Figure 5B). Increase in temperature might cause an increase in the kinetic energy of molecules which resulted in increase in the enzyme activity. Further increase in temperature caused a decline in the enzyme activity due to denaturation of the enzyme. Similar result was obtained by Dong et al. (2010) who obtained maximum endo-glucanase activity at 60°C. Bajaj et al. (2009) and Akita et al. (2005) also reported that maximum endo-glucanase activity from *Bacillus* strain M-9 and *Bacillus halodurans* C-125 was obtained at 60°C. These results are also in accordance with the work of Norita et al. (2010) who reported optimal endoglucanase activity of *Aspergillus*

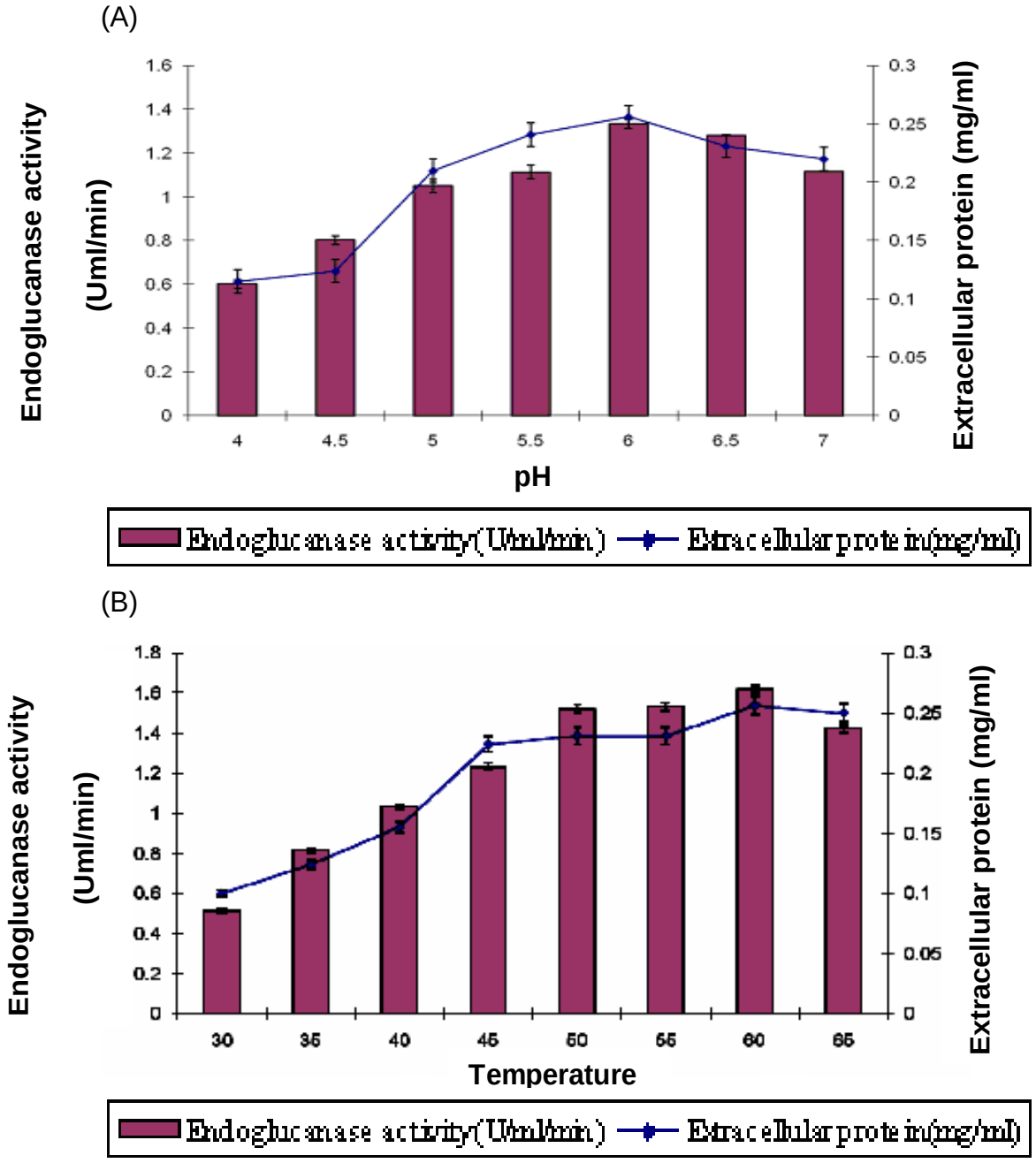

Figure 5. (A) Effect of different values of pH on production of endo-glucanase gene in shake flask. (B) Effect of different temperature values on production of endo-glucanase gene in shake flask. Y error bars indicates standard error of mean among three parallel replicates. These values are statistically significant at probability level of ≤ 0.05.

niger at 60°C. However, our results vary from the results obtained by Louimie et al. (2007) who reported 45°C to be an optimal temperature for endo-glucanase activity. The effect of incubation period on endo-glucanase production was determined by incubating the enzyme at different time intervals of 10 to 40 min (Figure 6). The enzyme production increased with time and reached it maximum value after 30 min of incubation. However, the activity of enzyme slowed down as the incubation proceeded. As far as the expression level of the endo-glucanase gene is concerned, our results are much better than the recently conducted studies on the expression of endo-glucanase of various organisms (Shyamala et al., 2011; Akita et al., 2005; Noronha and Ulhoa, 2000). Further, due to higher temperature tolerant ability of the endo-glucanase of *B. licheniformis*, this endo-glucanase could be used in various industrial processes that are preceded at higher temperatures.

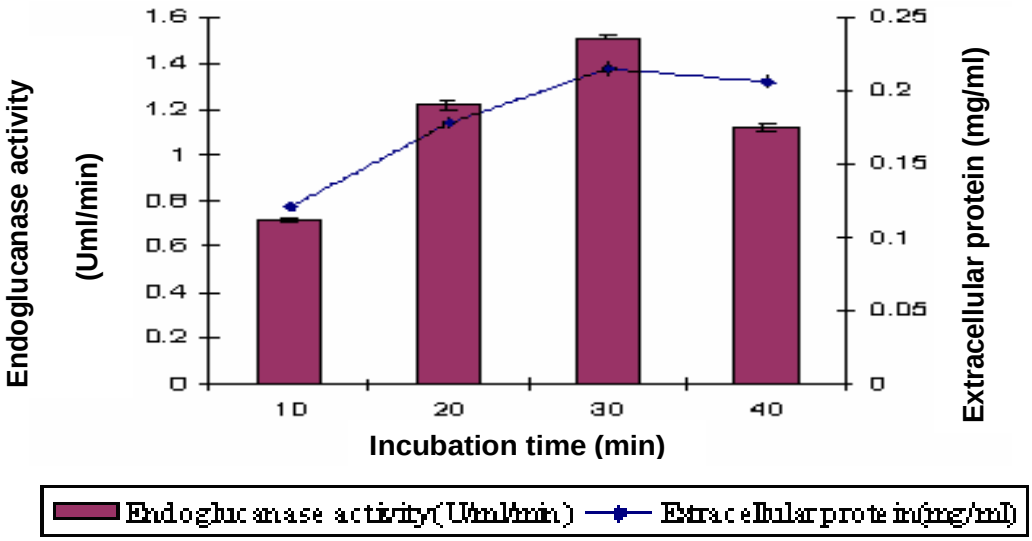

Figure 6. Effect of incubation time on production of endo-glucanase gene in shake flask. Y error bars indicates standard error of mean among three parallel replicates. These values are statistically significant at probability level of ≤ 0.05.

REFERENCES

Afzal S, Saleem M, Yasmin R, Naz M, Imran M (2010). Pre and post cloning characterization of a beta-1, 4-endoglucanase from *Bacillus* sp. Mol. Bio. Res. 37(4): 1717-23.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Akita M, Kayatama K, Hatada Y, Ito S, Horikoshi K (2005). A novel beta-glucanase gene from *Bacillus halodurans* C-125. FEMS Microbiol Lett. 248(1): 9-15.

Bajaj KB, Pangotra H, Wani MA, Sharma P Sharma A (2009). Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated *Bacillus* strain M-9. Indian J. Chem. Technol. 16: 382-387.

Bang ML, Villadsen I, Sandal T (1999). Cloning and characterization of an endo-beta-1,3(4)glucanase and an aspartic protease from *Phaffia rhodozyma* CBS 6938. Appl. Microbiol. Biotechnol. 51: 215-222.

Birnboim CH, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res.7: 1513-1515.

Bischoff KM, Rooney AP, Li X, Liu S Hughes SR (2006). Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis.* Biotechnol. Lett. 28(21): 1761-1765.

Blattel V, Larisika M, Pfeiffer P, Nowak C, Eich A, Eckelt J, Konig H (2011). β-1,3-Glucanase from *Delftia tsuruhatensis* strain MV01 and its potential application in vinification. Appl Environ. Microbiol. 77(3): 983-990.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res. 37: 233-238.

Celestino KRS, Cunha RB, Felix CR (2006). Characterization of a β-glucanase produced by *Rhizopus microspores* var. *microsporus*, and its potential for application in the brewing industry. doi:10.1186/1471-2091-7-23. BMC Biochem. 7: 23.

Choi JH, Lee SY (2004). Secretory and extracellular production of recombinant proteins using *Escherchia coli*. Appl. Microbiol. Biotechnol. 64: 625-635.

Cohen SN, Chang AC, Hsu L (1972). Non chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. 70: 3240-3244.

Dominguez JM, Acebal C, Jimenez J, Mata IDL, Macarron R, Castillon MP (1992). Mechanisms of thermo inactivation of endoglucanasel from *Trichoderma reesei* QM 9414. J. Biochem. 287: 583-588.

Dong J, Hong Y, Shao Z, Liu Z (2010). Molecular cloning, purification, and characterization of a novel, acidic, pH-stable endoglucanase from *Martelella mediterranea*. J. Microbiol. 48(3): 393-398.

Ekinci MS, McCrae SI, Flint HJ (1997). Isolation and overexpression of a gene encoding an extracellular beta-(1,3-1,4)-glucanase from *Streptococcus bovis* JB1. Appl Environ Microbiol. 63(10): 3752-3756.

Flint HJ, Martin J, McPherson CA, Daniel AS, Zhang JX (1993). A bifunctional enzyme, with separate xylanase and beta(1,3-1,4)-glucanase domains, encoded by the xynD gene of *Ruminococcus flavefaciens*. J. Bacteriol. 175: 2943-2951.

Gueguen Y, Voorhorst WG, van der Oost J, De Vos Wm (1997). Molecular and biochemical characterization of an endo-beta-1,3-glucanase of the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Biol. Chem. 272: 31258-31264.

Han SJ, Yoo YJ, Kang HS (1995). Characterization of a bifunctional cellulase and its structural gene. J. Biol. Chem., 270(43): 26012-26019.

Ishida T, Fushinobu S, Kawai R, Kitaoka M, Igarashi K, Samejima M (2009). Crystal structure of glycoside hydrolase family 55 {beta}-1,3-glucanase from the basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. 284: 10100-10109.

Jung ED, Lao G, Irwin D, Barr BK, Benjamin A, Wilson DB (1993). DNA sequences and expression in Streptomyces lividans of an exoglucanase gene and an endoglucanase gene from Thermomonospora fusca. Appl. Environ. Microbiol. 59: 3032-3043.

Khan FAB, Husaini AAS (2006). Enhancing *α*-amylase and cellulase in vivo enzyme expressions on sago pith residue using *Bacillus amyloliquefaciens* UMAS 1002. Biotechnol. (Pakistan), 5: 391-403.

Kronstad JW, Schnepf E, Whiteley RH (1983). Diversity of location for *Bacillus thuringiensis* crystal protein genes. J. Bacteriol. 154: 419-428.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lam KH, Chow KC, Wong WK (1998). Construction of an efficient *Bacillus subtilis* system for extracellular production of heterologous protein. J. Biotechnol. 63: 167-177.

Li W, Huan X, Zhou Y, Ma Q, Chen Y (2009). Simultaneous cloning and expression of two cellulase genes from *Bacillus subtilis* newly

isolated from Golden Takin. Biochem. Biophys. Res. Commun. 383(4): 397-400.
Liu B, Yang Q, Zhou Q, Song J, Chen D, Liu H. 2004. Cloning and expression of the endo-β-glucanase III cDNA gene from *Trichoderma viride* AS3.3711. Acta Environ. Sinica 25: 127-132.
Louime C, Abazinge M, Johnson E, Latinwo L, Ikediobi C, Clark AM (2007). Molecular cloning and biochemical characterization of a family-9 endoglucanase with an unusual structure from the gliding bacteria *Cytophaga hutchinsonii.* Appl. Biochem. Biotechnol. 141(1): 127-38.
Mawadza C Zvauya R (1996). Some factors affecting Endo-β -1, 4-Glucanase production by two *Bacillus* strains isolated from zimbabwean hot springs. J. Basic Microbiol. 36(3): 177-185.
Miller LG (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31: 426-428.
Norita S, Rosfarizan M, Ariff AB (2010). Evaluation of the activities of concentrated crude mannan-degrading enzymes produced by *Aspergillus niger*. Mal. J. Microb. 6(2): 171-180.
Noronha EF, Ulhoa CJ (2000). Characterization of a 29-kDa beta-1,3-glucanase from *Trichoderma harzianum*. FEMS Microbiol Lett. 183(1): 119-123.
Nurachman Z, Kurniasih SD, Puspitawati F, Hadi S, Radjasa OK Natalia D (2010). Cloning of the Endoglucanase gene from a *Bacillus amyloliquefaciens* PSM 3.1 in *Escherichia coli* revealed catalytic triad residues Thr-His-Glu. Am. Biochem. Biotech. 6(4): 268-274.
Onsori H, Zamani MR, Motallebi M, Zarghami N (2005). Identification of over producer strain of endo-1, 4-glucanase in *Aspergillus Species*: Characterization of crude carboxymethyl cellulase. Afr. J. Biotech. 4(1): 26-30.
Qiao J, Dong B, Li Y, Zhang B, Cao Y (2009). Cloning of a beta-1,3-1,4-glucanase gene from *Bacillus subtilis* MA139 and its functional expression in *Escherichia coli*. Appl Biochem Biotechnol. 152(2): 334-342.
Rabbani M, Sadeghi HMM, Ani M, Chegini KG, Etemadifar Z Moazen F (2009). Cloning and nucleotide sequence of a lipase gene from a soil isolate. Res. Pharm. Sci. 4(1): 25-32.
Schimming S, Schwarz WH, Staudenbauer WL (1992). Structure of the *Clostridium thermocellum* gene licB and the encoded beta-1,3-1,4-glucanase. A catalytic region homologous to *Bacillus* lichenases joined to the reiterated domain of clostridial cellulases." Eur. J. Biochem. 204: 13-19.
Shen SH, Chretien P, Bastien L, Slilaty SN (1991). Primary sequence of the glucanase gene from *Oerskovia xanthineolytica*. Expression and purification of the enzyme from *Escherichia coli*. J. Biol. Chem. 266: 1058-1063.
Shyamala S, Ravikumar S, Vikramathithan J, Srikumar K (2011). Isolation, Purification, and Characterization of Two Thermostable Endo-1,4-β-D glucanase Forms from *Opuntia vulgaris*. Appl Biochem Biotechnol. [Epub ahead of print].
Sneath PHA, Mair NS, Sharpe E, Holt JG (1986). Bergey's Manual of Sytematic Bacteriology. 1st Ed., The Williams and Wilkins Co., Baltimore. Vol. 2.
Snedecor GW, Cochrane WG (1980). Statistical Methods, 7th Ed., Iowa State University Press, Ames, Iowa, ISBN 0-81381560-6.
Spilliaert R, Hreggvidsson GO, Kristjansson JK, Eggertsson G, Palsdottir A (1994). Cloning and sequencing of a *Rhodothermus marinus* gene, bglA, coding for a thermostable beta-glucanase and its expression in *Escherichia coli*. Eur. J. Biochem. 224: 923-930.
Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.
Veith B, Herzberg C, Steckel S, Feesche J, Maurer KH, Ehrenreich P, Baumer S, Henne A, Liesegang H, Merkl R, Ehrenreich A, Gottschalk G (2004). The Complete Genome Sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J. Mol. Microbiol. Biotechnol. 7(4): 204-211.
Waldeck J, Rammes HM, Wieland S, Feesche J, Maurer KH, Meinhardt F (2007. Targeted deletion of genes encoding extracellular enzymes in *Bacillus licheniformis* and the impact on the secretion capability. J. Biotechnol. 130: 124-132.
Walsh GA, Murphy RA, Killeen GF, Headon DR, Power RF (1995). Technical note: detection and quantification of supplemental fungal β-glucanase activity in animal feed. J. Anim. Sci. 73: 1074-1076
Waterhouse AM, Procter JB, Martin DM, Clamp M Barton GJ (2009). Jalview Version 2 – a multiple sequence alignment editor and analysis workbench. Bioinformatics, 25: 1189-1191.
Wen Z, Liao W, Chen S (2005). Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresour. Technol. 96(4): 491-499.