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Cloning and Characterization of Beta-1, 4 Glucosidase 2 (*Bgl*2) Gene from A High Producer Cellulolytic Enzyme *Trichoderma Harzianum* (T7)

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Abstract: In this study *Trichoderma harzianum* (T7) was selected as high producer of beta glucosidase enzyme in optimum conditions among 30 isolates of *Trichoderma* speices. Genomic DNA from a native Iranian isolate of *Trichoderma harzianum* (T7) that has been isolated from Kerman province was extracted by CTAB method and specific primers (TFbgl2 & TRbgl2) were used for *bgl*2 amplification. The amplified DNA fragment approximately 1.4 Kb was digested with appropriate enzymes and cloned into plasmid vector pUC19 and designated as pUCSA1. The sequence obtained from this construct is under accession number EF426298. For cDNA synthesis the mRNA of *bgl*2 was extracted from induced culture with 1% lactose and 1% CMC and used. This cDNA (approximately 1.3 kb) was isolated, digested, then confirmed with appropriate restriction enzymes and cloned into pUC19 plasmid vector and designated as pUCSA2. The DNA sequence of *bgl*2 from this construct is under accession number EF426299. Comparison of the genomic DNA with the cDNA sequences showed that *bgl*2 gene contains one short intron, 79 bp in length and its open reading frame (ORF) with approximately 1.3 kb encodes a polypeptide with 454 amino acids and estimated molecular mass of 51 kDa. Multiple alignment of the deduced amino acid sequence of Bgl2 with those from *Hypocrea jecorina* (BAA74959), *T. viride* (AAQ21384), *Humicola grisea* (BAA74958) and *A. niger* (AAF74209) showed 89.6%, 80.7%, 72.8% and 83.6% homology, respectively.

Key words: *Trichoderma harzianum* . Beta-glucosidase . genomic DNA . cDNA construction

INTERODUCTION

Cellulose is a simple, linear polymer built up from glycosyl units connected by β(1-4) linkages. These linear chains can vary in length and often consist of many thousands of units [1]. Within the biosphere, there is an enormous of cellulose through large-scale production and degradation. Though chemically simple, cellulose is physically complex with both crystalline and amorphous regions and a number of different enzymes are required for its efficient hydrolysis [2]. Cellulose is degraded in nature by the concerted action of several synergistically functioning enzymes. Depending on their mode of action, cellulolytic enzymes fall into one of two main groups, endogluconase or cellobiohydrolase [1]. The complete degradation of cellulose to glucose requires the action of at least three types of enzymes: endo-ß-1,4-glucanase, exo-ß-1,4-glucanase (cellobiohydrolase) and ß-glucosidase [3].

ß-glucosidases play a number of different important roles in biology, including the degradation of cellulosic biomass by fungi and bacteria, degredation of glycolypids in mammalian lysosomes and the cleavage of glucosylated flavonoids in plants. These enzymes are therefore of considerable industrial interest, not only as constituents of cellulose-degrading system, but also in the food industry [1, 4].

One of the most extensively studied cellulolytic organisms is the soft rot fungus *Trichoderma* sp. whose hypercellulolytic mutant strains secrete large amount of cellulases. This fungus produces a complete set of cellulases including ß-glucosidase [5]. At present, different species of *Trichoderma* appear to be most promising microorganisms for industrial production of cellulases, specially the ß-glucosidase enzyme.

In this study, we have cloned and sequenced the genomic DNA and cDNA of *bgl*2 gene from a high producer cellulolytic enzyme Iranian isolate of *T. harzianum* (T7) to characterize gene structure which could be used for expression of Bgl2 in appropriate host.

MATERIALS AND METHODS

Microorganism, plasmid vector and culture conditions: Thirty isolates of *Trichoderma* sp. Used for

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cellulolytic enzyme production [6]. The stock culture was stored on agar (1.5%) slants of MY medium (2% malt extract, 0.2% yeast extract, 1% maltose). *Trichoderma harzianum* (T7) used for DNA and RNA isolation.

For chromosomal DNA preparation, the spores were inoculated in the liquid MY medium and grown for 2 days at 28°C with shaking and mycelia were harvested by filtration [7]. *Escherichia coli* DH5a as a host and pUC19 as a vector used for routine cloning. *E. coli* were cultured in LB medium (1% trypton, 0.5% yeast extract and 1% NaCl).

Production of ß-1,4 glucosidase: Fungal isolates were cultured in Mandels salt medium with optimum conditions for enzyme assay [8]. 0.5 L erlenmayer flask containing 200 ml of Mandels medium, CarboxyMethil Cellulose (CMC) as carbon source and lactose as inducer at pH 5 was inoculated with 10^6 conidia. Culture was incubated in an orbital shaker incubator at 28°C at 150 rpm. After 7 days of incubation samples of 8 ml were collected and centrifuged at 12000 rpm (4°C). Enzyme activity was determined by the dinitrosalicylic acid (DNS) method [9], where one unit is defined as the amount of enzyme releasing 1 µmol glucose per minute (U min^{-1}) [4, 10].

Genomic DNA PCR amplification: For the purpose of amplification of beta glucosidase (*bgl*2) gene from *T. harzianum* (T7), we designed two specific primers against known *bgl*2 sequences.

The two tailed primers, TFbgl2 and TRbgl2 (Table 1), were designed based on sequence similarity of existing *bgl*2 genes present in the database. To facilitate subsequent cloning of the PCR-derived fragments, *XbaI* restriction site (bolded) was added to the 5'-end of these primers (Table 1).

Fungal chromosomal DNA was prepared as described by Sun., (2002) [11]. Amplification of the DNA fragments encoding *T. harzianum* (T7) *bgl*2 gene was performed using polymerase chain reaction (PCR). PCR reactions contained 2.5 units of Fermentas *Pfu* DNA polymerase, 1X buffer, 200 µM of each deoxynucleotide triphosphate, 2 µM $MgSO_4$ and 0.5 µM primers. Reaction conditions for PCR amplification were 94°C for 1 min, 62°C for 1 min and 72°C for 1 min, for 34 cycles followed by a final extension of 10 min. PCR products were separated by electrophoresis on a 1% agarose gel and products were purified by High pure PCR product purification Kit (Roche Diagnostics GmbH). The purified fragments were cloned into pUC19.

Table 1: Oligonucleotides (primers) used in this study

Name	Oligonucleotides sequence
TFbgl2	5'-GCTCTAGAATGTTGCCCAAGGACTTTCAG-3'
TRbgl2	5'-GCTCTAGATCAAGCTCTTTGCGCTCTTCTTG-3'
M13f	5'-GCTAGTTATTGCTCAGCGG-3'
M13r	5'-GTAAAACGACGGCCAGT-3'

RNA Isolation and cDNA synthesis: RNA was isolated as described by Jain (2004) [12]. For RNA isolation *T.* harzianum (T7) was grown in 250 ml Shacked flasks at 28°C and 200 rpm in 50 ml medium which contained 0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.03% $CaCl_2.2H_2O$, 0.03% $MgSO_4.7H_2O$, 0.1% Tween 80 and 1 ml/Lit trace elements solution, contained 18 mM $FeSO_4.7H_2O$, 6.6 mM $MnSO_4$, 4.8 mM $ZnSO_4.7H_2O$ and 15 mM $CoCl_2$ [7]. CMC (1%) and lactose (1%) used as a carbon source and inducer, respectively. Cells were harvested after 48 h of growth and frozen in liquid nitrogen. Frozen mycelium was ground and used for RNA extraction by RNAX-plus kit (cinagen, Iran).

cDNA was produced in the following manner. Reverse transcriptase reaction using the total RNA from the extraction described above was carried out based on the method described by manufacturer (Fermantas). The reaction volume was 50 µl and contained: 5 µg of total RNA, 1 mg of oligo$(dT)_{18}$, 20 units of RNase Block Ribonuclease Inhibitor, 1 X buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$) 500 µM of each dNTP and 200 units reverse transcriptase. The RNA was denatured at 70°C, cooled slowly at room temperature to allow the annealing of primers before it was added to the reaction mixture. The reaction mixture was incubated at 42°C for 2 h and then incubated at 70°C for an additional 10 min. The cDNA from the reaction was kept at-70°C and used for a PCR reaction with specific primers. DNA amplification was carried out in a PCR reactions as described above.

The resulting cDNA gene fragment was recovered and purified from a 1% (w/v) agarose/Tris-borate-EDTA gel using the High pure PCR product purification Kit and cloned into pUC19 cloning vector.

General DNA procedures: Plasmid DNA preparation and electrophoresis of DNA fragments were performed by routine procedures [13]. Enzymatic treatments of DNA molecules were carried out as recommended by manufacturer (Fermentas, Germany). The bacteria were transformed by the $CaCl_2$ method [13].

Sequence and computer analysis: Beta glucanase sequences from different *Trichoderma* sp. were retired

from uniprot database (http://www.pir.uniprot.org). Multiple sequence alignment was generated using Clustal W (http://www.ebi-ac.uk/ClustalW). Cloned DNA and cDNA fragments in pUC19 (70-220 ng/µl) were sequenced by a Commercial Service (Seqlab, Gottingen, Germany) by primer walking sequencing method. Computer analysis of the sequences was carried out and the deduced amino acid sequence from *bgn2* gene was obtained by BLASTX Network Service (NCBI) and alignment of this amino acid sequence was done by clustal method with BLOSUM62 Service.

RESULTS

To compare the beta glucosidase activity, 30 isolates of *Trichoderma* sp. were grown for 7 days in shake flasks on CMC and lactose containing medium. Enzyme specific activity for 30 isolates are shown in Table 2. Results showed that in this condition *T. harzianum* (T7) has highest enzyme specific activity (44.8 U mg^{-1}) among all isolates.

In order to amplify beta glucosidase (*bgl2)* from *T. harzianum* (T7) as a high producer of beta glucosidase enzyme, we aligned two known beta glucosidase gene sequences from the GenBank database at the National Center for Biotechnology Information (NCBI). Based on this alignment, the specific oligonucleotide primers (TFbgl2 and TRbgl2) were designed (Fig. 1). Under experimental conditions, the genomic and cDNA of *bgl*2 were amplified from chromosomal and first-strand cDNA, respectively, using specific primers. A specific band about 1.4 kb was amplified from *T. harzianum* (T7) chromosomal DNA (Fig. 2). The PCR product was analyzed by restriction pattern analysis using *Bam*HI, *Pvu*II and *Xho*I enzymes (Fig. 2). Restriction pattern of

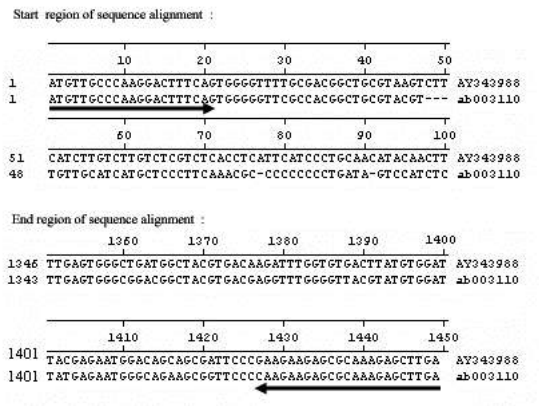

Fig. 1: Sequence alignment of the two known *bgl2* genes of *T. viride* (AY343988) and *T. reesei* (AB003110). The positions of specific primers are indicated by arrows

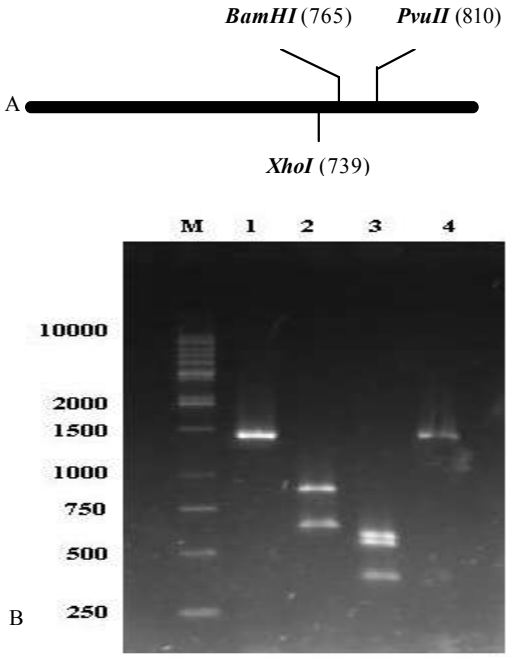

Fig. 2: A) Schematic representation of expected restriction pattern based of bgl2 sequence of *T. viride* (AY343988) from geneBank. B) Restriction pattern analysis of *bgl2* genomic DNA: PCR amplification of *bgl2* genomic DNA (line 1, approximately,1.4 Kb), digestion of PCR product using *Bam*HI (line 2), *Pvu*II (line 3) and *Xho*I (line 4), M= DNA size marker

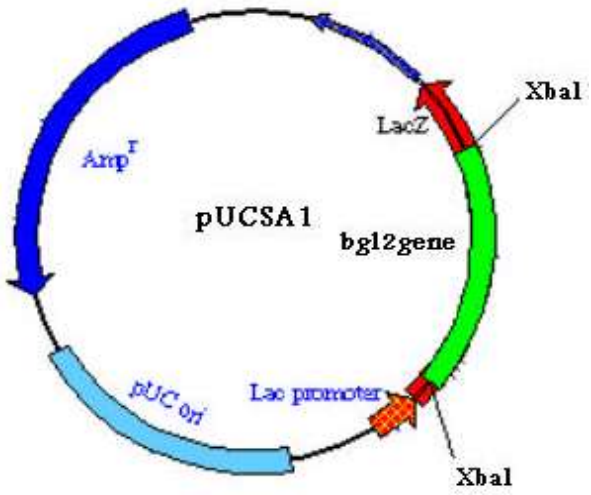

Fig. 3: Plasmid construction of *bgl*2 genomic DNA. The *bgl2* genomic DNA was cloned into pUC19 to create pUCSA1 plasmid

*Bam*HI enzyme confirmed the *bgl*2 sequence, while those of *Pvu*II and *Xho*I showed different patterns in compared with expected data from known sequences (Fig. 2).

Table 2: Beta-glucosidase production (specific activity, U ml^{-1}) of different isolates of *Trichoderma* speices

Isolates	Specific activity (U mg^{-1})	Isolates	Specific activity (U mg^{-1})
T. longibrachiatum (5)	42.8±0.7	*T. koningii* PTCC5139	42.4±0.9
T. viridae (1)	42.8±0.9	*Trichoderma sp* PTCC5138	43.7±0.5
T. harzianum (7)	42.9±1.0	*T. lebgibrachiatum* PTCC5140	42.6±0.7
T. viridae (2)	43.2±0.6	*Trichoderma* sp. (T1	43.1±0.5
T. hamatum (12)	42.6±0.7	*T. harzianum* (T2	43.1±0.7
T. Virens (9)	43.2±0.7	*Trichoderma* sp. (T3	42.6±1.0
T koningii (11)	43.1±0.8	*Trichoderma* sp. (T6	43.1±0.6
T. longibrachiatum (6)	44.2±0.4	*T. harzianum* (T7)	44.8±0.7
T. parceramosum (4)	43.3±1.0	*Trichoderma* sp. (T9	42.2±0.8
T. Parceramosum (3)	43.5±0.9	*T. harzianum* (T10	43.2±0.3
T. harzianum (8)	42.5±0.5	*Trichoderma* sp. (T11	42.3±0.5
T. virens (10)	43.3±0.4	*T. harzianum* (T12	43.2±0.6
T. tansarum PTCC5220	42.4±0.6	*Trichoderma* sp. (T13	42.4±0.9
T. reesei PTCC5142	45.2±0.7	*T. atroviride* (T24	43.4±1.0
T. viridae PTCC5157	43.2±0.6	*Trichoderma* sp. (T26	42.6±0.5

Results are averages of three replicates, ±standard error

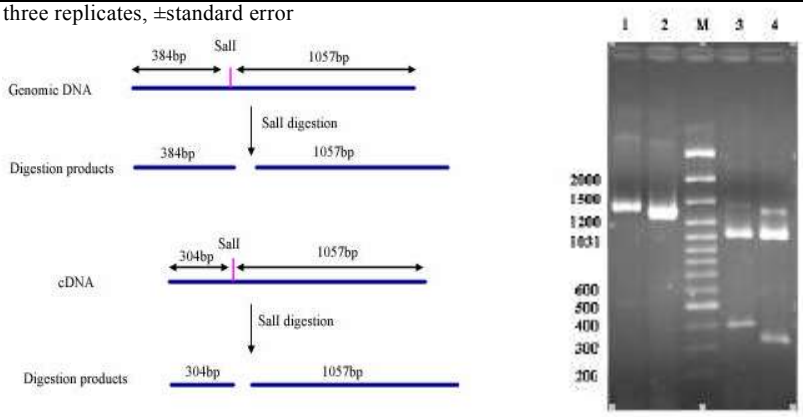

Fig. 4: A) Schematic representation of *Sal*I restriction pattern from *T. harzianum* (T7) *bgl*2 genomic DNA (top) and cDNA (bottom). B) Confirmation of constructed *bgl*2 cDNA: PCR amplification of *bgl*2 genomic DNA using specific primers (TFbgl2 & TRbgl2) (line 1, approximalty 1.4 kb), *bgl*2 cDNA amplification using specific primers (TFbgl2 & TRbgl2) (line 2, approximalty 1.3 kb), Confirmation of PCR product of genomic DNA (line 3, 1057 and 384 bp) and cDNA (line 4, 1057 and 304 bp), by restriction pattern using *Sal*I enzyme, M= DNA size marker

Following amplification of genomic DNA from *T. harzianum* (T7), DNA fragment was cloned in pUC19 and designated as pUCSA1 (Fig. 3). The cloned fragment was sequenced by M13 forward/reverse primers and submitted to the NCBI data base under accession number EF426298.

A homology search utilizing the computer program BLSTN, revealed high homology between *T. harzianum* (T7) *bgl2* and several beta glucosidase gene of other fungi, including *Hypocorea jecorina* (89%) [14], *Aspergillus niger* (81%) [15], *T. viride* (80%) [16] and *Humicola grisea* (71%) [14]. DNA sequence information confirmed that we had cloned PCR fragment with high homology to the previously reported *bgl*2 sequences of different *Trichoderma* sp.

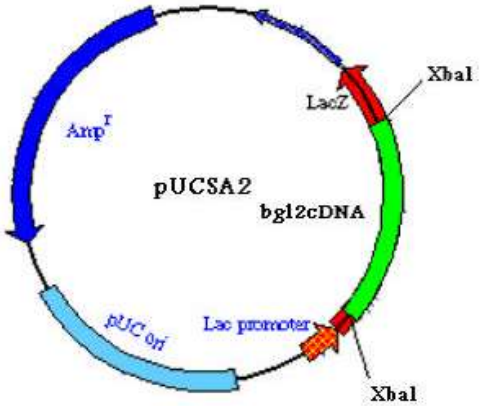

Fig. 5: Plasmid construction of *bgl*2 cDNA. The cDNA was cloned into pUC19 to create pUCSA2 plasmid

For the purpose of verifying gene structure, cDNA clone for *bgl*2 gene was isolated and characterized. We used the specific primers (TFbgl2 and TRbgl2) for cloning the full-length cDNA clone by reverse transcription (RT)-PCR. RNA was extracted from mycelium grown in the presence of both CMC (1%) and lactose (1%) which cause the highest level of beta glucosidase transcript.

Under conditions described in materials and methods, cDNA fragment approximately 1.3 kb was obtained. This PCR product was isolated and confirmed by restriction pattern analysis using *Sal*I enzyme which could easily differentiate between cDNA and genomic DNA of *bgl*2 (Fig. 4). This fragment ligated to pUC19 vector and designated as pUCSA2 (Fig. 5). The cloned cDNA fragment (1362 bp) was sequenced and submitted under accession number EF426299. Comparison of the cDNA sequence with the genomic *bgl*2 sequence demonstrated this gene is interrupted by one small intron, 79 bp in length (Fig. 6). The

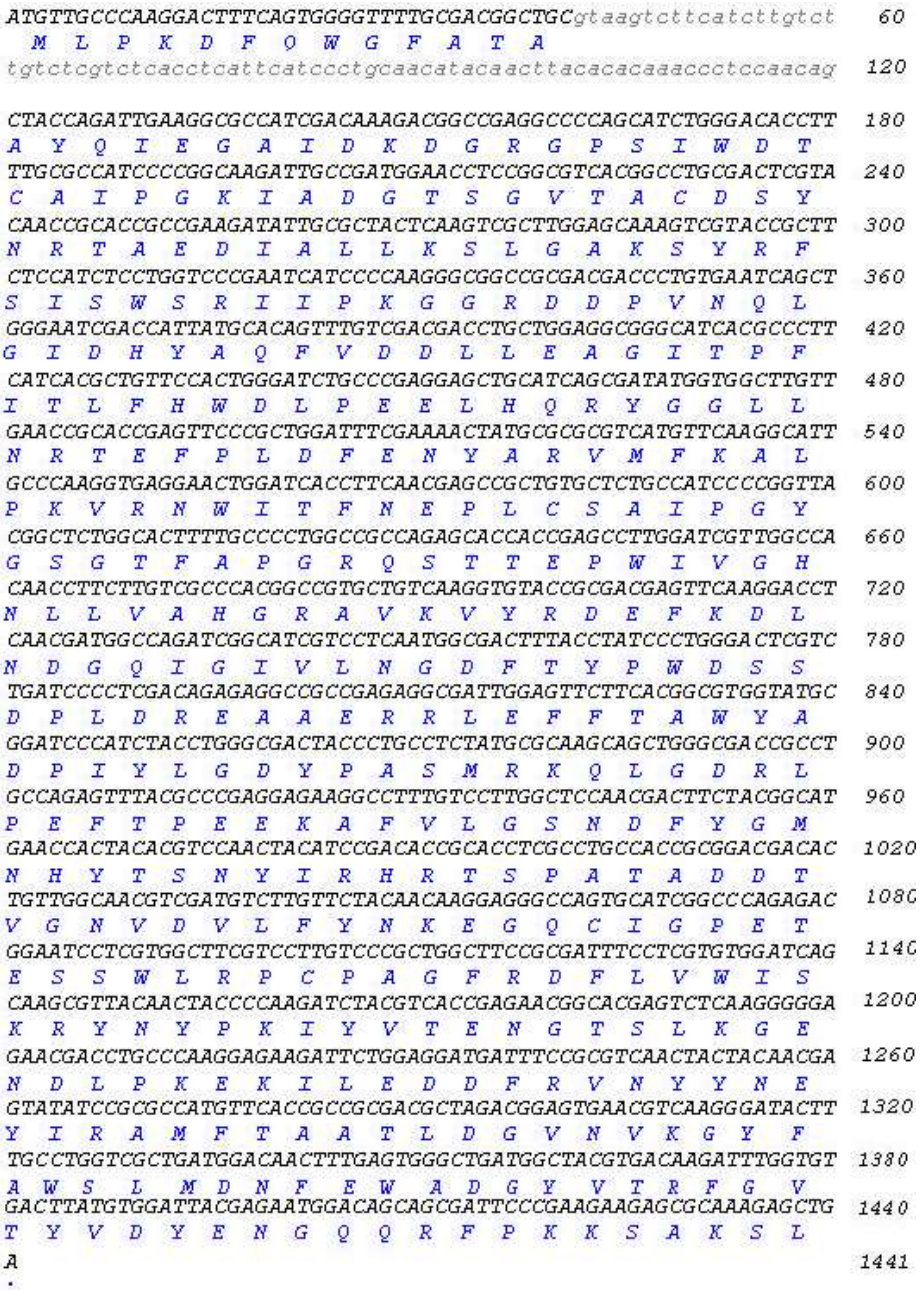

Fig. 6: Nucleotide and deduced amino acid sequences of *T. harzianum* (T7) *bgl2* gene. The intron sequence is presented with small characters. Stop codon is indicated by an asterisk

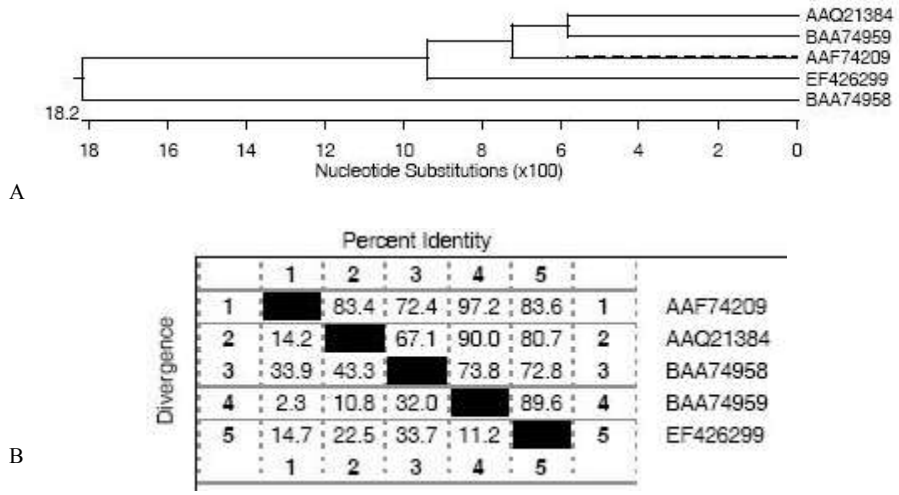

Fig. 7: A) Phylogenetic tree and sequence distances of the Bgl2 amino acid sequences comparing *T. harzianum* Bgl2 (T7)(EF426299) with those of *T. reesei* (BAA74959), *T. viride* (AAQ21384), *Humicola grisea* (BAA74958) and *Aspergillus niger* (AAF74209). B) percentage of sequence similarity and divergence between amino acid of *T. harzianum* Bgl2 (EF426299) and *T. reesei* (BAA74959), *T. viride* (AAQ21384), *Humicola grisea* (BAA74958) and *Aspergillus niger* (AAF74209) generated from multiple alignment

consensus sequences, GT on the 5' end and AG on the 3' end of the intron of the *bgl*2 gene were also observed. The cDNA contains an open reading fram encoding a protein of 454 amino acids (Fig. 6). The calculated molecular mass of the predicted product is 51 kDa.

Multiple alignment of the deduced amino acid sequence with related fungal proteins was performed with the CLUSTAL W program. When *T. harzianum* (T7) Bgl2 was compared with the previously reported beta glucosidase sequences of *Hypocrea jecorina* (anamorph: *T. reesei*) (BAA74959), *A. niger* (AAF74209), *T. viride* (AAQ21384) and *H. grisea* (BAA74958), it showed high homology to these sequences. Using pairwise alignment, the homology found between the *T. harzianum* (T7) Bgl2 and beta glucosidase protein from *A. niger, T.viride*, *H. grisea* and *T. reesei* was 83.6%, 80.7%, 72.8% and 89.6%, respectively (Fig. 7).

DISCUSSION

Lignocellulosic materials are most abundant organic compounds in biosphere which is produced by photosynthetic process. Annual production of cellulose is more than 4 billion tones. Cellulose has enormous potential as a renewable source of energy, feed, fuel and chemicals [4]. Enzymatic hydrolysis of cellulose is a complex process that perform by synergistic action of three enzymes (endoglucanase,

T. harzianum (T7)	MLPKDFQWGFATAAYQIEGAVDQDGR
T. reesei	MLPKDFQWGFATAAYQIEGAVDQDGR
H. grisea	MS LPPD FKWGFATAAYQIEGAVDQDGR
	* *

Fig. 8: Alignment of the N-terminal amino acid sequences of *T. harzianum* (T7) Bgl2, *T. reesei* Bgl2 and *Humicola grisea* Bgl4. Identical amino acids are indicated by asterisks

exoglucanase and beta-glucosidase) [3]. These enzymes are produced by the filamentous fungus *Trichoderma* species [17, 18].

To evaluate the production of beta glucosidase activity, *T. harzianum* (T7) was selected among 30 isolates of different *Trichoderma* spices at 28°C in shake flasks. The same conditions have been reported in other studies [19, 20].

To study the *bgl*2 gene structure, in this paper, we report the sequence and analysis of cDNA and genomic clone of the *bgl*2 gene, encoding beta glucosidase, from the high producer of beta glucosidase enzymes Iranian isolate of *T. harzianum* (T7). DNA sequence information of *bgl2* gene showed relatively high homology to the previously reported beta-glucosidase sequences from *T. reesei* [14], *Aspergillus niger* [15] and *Humicola grisea* [14]. The work on beta glucosidase gene was reported for first time by Takashima *et al*. (1999) [14] from *Humocola grisea*

and then they used this gene as a hybridization probe for isolation of *T. reesei bgl2* gene.

Analysis of the *bgl*2 sequence demonstrated that it contains a small intron which has also been reported in other glucosidase fungal genes [14, 15]. The consensus sequences, GT on the 5' end and AG on the 3' end of the intron of the *bgl2* gene are also observed. The coding region of *bgl2* from *T. harzianum* (T7) codes for a polypeptide with molecular mass of 51 kDa. On the basis of amino acid sequence comparisons, the ß-glucosidase enzyme (Bgl2) of *T. harzianum* (T7) is closely related to beta glucosidase enzyme of other fungi (Fig. 7).

The N-terminal amino acid sequence alignment of *T. harzianum* (T7) Bgl2 and *T. reesei* Bgl2 and *H. grisea* Bgl4 [14] demonstrated that they are highly similar (Fig. 8), but neither of them is recognized as a signal sequence by a signal sequence prediction program [21]. The finding of Takashima *et al*. (1999) [14] indicates that this sequence in Bgl4 of *H. grisea* is able to secrete this protein, while the Bgl2 containing this putative signal peptide is intracellular in *T. reesei* [22]. Therefore, it would seem likely that *H. grisea* Bgl4 was also originally an intracellular enzyme and that a mutation at its N-terminal region would have turned this region into a signal sequence.

REFERENCES

1. Begain, P. and J.P. Aubert, 1994. The biological degradation of cellulose. FEMS Microbiology.
2. Tomme, P., H. Ván Tilbeurgh, G. Pettersson, J. Van Damme, J. Vandekerckhove, J. Knowles, T. Teeri and M. Claeyssens, 1988. Study of cellulolytic system of *Trichoderma reesei* QM 9414: Analysis of domain function in two cellobiohydrolasees by limited proteolysis. Eur. J. Biochem., 170: 575-581.
3. Beguin, P. and M. Lemaire, 1996. The cellulosome: An exocellular, multi-protein complex specialized in cellulose degradation. Crit. Rev. Biochem. Mol. Biol., 31: 201-236.
4. Zaldivar, M., J.C. Velasquez, I. Contreras and L.M. Perez, 2001. *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: Its potential use in waste cellulose degradation and/or biocontrol. Elect. J. Biotechnol., 4: 160-168.
5. Nevalainen, H., I. Lavygina, D. Neethling and N. Packer, 1995. The biochemical nature of the cell envelope of a high cellulose-secreting mutant differs from that of the *Trichoderma reesei* wild type. J. Biotechnol., 42: 53-59.
6. Seyed Asli, N., M.R. Zamani and M. Motallebi, 2004. Study of chitinolytic enzyme production in Trichoderma isolates. Ir. J. Biol., 17: 227-246.
7. Takashima, S., H. Iikura, A. Nakamura, M. Hidaka, H. Masaki and T. Uozumi, 1998. Overproduction of recombinant *Trichoderma reesei* cellulase by *Aspergillus oryzae* and their enzymatic properties. J. Biotechnol., 65: 163-171.
8. Ghoujeghi, F., M.R. Zamani, M. Motallebi and S. Zahri, 2007. ß-glucosidase enzyme in *Trichoderma reesei*. Pajouhesh-va-Sazandegi. Acceptted.
9. Mandels, M., R. Andreotti and C. Roche, 1976. Measurment of sachcarifying cellulose. Biotechnol. Bioeng. Symp., 6: 21-34.
10. Kader, A.J., O. Omar and L.S. Feng, 1999. Isolation of Cellulolytic Fungi from the Bario Highlands, Sarawak, ASEAN Review of Biodiversity and Environmental Conservation (ARBEC).
11. Sun, C.B., Q.L. Kong and W.S. Xu, 2002. Efficient transformation of *Penicillium chrysogenum* mediated by *Agrobacterium tumnefaciens* LBA4404 for cloning of vitreoscilla hemoglobin gene. E. J. Biotechnol., 5: 163-171.
12. Jain, A.K., 2004. Cloning and structural analysis of a cDNA clone encoding glycinin (*Gly-1*) seed storage protein of peanut. Elect. J. Biotechnol., 7: 224-234.
13. Sambrook, J. and D.W. Russell, 2001. Molecular cloning. Cold Spring Harbor: New York.
14. Takashima, S., A. Nakamura, M. Hidaka, H. Masaki and T. Uozumi, 1999. Molecular cloning and exoression of the novel fungal beta-glucosidase gene from *Humicola grisea* and *Trichoderma reesei*. J. Biochem., 125: 728-736.
15. Ai, Y.C. and F.M. Meng, 2000. Molecular cloning and sequencing of a beta-glucosidase gene from *Aspergillus niger* AMS11. Submitted to NCBI under accession number of AAF74209.
16. Liu, B.D., Q. Yang and Q. Zhou, 2003. Cloning and sequencing analysis of the beta-glucosidase 2 gene from *Trichoderma viride* As3.3711. Submitted to NCBI under accession number of AAQ21384.
17. Fujita, Y., S. Takashima, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda and A. Kondo, 2002. Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. Applied and Environ. Microbiol., 68: 5136-5141.

18. Liming, X. and Xueliang, 2004. High-yield production by *Trichoderma reesei* ZU-02 on corn cob residue. Bioresource Technol., 91: 259-262.
19. Margolles-Clark, E., C.K. Hayes, G.E. Harman and M. Pentilla, 2000. Improved production of *Trichoderma harzianum* endochitinase by expression in *T. reesei.* Applied and Environ. Microbiol., 62: 2145-2151.
20. Ulhoa, C.J. and F.J. Peberda, 1991. Purification and characterization of an extracellular chitobiase from *Trichoderma harzianum.* Curr. Microbial., 23: 285-289.
21. Von Heijne, G., 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acid Res., 14: 4683-4690.
22. Saloheimo, M., Kuja-Panula, E. Ylosmaki, M. Ward and M. Penttila, 2002. Enzymatic properties and intracellular localization of the novel *Trichoderma reesei* beta glucanase BGLII (CellA). Applied and Environ. Microbiol., 68: 4546-4553.