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Research Article

Genes expression of cellulase enzyme in four *Aspergillus* species

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Abstract: Production of cellulase is of great importance for industrially processes and developing the metabolic microbes used widely in different fields. *Aspergillus*, as one of fungal genera, serve to produce many enzymes such as cellulase. Twenty-three isolates of fungi belong to four species of *Aspergillus* were obtained from central lab veterinary medicine in Basra, Iraq and screened for maximum cellulolytic activity. Screening of fungal species was done on Petri plate containing carboxymethyl cellulase growth media. Among 23 isolates of fungi, 20 fungal isolates revealed cellulolytic activity and three depicted zero cellulase activity. All isolates of *A. niger* showed strong secretion of cellulolytic activity on agars followed by *A. fumigatus,* with two isolates of *A. flavus* and one isolateof *A. terreus* revealed zero cellulytic activity. The results of relative gene expression of *Aspergillus* species to five cellulase genes cbhb, exogluconase, endogluconase A, endogluconase B, endogluconase C and control B actin gene revealed over expression of *A. niger* compared to other species.

Keywords: Fungi, *Aspergillus*, Cellulolytic enzyme, Molecular assay.

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Introduction

Cellulose is one of mostly abundance biological materials deriving from many organisms worldwide. Plants are generally contributing in cellulose pool of biosphere as it synthesis by a process known as the photosynthesis. Hence, cellulose mostly act as a part of vegetal biomasses in addition to lignin and hemicellulose (Saxena et al. 2009; Nidhi et al. 2017). Chemical composition of cellulose involved the units of β-D-glucopyranoside that attached by the bonds of β-D-glucosyl (Ahmed et al. 2017). In nature, this material degraded through an enzyme known as cellulose that consisting principally from 3 enzymes known as β-glucosidase, cellobiohydrolase and endoglucanase, which work synergistically for degrading the units of cellulose to glucose. This process of degradation applies widely among different biological and technological applications like synthesis of amino acids, production of bio-fuel and laundry, pulp, paper and textile industry (Sun & Cheng 2002; Dashtban et al. 2010; Imran et al. 2016). In addition, it can be applied for industrially animal nutrients, pre-treating of agricultural silages and grains used to fed animals through enhancing the nutritional values and performance (Kuhad et al. 2011).

Fungus is crucially contributing for decompressing of cellulose as it accounts about 80% of cellulose degradation among natures in particular in forest ecosystem, in which, fungus plays an important activity for decomposing of biomass. Many species of fungus have the ability for degrading of cellulose (Timo et al. 2017; Hernandez et al. 2018). The most important fungi that capable for production of cellulase are *Aspergillus* (Bansal et al. 2012). Naturally, saprophytic filamentous fungus

Table 1. Primers designated and applied for detection of cellulose genes in *Aspergillus* spp.

No	Gene		Primer sequence (5′-3′)	Product size	NCBI reference
1	cbhB	F	TCCAGCAACAACGTCAACAC	86 bp	AKR052992.1
		R	TTGGAGATGCTGTTCGCTTC		
2	exo	F	ATGCAGCAGAGTGAAACACC	148 bp	XM-001400412.2
		R	TGCGTTCGAACAATGCCATG		
3	eglA	F	TGGAATGGAAGCAGGACAAC	70 bp	GU724764.1
		R	ACATTCGCTGCGGTGAAAAG		
4	eglB	F	AGGGCGTCTGTGTTTGAATG	149 bp	AJ224452.1
		R	ACGCGGAAGAAGTTCATTCC		
5	eglC	F	AACAAACGGCATCGACTACG	110 bp	AY040839.1
		R	ACCAAGATTCGCCGAAACTG		
6	actin	F	ACAACGAGCTCAGGAATTGC	126 bp	M-001389294.2
		R	AGCGTTTTCAGCTCGTTCTC		

has the ability for secretion great quantity of cellulolytic enzymes like cellulase and hemicellulase that degrading the vegetal wall. These degradation processes play a great effect in recycling of vegetal biomass in ecosystem (Kubicek et al. 2009, 2013; Glass et al. 2013; Mäkelä et al. 2014). Therefore, the current study aimed for detection the genes responsible for production of cellulose in some *Aspergillus* species using the technique of real-time polymerase chain reaction (qPCR).

Material and methods

Cellulase activity: Cellulase activity of fungal isolate was detected through the method of plate screening medium (PSM) (Mandels 1974). The blocks of agar containing the fungal colonies were grown on MEA plates that stained with Congo red dye, incubated for 1 week, and the cellulolytic fungi were harvested according to diameters of hydrolysis zone around the fungal colonies. The activity of cellulose on carboxymethyl agar was reported as an index of relative enzyme activity and measured as the Clear Zone Ratio = Clear Zone Diameter / Colony Ciameter (Teather & Wood 1982; Bradner et al. 1999; Dale Peciulyte 2007). Fungal isolates grown on MEA were used as control.

Molecular experiments: Following the manufacturer instructions of Easy-BLUE™ for Total RNA extraction kit (Intron, Korea), total RNAs were extracted from the fungal isolates. According to Mahmod et at. (2016), qPCR assay was performed to detect the quantity and analysis of relative gene-expression responsible for cellulase production in isolates of *Asperigellus* spp. using of housekeeping (actin) gene to normalize the assay (Table 1).

The tubes of Master Mix were at a total volume of 20µl using a ready to use RealMOD™ Green SF 2X qPCR Mix Kit (Intron, Korea). Subsequently, the Master mix tubes were subjected for amplification under the thermal cycler conditions obtained the by Optimase Protocol Writer™ online as following; 1 cycle for initial denaturation (95°C/10min), 40 cycles for denaturation (95°C/20sec) and annealing / extension (58°C/30sec), and 1 cycle for melting (65-95°C). The data of qPCR were analyzed as described by Livak & Schmittgen (2008).

Results

The cellulase activity of some species of *Aspergillus* isolates was detected through the carboxymethyl cellulose as a substrate. Twenty-three isolates of fungi belong to four species of *Aspergillus* were obtained and screened for maximum cellulolytic activity. Screening of fungal species was done on Petri plate containing carboxymethyl cellulase CMC growth media. Among 23 isolates, 20 isolates revealed cellulolytic activity and three depicted zero cellulase activity. All isolates of *A. niger* were showed a strong secretion of cellulolytic activity on agar plates followed by *A. fumigatus,* two isolates of

Table 2. Cellulase activity for fungal isolates on CMC agar.

No. of isolate	Fungal Species	Activity of cellulase
A1	*A. flavus*	+ +
A2	*A. flavus*	+ +
A3	*A. flavus*	+ + +
A4	*A. flavus*	+
A5	*A. flavus*	-
A6	*A. flavus*	-
B1	*A. fumigatus*	+ + +
B2	*A. fumigatus*	+ +
B3	*A. fumigatus*	+ + +
B4	*A. fumigatus*	+ + +
B5	*A. fumigatus*	+ + +
B6	*A. fumigatus*	+ +
C1	*A. niger*	+ + +
C2	*A. niger*	+ + +
C3	*A. niger*	+ + +
C4	*A. niger*	+ + +
C5	*A. niger*	+ + +
C6	*A. niger*	+ + +
D1	*A. terreus*	+ +
D2	*A. terreus*	+ +
D3	*A. terreus*	+ + +
D4	*A. terreus*	+
D5	*A. terreus*	-

No secretion (-); Weak secretion, 1-3 mm (+); Medium secretion, 3-5 mm (+ +); Strong secretion, 8-11 mm (+ + +).

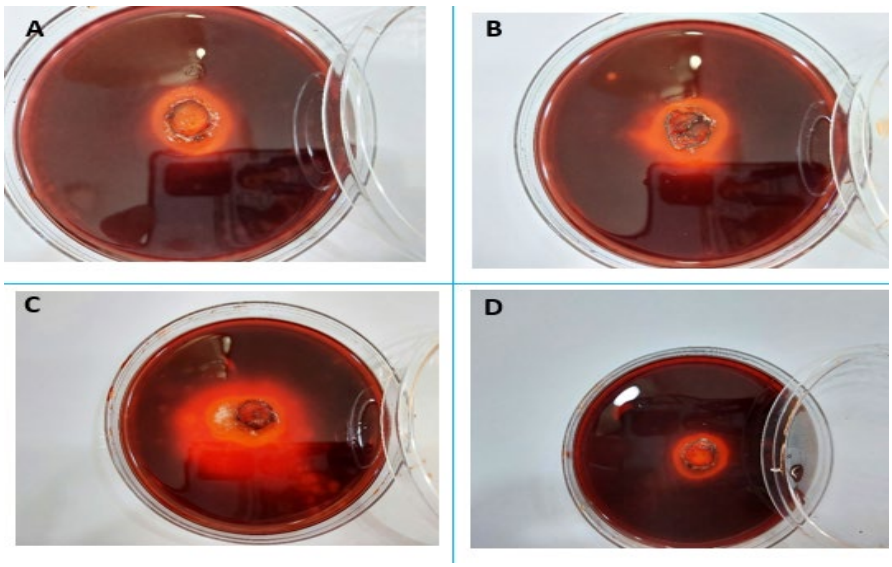

Fig.1. Inhibition zones (Yellow hallow) of *Aspergillus* spp. in CMC agar ((A): *A. flavus*, (B): *A. fumigates*, (C): *A. niger* and (D): *A. terreus*)

A. flavus and one isolate of *A. terrus* revealed zero cellulytic activity (Fig. 1, Table 2).

The profiles of genes (Eg1A, Eg1B, Eg1C, and Exo) responsible for cellulase activity of *Aspergillus* isolates were detected by qPCR. Gene expression of cbhb gene revealed that *A. niger* with overexpressed 31.10 followed by *A. fumigatus* in 23.82 with no significant differences between *A. flavus* and *A. terreus* (Figs. 2, 3, Table 3). The findings showed that the isolates of *A. niger* had high expression in

Table 3. Gene expression (Fold change) of cbhB gene in different type of *Aspergillus*.

Type of fungus	Group	
	Test	Control
A. niger	31.10±8.12	1.00±0.05
A. flavus	17.06±4.22	1.18±0.75
A. fumigatus	23.82±3.89	1.26±0.94
A. terreus	17.40±2.12	1.21±0.82
$LSD_{0.05}$	1.28	

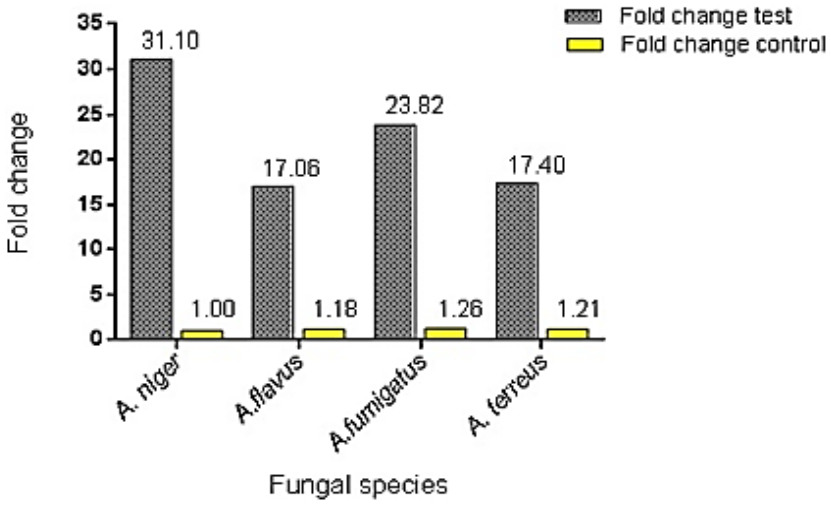

Fig.2. Fold changes of Aspergillus species to cbhB and control gene.

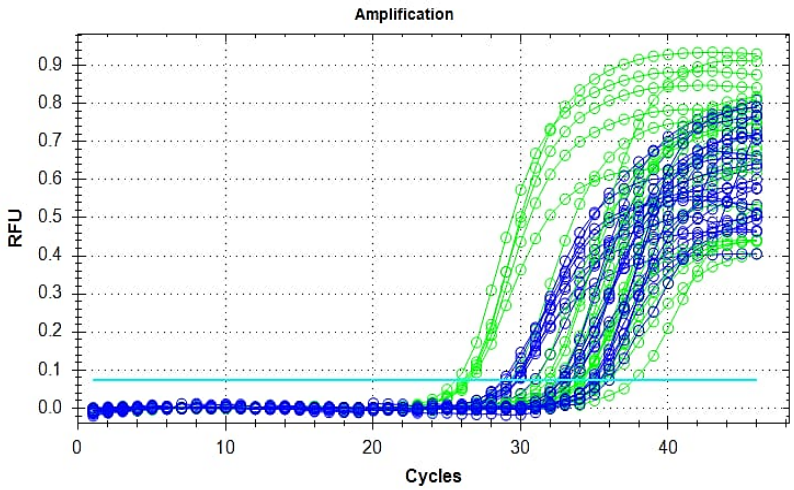

Fig.3. qPCR amplicon plots of celliobiohydrolase (cbhb) and exonuclease (exo) gene.

exonuclaese gene followed by *A. terrus, A. fumigatus* and low expression of *A. flavus* (Fig. 4, Table 4).

The results of endogluconase A showed a significant differences of *A. niger* (28.78) compared to other species (Fig. 5, Table 5). The results of endogluconase B gene expression revealed a high expressed value of *A. niger* (24.79) followed by *A. flavus* and *A. terreus* and less expression in *A. fumigates* showing in amplification blot (Figs. 6, 7, Table 6). Endogluconase C gene expression

Table 4. Gene expression (Fold change) of exo gene in different type of *Aspergillus*.

Type of fungus	Group	
	Test	Control
Aspergillus niger	18.25±1.28	1.01±0.04
Aspergillus flavus	5.90±0.98	1.18±0.28
Aspergillus fumigatus	9.45±1.04	1.03±0.16
Aspergillus terreus	13.96±1.36	1.25±0.34
$LSD_{0.05}$	1.08	

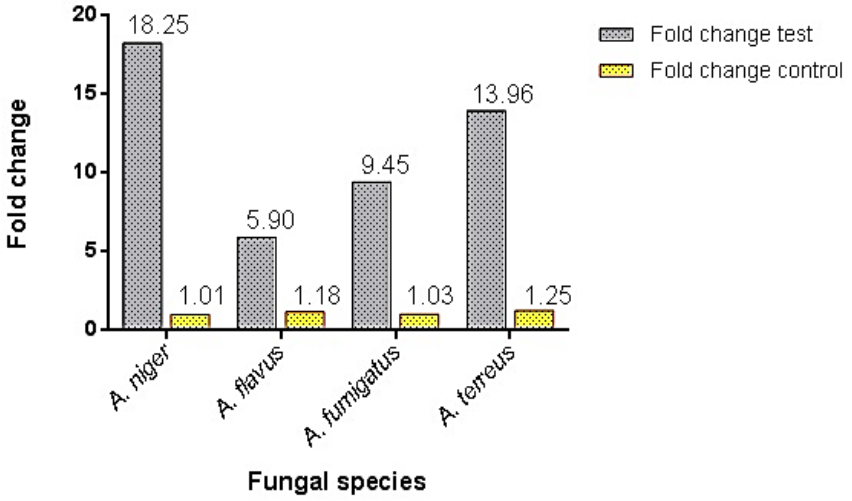

Fig.4. Gene expression of *Aspergillus* species to exonuclease (exo) gene.

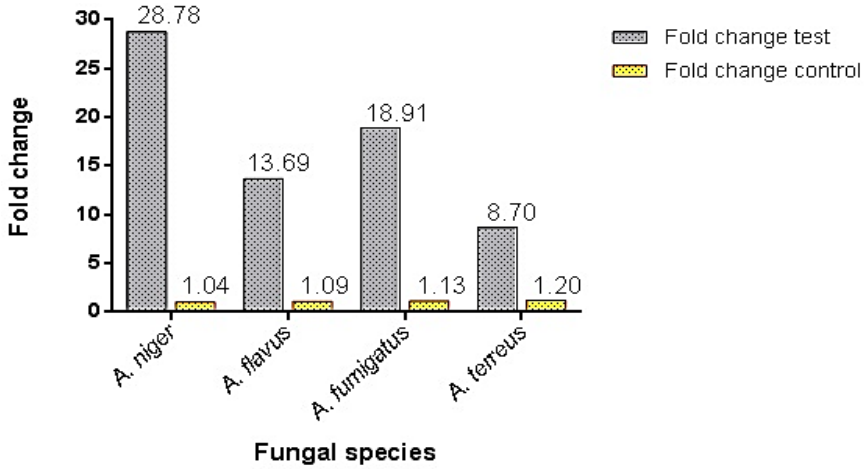

Fig.5. Gene expression of Aspergillus species to endogluconase eglA gene of *Aspergillus* species compared with control group.

showed highly expressed of *A. niger* (22.33) followed by *A. fumigatus*, *A. terrus* and *A. flavus* (Figs. 8, 9, Table 7).

Discussion

Cellulases, a group of enzymes capable for degradation of cellulose, belong to a superfamily of glycoside hydrolases (GH) involving many enzymes that have the ability for degradation of vegetal cell walls like xylanase and laccase (Cantarel et al. 2009; Gilbert 2010). Identity between the protein sequences is one of the method which applied to identify the GH

Table 5. Gene expression (Fold change) of eglA gene in different type of *Aspergillus*.

Type of fungus	Group	
	Test	Control
Aspergillus niger	28.78±2.45	1.04±0.3
Aspergillus flavus	13.69±1.7	1.09±0.24
Aspergillus fumigatus	18.91±1.44	1.13±0.08
Aspergillus terreus	8.70±0.78	1.20±0.42
$LSD_{0.05}$	1.61	

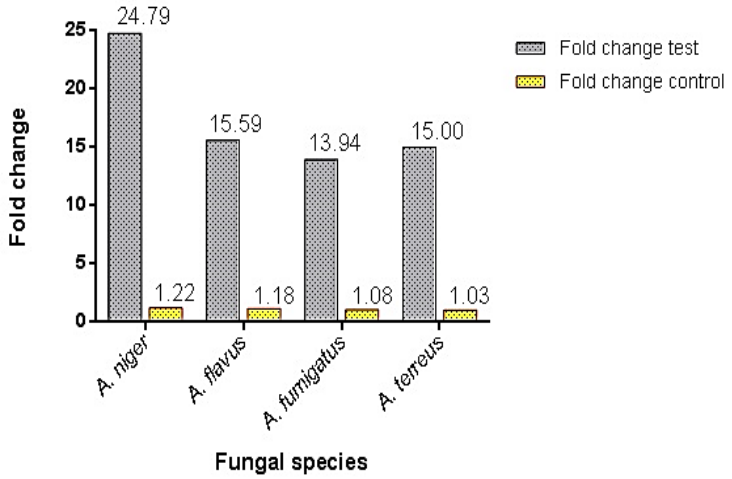

Fig.6. Gene expression of *Aspergillus* species to endogluconase B (eglB) gene and control group.

Table 6. Gene expression (Fold change) of eglB gene in different type of *Aspergillus*.

Type of fungus	Group	
	Test	Control
Aspergillus niger	24.79±3.45	1.22±0.22
Aspergillus flavus	15.59±2.34	1.18±0.18
Aspergillus fumigatus	13.94±2.08	1.08±0.32
Aspergillus terreus	15.00±3.6	1.03±0.21
$LSD_{0.05}$	2.12	

family as a given GH family can involve a particular protein with differences in activity of enzymes (Henrissat 1991). Consequently, activity of enzymes can be found in many families of GH. Degrading of cellulase to cellulose polymers can occur throughout hydrolysing the bonds of b-1,4 glycosidic at 3 variable ways that mentioned among at least 10 different GH families (1, 3, 5, 6, 8, 9, 12, 44, 45, 48).

Genomes of many *Aspergillus* spp. are usually contained many genes responsible for the cellulolytic enzymes that showed variable expression according to conditions of culture in addition to many factors (Ward et al. 2006). Genetically, transcriptomic and proteomic reports confirmed that many genes and regulatory circuits can be activated throughout the production of enzymes (Al-Sheikh et al. 2004).

The patterns of transcription in cellulolytic enzymes can be tested by different biological and molecular techniques as the Northern Blotting (Marui et al. 2002), PCR (Mahmood et al. 2014; Bak

Table 7. Gene expression (Fold change) of eglC gene in different type of *Aspergillus*.

Type of fungus	Group	
	Test	Control
Aspergillus niger	22.33±3.02	1.36±0.51
Aspergillus flavus	12.61±1.74	1.18±0.32
Aspergillus fumigatus	19.70±2.08	1.08±0.12
Aspergillus terreus	11.91±1.56	1.05±0.18
$LSD_{0.05}$	1.21	

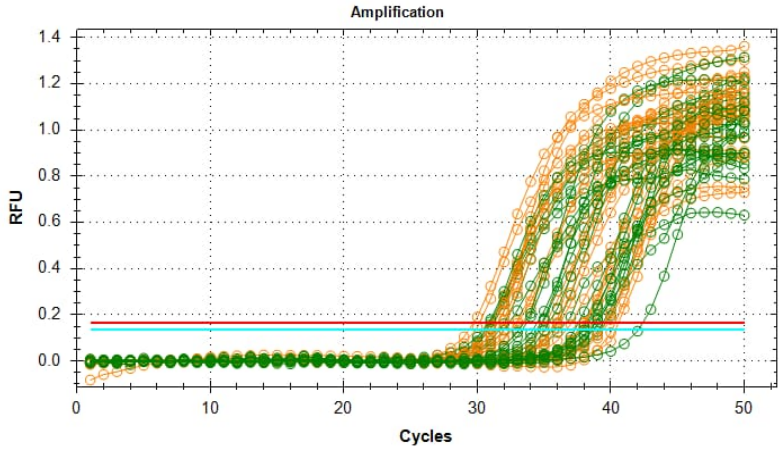

Fig.7. Relative PCR amplification plot of eglB and eglA genes of *Aspergillus* species.

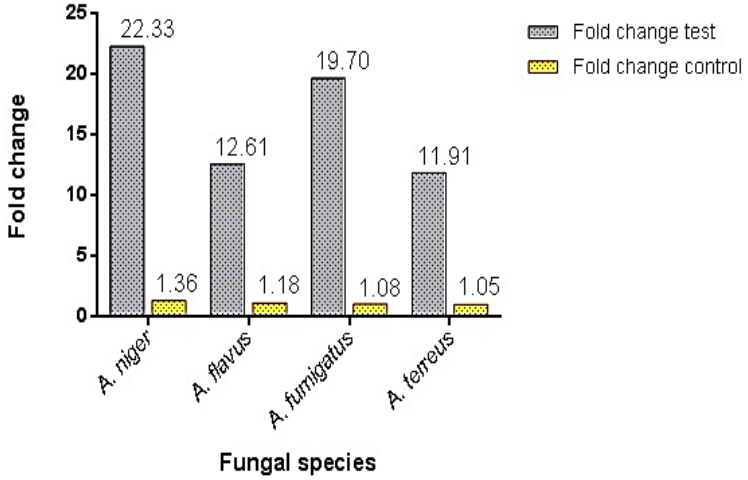

Fig.8. Gene expression of endocluconase C (eglc) gene of *Aspergillus* species.

2015), expressed sequence tag analysis (Todaka et al. 2010) and DNA microarray analysis (Ogawa et al. 2013). The gene profile of EGs (eglA, eglB, and eglC), EXGs (cbhA, cbhB, and exo), and bGs (β-actin) could be used for distinguishing between the different fungi in particular *A. niger* and *Trichoderma reesei* (Mahmood et al. 2014). Many genes in cellulase in addition to its expression pattern were detected gnomically by sequence. Gene expression and secretion of fungal cellulase might severely managed at the level of transcription (Stricker et al. 2008). The stability of mRNA is other great importance element to regulate the expression of genes. Balancing between mRNA synthesis and degradation is not only important for gene expression, but is also vital for cell adaptation

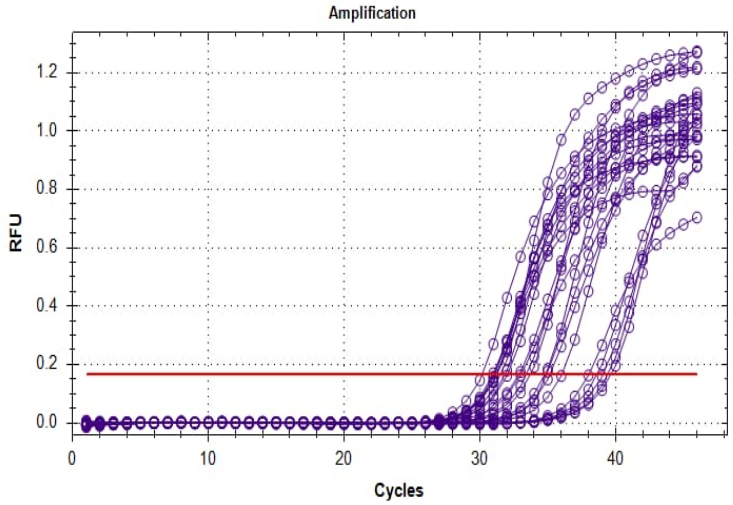

Fig.9. qPCR amplification plot of eglc gene of *Aspergillus* species with control group.

(Caddick et al. 2006). The production of cellulolytic enzymes in microbes can be estimated (Renge et al. 2012). Many different cellulase isoform genes can be expressed in responses to carbon sources were confirmed (Amore et al. 2013). There were many molecular mechanisms, by which, expression can be managed among several forms of cellulase, as well as different sources of carbon might be affected on amounts and forms of expression which are not well initiated due to disability for genetic engineering of these fungi in different industrial purposes (Coradetti et al. 2012). Thus, knowing the mechanism of cellulase expression can support and provide a critical data for enhancing the production of cellulase, and for investigating *Trichoderma* and *Aspergillus* (Gautam et al. 2011).

Many fungi have the ability to produce the extracellular cellulase throughout their growing on a suitable media contain the polymers of plant or a short oligosaccharide, which act as a source of energy. Cultivation of these fungi on media having easily metabolized sugars like glucose resulting in repressing the expression of cellulase. Carbon catabolite repression considers one of the almost acceptable mechanisms for repressing the production of cellulase during cultivation of fungi on easily metabolizable sugar (Antonella et al. 2013).

Cellulase can be used additionally in agricultural fields to hydrolyze the cell walls of vegetal pathogenic agents, thus, they can be applied to control vegetal infections. Several cellulolytic fungi such as *Aspergillus* known to play a key role in agriculture by increased crop yields, improved root system, rapid plant growth and flowering and enhancing the seed germination (Kuhad et al. 2011; Behera et al. 2016)

Conclusion

Our findings suggest that *A. niger* may be a useful novel gene source for cellulose. However, further studies must focus on manipulation of cellulase throughout engineering of genes and proteins to support the efficacy of biomass degradation and bioconversion.

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