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Molecular Study of Cellulase Gene Transcription Regulatory Elements via EMS Mutagenesis in a Novelthermophilic Cellulytic *Bacillus* sp.

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Abstract: ***Cellulose is the most common biomass on the globe and is available as huge amounts of agricultural and forestry natural residues. Microbial cellulases are the major enzymes widely used in degrading cellulose. Naturally cellulytic microorganisms need to be induced to increase their cellulytic activity. In this study, EMS chemicalmutagen was used to increase cellulytic activity in the novel thermophilic Bacillus sp. isolate isolated from Iraqi soil. Sequencing results of cellulase gene transcription regulatory elements post-EMS mutagenesis showed a change in the nucleotides sequence. This change was accompanied by a significant increase in cellulase quantitative activity and total cellulase activity.***

Keywords: Cellulase gene, transcription regulatory element, thermophilic cellulytic *Bacillus*

1. Introduction

Cellulose constitutes the most common organic polymer. It represents about 1.5 $X10^{12}$ tons of the annually total biomass production through photosynthesis, especially in the tropical areas. It is considered to be the most abundant and renewable biopolymer on earth and the dominating waste material from agriculture (Klemm *et al*., 2002).

The enzymatic depolymerization is environmental friendly (Juturu and Wu, 2014). Therefore, attention was focused on the sources of microbial cellulases, i.e. bacterial and fungal cellulases (Abdullah *et al*., 2016). Biodegradation of cellulose by bacteria and fungi is accomplished by these extracellular cellulolytic enzymes which are encoded by genes which are subjected to transcriptional control (Ali *et al*., 2014).

The most common cellulolytic bacterial genera are *Bacillus* spp., *Cellulomonas*spp., *Clostridium* spp., *Thermonospora*spp., *Pseudomonas* spp., *Streptomyces* spp. and *Salmonella* spp. (Menendez *et al*., 2015). Thermophilic *Bacillus*spp. is a well-studied group due to its potency in producing enzymes of industrial importance and is one of the most studied genera among the thermophilic microorganisms (Ibrahim and El-diwany, 2007 ; Kazeem*et al*., 2016).

Ethyl methane sulfonate was reported as a potent mutagen in *Bacillus* spp. (Karanam and Medicherla, 2008). It can induce mutations at a rate of $5x10^{-2}$to $5x10^{-4}$per gene without substantial killing (Haq *et al*., 2009).

In this study, a novel thermophilic *Bacillus* sp. isolate isolated from Iraqi soil was treated with EMS in different concentrations to improve its cellulytic ability. The developed mutants were screened and evaluated for enzyme production level.

2. Methods

2.1 Isolation of organisms

Thermophilic *Bacillus* sp. was isolated from soil samples which were collected from the soil beneath plant wastes. The isolate was identified as *Bacillus* sp. biochemically by reference to Acharya *et al*. (2012).The Vitek2 tests were also performed for more precise identification confirmation.

2.2 Screening of *Bacillus* isolate for thermophilic cellulase degradation ability

The experiments were set up as described by Kauri and Kushner (1985).

The pH of the medium was adjusted to (7.3-7.5).Five grams of each sample was inoculated into a (250) ml erylynmyer flask containing 100ml of sterilized CMC broth, then incubated at (55)C° for (24) hrs. After that, (0.2) ml was taken from each flask and spreaded onto CMC agar plates then incubated at (55)C° for (24) hrs.

2.3 Qualitative screening

The ability of the selected isolates to produce cellulase on a solid medium was examined according to Yeoh *et al*. (1985) and Bai *et al*. (2012) with modifications. The diameter of the hydrolysis halo which indicates the production of cellulase was measured.

2.4 Selection and preparation of the chosen bacterial isolate for mutagenesis

After statistically analyzing the hydrolysis halo measurements of all samples by ANOVA test using (SPSS) program; the best isolate in cellulase production, named B16, was molecularly characterized using

16SrRNA typing table (6).This isolate was further tested for its semi-quantitative activity which was performed according to Ponnambalam *et al.*, (2011).Quantitative cellulytic activity and total cellulase activity or what is termed filter paper assay (FPA or FPase) assays were carried out according to Samira *et al*. (2011).

2.5 Mutagenesis of thermophilic cellulytic bacterial isolate

The chosen thermophilic isolate B16, was exposed to EMS chemical agent in an attempt to increase its total cellulytic and cellulytic quantitative activity. Mutagenesis of the selected isolate with EMS was carried out according to Haq *et al.* (2009) with modifications. The concentrations used were (0, 50, 100, 150) µg/ml. The qualitative, semi-quantitative, quantitative and FPase activity assays were performed to find out the most effective concentration in increasing cellulytic activity and then, via primers especially designed for this study by Dr. Ehab D. Salman, sequencing was performed on the transcription regulatory elements of cellulase gene for the best cellulytic mutated isolate and was compared with the sequence of regulatory elements ofB16 control (wild) isolate.

3. Results and Discussion

3.1 Isolation and identification of isolates

Fifty one samples were isolated from soil beneath the plant wastes, characterized and identified based on their morphological and cultural properties. According to statistical analysis by ANOVA processed on the qualitative screening values, four of the isolates were chosen to be subjected to biochemical tests; the results in table (1) showed that they were *Bacillus* spp. Subsequently, the isolates were subjected to VITEK 2 identification for more precise identification of the isolates. The results in tables (2, 3, 4 and 5) showed that two isolates (B16 and B39) had an identification with *Bacillus licheniformis* by the percentage (89)% and (93)%, respectively. To certify the identification, the two isolates B16 and B39 were molecularly characterized depending on 16SrRNA gene and more specifically via *gyrase b* gene. The primers for 16S rRNA gene of *Bacillus* sp. were designed according to Waldeck *et al*., (2006) table (6), while the primers specific to *Bacillus licheniformis* gyrase b gene were designed according to Huang *et al.*, (2012) table (6).Both isolates had been amplified for 16S rRNA gene while had not been amplified for *gyrase b* gene which means that both isolates are *Bacillus* spp. while not *B.licheniformis.* This agrees with Huang *et al.* (2012).After additional tests, the (B16) isolate was chosen to be mutated by EMS chemical agentmutagen.

3.2 Mutagenesis

To study the effects of EMS on the regulatory elements i.e., the promoter and the terminator of the cellulase gene, B16 isolate was subjected to different concentrations of this chemical mutagen; these treatments induce random mutations which may enhance or decrease the thermophilic cellulase production. This was deduced from estimating the enzyme activity by the three methods qualitative, semi-quantitative and quantitative activity in addition to estimating FPase activity, all of these tests, pre- and post-treatment as illustrated in the table (7).

After sequencing, the alignment results for the wild B16 isolate and the B16 EMS treatment had showed, for the promoter region [that contains Shine-Dalgarno (SD)which is a sequence of ribosomal binding site in bacterial and archaeal messenger RNA, generally located around 8 bases upstream of the start codon AUG; the RNA sequence helps recruit the ribosome to the messenger RNA (mRNA) to initiate protein synthesis by aligning the ribosome with the start codon, it has the general consensus sequence AGGAGG, Malys, 2012)], a deletion in the locus (-13) which lies in the (SD) sequence, figure (1).Also, the promoter contains the -10 region (Pribnow box) and -35 region. For the Pribnow box region, there were no changes in the B16 isolate sequence alignment post-EMS treatment. The same result was noticed for -35 region. For terminator region, no changes were observed in the sequence alignment post- EMS treatment, figure (2).The table (8) illustrates post-mutagenesis changes in the promoter and terminator regions briefly.

Statistical analysis results, which indicate to an increase in the different types of enzyme activity assays values in the EMS treatment at (50) µg/ml with a significant difference ($P< 0.05$), refer to the positive role of EMS mutagen on thermophilic cellulase production and agrees with Mala *et al*., (2001) who found an over-production of the bacterial products randomly in some colonies of the mutant post-EMS treatment.

The EMS is a mutagenic alkylating agent whose mode of action is attributed to the alkylation of nitrogen position 7 of guanosine of the DNA molecule (Ho and Chor, 2015), this agent carries one, two or more alkyl groups in reactive form. The transfer of ethyl or methyl groups to the bases leads to the alteration of their base-pairing resulting in transition mutation. The majority (99%) of the mutations caused by EMS induces C- to T- changes resulting in C/G to T/A substitutions. At a low frequency, EMS generates G/C to C/G or G/C to T/A transversions by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors. Therefore, it induces all types of mutations i.e. transition, transversion, frame shift and even chromosome aberrations with different frequencies (Kim *et al*., 2006; Karanam and Medicherla, 2008). The point mutations (transition, transversions and frameshifts) are minor changes in the primary structure of DNA resulting in occasional base-pairing mistakes ; while segment mutations (deletions, inversions, translocations, and duplications) leading to inactivation, chromosomal rearrangements, and chromosomal breaks, are believed to cause lethal DNA alterations because of the severe changes in the primary and secondary structure of DNA (Rhaese and Boetker, 1973).The point mutation which has the effect of an amino acid substitution may produce a mutant protein with a partial loss of activity, or occasionally an increase in activity (Minde *et al*., 2011).

The increase in the enzyme activity leads to the conclusion that the EMS might had induced a point mutation of an amino acid substitution which might be because of the insertion of a new amino acid in the peptide chain resulted from the mutant coding sequence of cellulase gene which had led to an improvement of cellulase activity or it might be because of a point mutation in another locus of the bacterial chromosome which had a positive effect on the cellulase gene regulation or performance. These results match with results of Agrawal *et al.*, (2013) and Raju, (2013) who found an obvious increase in cellulase activity and fibrinolytic protease activity after EMS treatment of the *Bacillus subtilis* and *Bacillus cereus* respectively.

4. Conclusions

The isolate B16, which was isolated from Iraqi soil and characterized as *Bacillus* sp., is an efficient bacteria in producing thermophilic cellulase. From the values of enzyme activity assays carried on the control (wild) and mutated B16 isolate, it could be concluded that EMS is an active chemical mutagen since it was capable of enhancing thermophilic cellulase production in the mutant sample compared with control. The change in SD region might have enhanced the cellulase gene expression.

5. Future Scope

Iraq has a hot desert climate in 70% of its lands. Therefore, its soil contains a variety of thermophilic microorganisms which wait to be discovered and exploited in many industrial fields including enzymatic cellulose degradation, the growing renewal power field. New thermophilic species and strains of microorganisms economically desirable in the non-fossil fuel possessing countries, could be discovered, genetically modified and being exported to such countries which will represent a new financial source to the Iraqi financial revenues, especially to poor countries which cannot afford the expenses of importing fossil fuel.

Table 1: The biochemical tests results of the bacterial isolates (B16, B37, B39, B44)

The biochemical test	B16	B37	B39	B44
Gram stain	Gm+ve	Gm+ve	Gm+ve	Gm+ve
Catalase	positive +	positive +	positive +	positive +
Starch	positive +	positive +	positive +	positive +
Gelatin	positive +	positive +	positive +	positive +
Motility	positive +	positive +	positive +	doubted
pH				
5.7	positive +	positive +	positive +	positive +
6.8	positive +	positive +	positive +	positive +
Salinity				
5%	positive +	positive +	positive +	positive +
7%	positive +	positive +	positive +	positive +
VP mixed sugar hydrolysis	positive +	positive +	positive +	positive +
MR complete g hydrolysis	positive +	positive +	positive +	positive +
Lecithinase	positive +	positive +	positive +	positive +
Anaerobic growth	positive +	positive +	positive +	positive +
Indole	negative -	negative -	negative -	negative -
Temperature (C^o)				
30	positive +	positive +	positive +	positive +
40	positive +	positive +	positive +	positive +
50	positive +	positive +	positive +	positive +
55	positive +	positive +	positive +	positive +
65	negative -	positive +	positive +	negative -
Spore stain	terminal	terminal	terminal	terminal
Urea	positive +	negative -	positive +	positive +
H2S	negative -	negative -	negative -	negative -
Carbohydrate fermentation				
Arabinose	positive +	positive +	positive +	positive +
Glucose	positive +	positive +	positive +	positive +
Mannitol	positive +	positive +	positive +	positive +
Xylose	positive +	positive +	positive +	positive +
Simmon's citrate	positive +	positive +	positive +	positive +

Table 2: VITEK 2 results for sample (16) identified according to this test as *Bacillus licheniformis* by the ratio 89%

No. of test	Test	Result	No. of test	Test	Result
1	BXYL	-	24	dMAN	+
2	LysA	-	25	dMNE	+
3	AspA	-	26	dMLZ	-
4	LeuA	-	27	NAG	+
5	PheA	+	28	PLE	+
6	ProA	-	29	IRHA	-
7	BGAL	+	30	BGLU	+
8	PyrA	+	31	BMAN	-
9	AGAL	-	32	PHC	-
10	AlaA	-	33	PVATE	+
11	TyrA	+	34	AGLU	-
12	BNAG	-	35	dTAG	+
13	APPA	-	36	dTRE	+
14	CDEX	+	37	INU	-
15	dGAL	-	38	dGLU	+
16	GLYG	-	39	dRIB	-
17	INO	+	40	PSCNa	-
18	MdG	+	41	NaCl 6.5%	+
19	ELLM	+	42	KAN	-
20	MdX	-	43	OLD	+
21	AMAN	-	44	ESC	+
22	MTE	+	45	TTZ	-
23	GlyA	+	46	POLB_R	+

Table 3: VITEK 2 results for sample (37) identified according to this test as *Bacillus cereus/thuringiensis/mycoides* by the ratio 86%

No. of test	Test	Result	No. of test	Test	Result
1	BXYL	-	24	dMAN	-
2	LysA	-	25	dMNE	-
3	AspA	-	26	dMLZ	-
4	LeuA	-	27	NAG	+
5	PheA	+	28	PLE	-
6	ProA	-	29	IRHA	-
7	BGAL	-	30	BGLU	-
8	PyrA	+	31	BMAN	-
9	AGAL	-	32	PHC	-
10	AlaA	-	33	PVATE	+
11	TyrA	-	34	AGLU	-
12	BNAG	+	35	dTAG	-
13	APPA	-	36	dTRE	+
14	CDEX	-	37	INU	-
15	dGAL	-	38	dGLU	+
16	GLYG	-	39	dRIB	+
17	INO	-	40	PSCNa	-
18	MdG	-	41	NaCl 6.5%	+
19	ELLM	-	42	KAN	-
20	MdX	-	43	OLD	-
21	AMAN	+	44	ESC	+
22	MTE	+	45	TTZ	-
23	GlyA	-	46	POLB_R	+

Table 4: VITEK 2 results for sample (39) identified according to this test as *Bacillus licheniformis* by the ratio 93%

No. of test	Test	Result	No. of test	Test	Result
1	BXYL	-	24	dMAN	+
2	LysA	-	25	dMNE	+
3	AspA	-	26	dMLZ	-
4	LeuA	+	27	NAG	(+)
5	PheA	+	28	PLE	+
6	ProA	-	29	IRHA	-
7	BGAL	+	30	BGLU	+
8	PyrA	+	31	BMAN	-
9	AGAL	-	32	PHC	-
10	AlaA	-	33	PVATE	+
11	TyrA	+	34	AGLU	-
12	BNAG	-	35	dTAG	+
13	APPA	+	36	dTRE	+
14	CDEX	+	37	INU	-
15	dGAL	-	38	dGLU	(-)
16	GLYG	+	39	dRIB	-
17	INO	+	40	PSCNa	-
18	MdG	+	41	NaCl 6.5%	+
19	ELLM	+	42	KAN	-
20	MdX	-	43	OLD	+
21	AMAN	-	44	ESC	+
22	MTE	+	45	TTZ	+
23	GlyA	+	46	POLB_R	+

Table 5: VITEK 2 results for sample (44) identified according to this test as *Lysinibacillussphaericus / Lysinibacillusfusiformis* by the ratio 98%

No. of test	Test	Result	No. of test	Test	Result
1	BXYL	-	24	dMAN	-
2	LysA	-	25	dMNE	-
3	AspA	+	26	dMLZ	-
4	LeuA	+	27	NAG	-
5	PheA	+	28	PLE	-
6	ProA	-	29	IRHA	-
7	BGAL	-	30	BGLU	-
8	PyrA	+	31	BMAN	-
9	AGAL	-	32	PHC	-
10	AlaA	+	33	PVATE	+
11	TyrA	(+)	34	AGLU	-
12	BNAG	+	35	dTAG	-
13	APPA	+	36	dTRE	-
14	CDEX	-	37	INU	-
15	dGAL	-	38	dGLU	-
16	GLYG	-	39	dRIB	-
17	INO	-	40	PSCNa	-
18	MdG	-	41	NaCl 6.5%	-
19	ELLM	+	42	KAN	-
20	MdX	-	43	OLD	-
21	AMAN	-	44	ESC	-
22	MTE	-	45	TTZ	-
23	GlyA	-	46	POLB_R	-

Table 6: Primers used in this study

Primer type	Primer sequence	Conc. Pmol/ µl	Product size
F-PR	5'-GTTCGTGCTACAGGCAAGGA-3'	100.9	170 bp
R-PR	5'-TTCGGTGCAATGATACCGCT-3'	107.1	
F-TR	5'-GGAAAGCTGATTTGGGGCAC-3'	123.1	240 bp
R-TR	5'-CCGGTCCATTGATCGTGTCATA-3'	114.5	
F-16S rRNA	5'-AGAGTTTGATCCTGGCTCAG-3'	104.1	1512 bp
R-16S rRNA	5'-TACGGCTACCTTGTTACGACTT-3'	149.4	
F- *Gyrase B*	5´-AK*ACGGAAGTCACGGGAAC-3´	100.8	550 bp
R-*Gayrase B*	5´-AGAAACTTTTCR**AGCGCTT-3´	109.8	

(PR): Refers to cellulase gene promoter region
(TR): Refers to cellulase gene terminator region
*K means G or T according IUPAC codes
**R means A or G according IUPAC codes

Table 7: The cellulase activity pre- and post EMS treatment at (50) µg/ml

The assay type	Pre-treatment	Post-treatment
Qualitative assay	17.4mm	18mm
Semi-quantitative assay	24.3mm	17.5mm
Quantitative assay	0.59 U/ml	1.21U/ml
FPase assay	1.39U/ml	1.85U/ml

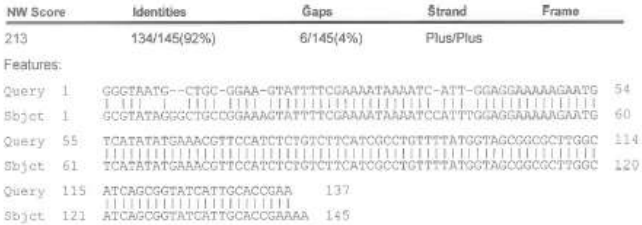

Figure 1: Alignment results of B16 isolate promoter region post-EMS treatment

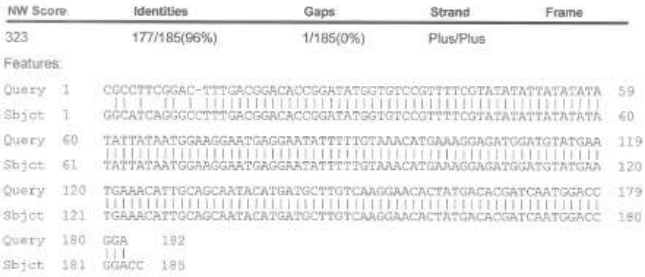

Figure 2: Alignment results of B16 isolate terminator region post-EMS treatment

Table 8: Changes in the sequence of transcription regulatory elements of cellulase gene post-EMS

Regulatory element	Location	Sequence	Isolate type
Shine-Dalgarno (SD)	(-8)- (-13)	5'TGGAGG'3	B16 wild isolate
		5' (-)GGAGG'3	B16 EMS treatment
Pribnow box (-10 region)	(-19)- (-24)	5'TAAAAT'3	B16 wild isolate
		5'TAAAAT'3	B16 EMS treatment
-35 region	(-27)- (-32)	5'TTCGAA'3	B16 wild isolate
		5'TTCGAA'3	B16 EMS treatment
Terminator	(3200)- (3225)	5'CGGACACCGGATATGGTGTCCGTTT'3	B16 wild isolate
		5'CGGACACCGGATATGGTGTCCGTTT'3	B16 EMS treatment

(-): refers to nucleotide deletions

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