Current Research in Microbiology and Biotechnology
Vol. 5, No. 6 (2017): 1317-1322
Research Article
Open Access

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ISSN: 2320-2246

Enhancing cellulase activity in a novel thermophilic *Bacillus* sp. isolated from Iraqi soil by using chemical and physical mutagens

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Received: 04 October 2017 Accepted: 21 October 2017 Online: 01 November 2017

ABSTRACT

Cellulose is the most common biomass on the globe and is available as huge amounts of agricultural and forestry natural residues. Cellulases are the major enzymes capable of degrading cellulose biomass. Microbial cellulases are widely used in this field. Industrially, cellulases producing microorganisms need to be enhanced to increase their ability in degrading cellulose. In this study, chemical and physical enhancers were tested on a novel thermophilic *Bacillus* sp. isolate isolated from Iraqi soil. The chemical agent (EMS) was found to enhance the isolate cellulase quantitative ability from 0.59U/ml to 1.21U/ml. The physical agent atmospheric non thermal plasma (ANTP) had decreased cellulase quantitative ability to 0.21U/ml so did green pulsed laser (532)nm which decreased the quantitative ability to 0.49U/ml. When the chemical and physical agents were merged to treat the same isolate, it was found that (EMS+ANTP) treatment had given cellulase quantitative ability of 0.51U/ml while (EMS+laser) had given quantitative ability 1.05U/ml compared with 0.59U/ml cellulase quantitative ability of the control isolate.

Keywords: Cellulase, cellulytic bacteria, cellulase chemical enhancing, cellulase ANTP enhancing.

1. INTRODUCTION

Cellulose constitutes the most common organic polymer, it represents about 1.5 X 10^{12} tons of the annually total biomass production through photosynthesis, especially in the tropical areas, and is considered to be nearly inexhaustible source of raw material for different products. It is the most abundant and renewable biopolymer on earth and the dominating waste material from agriculture [1].

Cellulase is a designation for the enzyme complex secreted by the cellulose –degrading organisms which can cope the resistance of the highly crystalline cellulose substrate catalyzing cellulose hydrolysis into dissolved sugars, these enzymes work synergistically, which enables a more rapid and efficient cellulose hydrolysis [2, 3].

Therefore, the employment of cellulases to hydrolyze cellulose at moderate conditions without generating byproducts is very attractive because the enzymatic depolymerization is environmental friendly [4]. Therefore, attention was focused on the sources of microbial cellulases, i.e. bacterial and fungal cellulases. Microbial cellulases have low manufacturing costs, easiness of genetic doctrinaire, no seasonal impacts, and quick culture growth [3, 5].

The most common cellulolytic bacterial genera are *Bacillus* spp., *Cellulomonas* spp., *Clostridium* spp., *Thermonospora* spp., *Pseudomonas* spp., *Streptomyces* spp.and *Salmonella* spp.[6]. 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. The thermophilic strains of genus *Bacillus* produce considerable number of extra cellular thermostable enzymes including cellulases,e.g .*Bacillus licheniformis*, *Bacillus subtilis* K-18, *Bacillus sp*. SMIA-2, *Bacillus* sp CH43 and *Bacillus* sp. HR68 [7, 8, 9].

In this study, a selected *Bacillus* sp. isolate was improved with both chemical (EMS) and physical (ANTP) and (Nd:Yag) laser (532)nm agents. The developed colonies were screened and evaluated for enzyme production level.

2. MATERIALS AND METHODS

2.1 Isolation of organisms

Bacillus sp. was isolated from soil samples which were collected from the soil beneath the plant wastes. The isolate was identified as *Bacillus* biochemically by reference to Acharya *et al*.(2012)[10]. The following biochemical tests were carried out for identification: Gram Stain, Simon's citrate test, Catalase test, Starch hydrolysis, Motility test, Indole test, Voges Proskauer (VP) test, Methyl Red test, Carbohydrate Fermentation test, Gelatinase detection test, Growth at different temperatures, Lecithinase Test, Urease test in addition to many other biochemical tests. 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 using 0.5% v/v carboxy methyl cellulose(CMC) as carbon source in both broth and agar media as described by Kauri and Kushner (1985)[11].The composition of the CMC media used in this study was as follows (counted as g/L): NH4 NO3 (2.0), K 2 HPO4 (0.5), MgSO4.7H2O (0.2), CaCl2.2H2O (0.02), $FeSO_4.7H_2O$ (0.02), MnSO4.H2O (0.02), Yeast extract (0.2), Peptone (0.2), CMC (5.0) and agar (15) added in case of CMC agar medium.

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)Cº for (24) hrs. After that, (0.2) ml was taken from each flask and spreaded onto CMC agar plates then incubated at (55)Cº 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)[12] and Bai *et al* (2012)[13] with modifications by transferring a heavy inoculum from each bacterial isolated colony(one isolated colony per sample plate) onto the central portion of a new CMC agar plate in a circle of 5mm diameter ,the plates then were incubated at 55°C for 24 hrs. After that the surface of each plate was flooded with Iodine solution for (20-30) sec. then the solution was spilled and the plates were left for (10) min. The diameter of the hydrolysis halo which indicates the production of cellulase was measured.

2.4 Selection and preparation of the bacterial isolate for the enhancement of cellulases production

After statistically analyzing the hydrolysis halo measurements of all samples by ANOVA test using (SPSS) program; the best isolate in cellulase production was chosen to be tested for its semi-quantitative and quantitative cellulytic activity according to Samira et al (2011)[14]. Then this isolate was exposed to chemical and physical agents in attempt for increasing its cellulytic quantitative activity. A loopful of fresh active culture of *Bacillus* sp. grown on CMC-Nutrient agar plate (CN-agar) (prepared according to Stephan, 2011)[15] at 55°C for 24 hrs was transferred into a (100) ml cotton plugged Erlenmeyer flask containing pre-sterilized (50) ml CN broth ,incubated at (55)°C in a shaker incubator at (120) rpm for (72) hrs. Then, Two ml of the inoculum was transferred to a hundred ml cotton plugged Erlenemyer flask containing presterilized (50) ml CMC broth and incubated in a shaker incubator for (24) hrs. at (55) C o in chemical agent enhancement and for (18)hrs in physical agents treatments.

2.5 Enhancement of bacterial isolate ability in cellulase production

The enhancement of the selected isolate with (EMS) was performed according to Haq et al (2009) [16] with modifications; the concentrations used were (0,50,100,150) µg/ml. The physical enhancement via Nd:Yag laser (neodymium-doped yttrium aluminium garnet; Nd:Y3Al5O12) which is considered as pulsed laser; was implemented according to Al Aamirry, (2003)[17] with modifications ;the laser generating system parameters had been fixed which were : the wavelength (532) nm, the power (500) mJ, the frequency(6) Hz, the distance between the laser source and the sample (20) cm, beam spot size (0.2) cm and pulse duration (10)ns. The changing parameter was the isolate exposure time intervals which were (2) and (4) min. The enhancement of the cellulytic activity via (ANTP) was achieved in a way similar to that of laser. (ANTP) generating system parameters had been fixed which were: the sag of the special plate of the (ANTP) generating system which had the dimensions (2.5 X 1.5 X 0.1cm), the (ANTP) spot size (5 cm), the distance between the (ANTP) source and the sag of the plate had been adjusted to (1)mm and the voltage (550 volt). The changing parameter was the isolate exposure time intervals which were (0, 2, 4, 6 and 8) min. After that, the best enhancing (EMS) concentration was combined with the best enhancing exposure time interval of (Nd:Yag) laser. The same was accomplished with (ANTP).The semi-quantitative , quantitative and total cellulase activity or what is termed filter paper assay (FPA or FPase) assays were carried out according to Samira et al(2011)[14].

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 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. Therefore, they were considered as *Bacillus* spp., after additional tests, the (B16) isolate was chosen to be enhanced by chemical and physical enhancers.

Table 1: Isolates identification biochemical tests

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 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 identification tests for (B16) isolate

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 identification tests for B39 isolate

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	+

3.2 Cellulose degradation enhancing experiments

Tables (4,5,6) show comparison between control (B16) isolate and post (EMS), (Nd:Yag) laser and (ANTP) treatments separately whence qualitative ,semi-quantitative , quantitative and FPase assays values. The highest values were obtained in (EMS) treatment while there was a decrease in (Nd:Yag) laser treatment and a significant decrease in (ANTP) treatment. The increase in quantitative activity in (EMS) treatment might be attributed to the mutagenic alkylating ability of this chemical agent which might have induced a positive mutation in the cellulase coding gene. This result is in an agreement with [18] .The reduction of the cellulytic activity in (Nd:Yag) laser treatment in the exposure duration (2 min). and the lack of the enzyme production at (4 min) might be due to the negative action of the green light (the pulsed laser) on the general cellular bioprocesses. The green light is absorbed by porphyrins ,mostly by cytochromes which alternately accepts and releases an electron at a slightly lower energy level [19], hence, it might affect or damage the respiratory chain ,consequently ,the total performance of the bacterial cell. This result is in agreement with [20].The reduction of cellulytic activity in (ANTP) treatment in the exposure duration of (4 min.) which had statistically given the best cellulytic activity among the other durations might be due to the random mutagenesis in the bacterial genetic material due to plasma exposure.(ANTP) important feature is its ability to produce a mixture of biologically active agents such as reactive oxygen species (ROS) from which the hydroxyl radical reacts with all the purine/pyrimidine bases as well as deoxyribose backbone [21] ; leading to a damage in the genetic material subsequently, the resulting protein activity. Up to date, no available study had dealt with enhancing cellulytic activity via (ANTP).

Table 4: Enzyme activity values of (EMS) treatment compared with control

The assay type	The B16 control isolate	Post-EMS treatment
Qualitative activity	17.4mm	18mm
Semi-quantitative activity	24.3mm	17.5mm
Quantitative activity	0.59 U/ml	1.21U/ml
FPase activity	1.39U/ml	1.85U/ml

Table 5: Enzyme activity values of (Nd:Yag) laser treatment compared with control

The assay type	The B16 control isolate	Post-Nd:Yag laser treatment
Qualitative enzyme activity	17.4mm	16.7mm
Semi-quantitative enzyme activity	24.3mm	22mm
Quantitative enzyme activity	0.59 U/ml	0.49U/ml
FPase activity	1.39U/ml	1.47U/ml

Table 6: (ANTP) treatment enzyme activity values compared with control

The assay type	The B16 control isolate	Post-(ANTP)treatment
Qualitative enzyme activity	17.4mm	34.9mm
Semi-quantitative enzyme activity	24.3mm	22mm
Quantitative enzyme activity	0.59 U/ml	0.21U/ml
FPase activity	1.39U/ml	1.02U/ml

Afterwards, the best enhancing (EMS) concentration in increasing the (B16) cellulytic activity was combined with physical enhancing agents in the durations which gave the best cellulytic induction values; it can be noticed from table (7) that the (EMS+Nd:Yag laser) quantitative value had raised comparing with the separated laser treatment which means that there is a positive synergistic action of the two agents together which cancels the negative reduction action of the (Nd:Yag) laser alone in the tested dose (2 min.) .This result is in agreement with[16]. A similar result was noticed in table (8) when (EMS) was merged with (ANTP)in the exposure duration (4 min.).This result is in agreement with [22].

Table 7: (EMS+Nd:Yag laser) treatment enzyme activity values compared with control

The assay type	The B16 control isolate	EMS+Nd:Yag laser treated isolate
Qualitative enzyme activity	17.4mm	14.67mm
Semi-quantitative enzyme activity	24.3mm	13mm
Quantitative enzyme activity	0.59 U/ml	1.05U/ml
FPase activity	1.39U/ml	1.68U/ml

Table 8: (EMS+ANTP) treatment enzyme activity values compared with control

The assay type	The B16 control isolate	EMS+ANTP treated isolate
Qualitative enzyme activity	17.4mm	20mm
Semi-quantitative enzyme activity	24.3mm	13mm
Quantitative enzyme activity	0.59 U/ml	0.51U/ml
FPase activity	1.39U/ml	1.62U/ml

From the study it was concluded that *Bacillus* sp. (B16) isolated from Iraqi soil is capable of producing thermophilic cellulase which can be enhanced by being treated with chemical agent (EMS) separately and collectively with physical agents (Nd:Yag laser) and (ANTP) used in this study as seen by quantitative and FPase activity tests.

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