Isolation and Screening of Thermophilic Bacteria for Producing Cellulase Enzyme Using Agricultural Waste

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Abstract

Wheat straw was used as a source for isolating thermophilic bacteria and as a substrate for cellulase production. Twenty five of thermophilic bacterial isolates were isolated from agricultural waste samples on Luria-Bertani (L.B) agar plates. These bacterial isolates were qualitatively tested at 50°C for cellulose production by flooding plates containing mineral salts medium (MSM) with 2% of wheat straw and 0.5% Congo red dye for 15 min followed by repeating wash with 1M NaCl. Results showed that 12 isolates gave a clear zone, after Congo red dye staining of plates; this indicated that the colonies produce cellulase. Bacterial isolates were screened for enzyme activity in submerged liquid MSM with 2% of wheat straw at 50°C. The produced enzyme is then examined for reducing sugar release in liquid medium. Results revealed that $2T_5$, $3T_{13}$ and $3T_{18}$ isolates were the highest in producing cellulase enzyme 5.9, 13.7 and 14.3 Unit/ml, respectively. The most active isolates $2T_5$, $3T_{13}$ and $3T_{18}$ were identified on the basis of morphological, biochemical characteristics and API identification kits, they were identified as *Bacillus subtilis, Bacillus megaterium* and *Bacillus licheniformis,* respectively. [DOI: 10.22401/JNUS.20.2.14]

Keywords: Thermophilic bacteria cellulase, *Bacillus* sp., wheat straw, thermophilic bacteria.

Introduction

Agricultural wastes generates in fields and processing sites are generally discarded without being further used. The huge amount of residual plant waste can potentially use to produce various value added products like biofuels, animal food, chemicals, enzymes …etc. Cellulose is the major component of agricultural waste. The cellulosic waste substance can be hydrolyzed to glucose and further soluble sugars using cellulase enzymes [1].

Cellulase enzyme complex consists of three modules of soluble extracellular enzymes: 1, 4-β-endoglucanase, 1, 4-β-exoglucanase, and β-glucosidase(β-D-glucoside glucohydrolase or cellobiase) [2]. The most important agents of cellulase enzymes are bacteria and fungi, these microorganisms are commonly found in soil. The potential cellulase forming bacteria were *Cellulomonas, Pseudomonas, Thermoactinomycetes* and *Bacillus* spp. [3].

Materials and Methods

Samples collection

Four samples of wheat straw were obtained from diverse sources of agricultural fields, and then the wheat straw samples were air dried and heated at 50°C for 2 days, then milled the samples into small pieces (3-5 mm). The milled wheat straw samples were used source for isolation of thermophilic bacteria [4].

Isolation of thermophilic bacteria

Thermophilic microorganisms were isolated directly from wheat straw samples by serial dilution technique on L.B agar plates, pH 7. Ten grams from each sample of milled wheat straw were suspended with 90 ml sterile saline in 250 ml flasks. The flasks were shaked vigorously for 15 min. and the suspensions of wheat straw were serially diluted. From each dilution of 10^{-3}, 10^{-4} and 10^{-5}, 0.1 ml was used and directly spreaded onto surface of L.B agar plates. The L.B medium consisted of 10 g tryptone, 5 g yeast extract, 5 g NaCl and 20g agar in 1L of Distilled Water. All plates were incubated at 50°C for 3 days. The isolates appeared on the plates were selected and kept on L.B agar slants at 4°C [4].

Qualitative screening

Samples of wheat straw were dried at 50°C for 2 days, and grounded to powder using a blender.

Besides, mineral salt medium (MSM) consisted of (g/l): wheat straw powder 20; $NaNO_3$ 0.5; K_2HPO_4 1.0; MgSO4.7H2O 0.5; $FeSO_4.7H_2O$ 0.01; and yeast extract 1.0 at pH 7.0., the medium was sterilized using autoclave at 121°C for 15 min. MSM substance was solidified before sterilization by 2% agar and poured in plates. The plates were individually inoculated with different isolates on the middle of surface plates, and incubated at 50°C for 3 days. After period of incubation, all plates are flooding with 1% Congo red indicator and left for 15 min at 25°C, then destining with 1M NaCl solution and left for another 15 min. Clear zones are resulted around growing bacteria colonies indicating cellulose hydrolysis. The colonies of bacteria showing better clear zone are selecting for further screening [5].

Quantitative screening

The promising thermophilic isolates were examined for cellulase production in liquid medium. Isolates were grown in 250 ml conical flasks containing 100 ml of the liquid MSM with 2% wheat straw powder. These flasks were inoculated with 1% (v/v) inoculum cultures and incubated for 3 days at 50ºC with shaking at 150 rpm. Crude enzyme was extracted from the fermented flasks using centrifugation at 10,000 rpm for 15 min at 4ºC, and clear cell-free supernatant (crude extract) was collected and assayed for cellulase activity using 3, 5-dinitrosalicylic acid [6].

Enzymatic assay

Cellulase activity was assayed using Dinitrosalisic acid (DNS) reagent by estimation the reducing sugars resulted from carboxymethylcellulose (CMC) as a substrate. Crude enzyme of 0.5 ml was added to 0.5 ml of 1% CMC 0.05M Phosphate Buffer and incubated at 50°C for 30 min., and then the reaction was ended by adding 1.5 ml of DNS reagent and boiled at 100 °C in a water bath for 10 min. The quantity of reducing sugar resulted by the hydrolysis of CMC was measured at 540nm using UV-Vis spectrophotometer. Cellulase fabrication was estimated using Glucose standard curve. The unit of enzyme activation was defined as the amount of enzyme that necessary to release 1µmol of glucose per min under standard assaying circumstances [6].

Identification of cellulolytic isolates

The most active isolates were identified depending on classical methods (morphological, cultural and biochemical characterizations) and API identification kits (API 20E and API CHB50) of pure cultures [7].

Results and Discussion

Isolation of thermophilic bacteria

Four wheat straw samples, with high content of cellulose were collected from different sites and used to isolate thermophilic bacteria. Twenty five isolates of thermophilic bacteria were isolated depending on morphology characterization from all samples, as listed in Table (1). Results showed a difference in the numbers of microbes isolated from each sample and most of the isolates were obtained from the samples collected from Babylon city. Cellulose is the most important polysaccharide in agriculture wastes, since it can be converted into various types of raw materials by microorganisms, which live in habitats where cellulosic waste presents [8].

Table (1)
Isolation of thermophilic bacteria from different wheat straw samples.

Sample	Date of collection	Site of agricultural field	Numbers of isolates
1T	22/5/ 2013	North of Baghdad	3
2T	22/5/ 2013	South of Baghdad	4
3T	28/5/ 2013	North of Babylon	11
4T	28/5/ 2013	South of Babylon	7
∑			25

Degradation of cellulosic materials is caused by various mesophilic and thermophilic microorganisms which have cellulolytic enzymes. Best sources of isolation of cellulolytic microorganisms are the habitats where the cellulosic waste presents and some of microorganisms are discovered during last periods that have ability to change cellulose into simple sugars [9].

Primary screening:

In order to detect the abilities of bacterial isolate to produce cellulase enzyme, twelve bacterial isolates among of twenty five had cellulase activity. Three of the twelve bacterial isolates namely $2T_5$, $3T_{13}$ and $3T_{18}$ showed a maximum enzyme fabrication after 3 days of incubation in 50°C. Results in Table (2) show the primary screening for bacterial isolates which measured according to diameter of clear zone. The results in this study

Table (2)
Qualitative screen of thermophilic bacterial isolates for cellulase production.

S.N.	Isolates code	Clear zone diameter (mm)	S.N.	Isolates code	Clear zone diameter (mm)
1	$1T_1$	11	14	$3T_{14}$	12
2	$1T_2$	-	15	$3T_{15}$	-
3	$1T_3$	10	16	$3T_{16}$	10
4	$2T_4$	-	17	$3T_{17}$	-
5	$2T_5$	14	18	$3T_{18}$	20
6	$2T_6$	-	19	$4T_{19}$	9
7	$2T_7$	11	20	$4T_{20}$	-
8	$3T_8$	-	21	$4T_{21}$	-
9	$3T_9$	-	22	$4T_{22}$	12
10	$3T_{10}$	9	23	$4T_{23}$	-
11	$3T_{11}$	-	24	$4T_{24}$	-
12	$3T_{12}$	-	25	$4T_{25}$	10
13	$3T_{13}$	16	-	-	

(-): Negative result

were in agreement with those reported by Acharya *et al*. [10], who found that *Bacillus* species are the most active cellulolytic isolates. A total of 25 thermophilic bacterial were examined for their cellulytic activity using 1% Congo red indicator, results exhibited that most potent thermophilic cellulolytic isolates were identified as *Bacillus* species and the isolate *Bacillus subtilis* showed the highest zone of hydrolysis (26 mm). Ray *et al*. [11] used four different cellulose basal media to test the ability of *Bacillus subtilis* to produce cellulase enzyme, results showed that maximum zone of hydrolysis was 30 mm. Whereas Attri and Garg [12] tested 33 bacterial isolates producing a clear zone on solid media, results appeared that 14 isolates gave a zone of hydrolysis on a medium contains wheat bran, 10 isolates displayed a zone of hydrolysis on a medium with xylan and 12 isolates gave zone on a medium contains cellulose.

Secondary screening

The twenty two isolates exhibited cellulolytic activity at the primary screening were selected to study their ability to produce cellulase enzyme in liquid medium when wheat straw was used as substrates.

The results in Table (3) revealed that all isolates gave difference productions for cellulose enzyme in liquid medium and three of them $2T_5$, $3T_{13}$ and $3T_{18}$ showed maximum enzyme yield 5.9, 13.7 and 14.3 unit/ml respectively.

Table (3)
Quantitative screening for cellulase production of most active isolates in liquid MSM supplemented with 2% of wheat straw powder. Flasks were incubated at 50°C and 150 rpm for 3 days.

S.N.	Isolates code	Cellulase activity (unit/ ml)	S.N.	Isolates code	Cellulase activity (unit/ ml)
1	$1T_1$	1.2	7	$3T_{14}$	2.4
2	$1T_3$	0.98	8	$3T_{16}$	1.8
3	$2T_5$	5.9	9	$3T_{18}$	14.3
4	$2T_7$	1.33	10	$4T_{19}$	0.89
5	$3T_{10}$	0.65	11	$4T_{22}$	2.1
6	$3T_{13}$	13.7	12	$4T_{25}$	1.9

Verma *et al*. [13] isolated and screened thermophilic *Bacillus* species from agricultural soil samples, the maximum cellulase yield was achieved after 48 h of incubation at 45°C in a medium of 1.5% CMC as substrate and the isolate *Bacillus subtilis* showed the highest enzyme production. In another study done by Abo-State *et al*. [4] different cellulosic wastes were used to isolate mesophilic and thermophilic bacteria with cellulolytic enzymes, fifty-nine bacterial isolates were isolated from seven different agricultural wastes and only thirty-seven isolates had cellulolytic activity and the isolate *Bacillus* sp. MAM-38 gave the highest CMCase and avicelase production 360 and 354 U/ml respectively. Also Patagundi *et al.* [14] isolated a total of 57 isolates from four different agricultural soil samples, three isolates dipicted maximum enzyme activity, they were identified as *Bacillus cereus*

(0.440 U/ml), *Bacillus subtilius* (0.357 U/ml) and *Bacillus thuringiensis* (0.334U/ml). While Chang *et al.* [15] isolated five isolates of thermophilic bacteria from cellulosic waste, and two of them (TA-1 and TB-2) were identified as *Thermoactinomyces* and *Bacillus* spp. Which showed maximum enzyme activity 2.07 and 2.3 U/ ml respectively.

Identification of bacterial isolates

The three active isolates ($2T_5$, $3T_{13}$ and $3T_{18}$), exhibiting high enzyme production, were identified as *Bacillus* species depending on classical methods (morphological, cultural and biochemical tests) explained in Bergey's Manual of Systematic Bacteriology [7]. However, further results from API identification kits (API 20E and API CHB50) revealed that the isolates $2T_5$, $3T_{13}$ and $3T_{18}$ were identified mainly as *Bacillus subtilis*, *Bacillus megaterium* and *Bacillus licheniformis*, respectively.

References

[1] Camassola M., Dillon A. "Production of cellulases and hemicellulases by Penicillium echinulatum grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation". J Appl Microbiol, 103, 6, 2007.

[2] Bansal N., Tewari R., Gupta J.K., Soni S.K., Soni R. "A novel strain of *Aspergillus niger* producing a cocktail of industrial depolymerising enzymes for the production of second generation biofuels". Bioresour Technol; 6, 1, 2011.

[3] Godana B., Mitra R., Singh S. "Production of Enzymes for application on animal feeds". Submitted in partial fulfillment of the requirements for the degree of Master of Technology (Biotechnology), Department of Biotechnology, Faculty of Science, Engineering and the Built Environment, Durban University of Technology, Durban, South Africa

[4] Abo-State M.A.M., Ghaly M.F., Abdellah E.M. "Production of cellulases and xylanase by thermophilic and alkaliphilic bacterial strains isolated from agricultural wastes". World Appl. Scien. J.; 22 (11), 1603-1612, 2013.

[5] Padilha I. Q. M., Carvalho L. C. T., Dias P. V. S., Grisi T. C., Honorato F. L., Santos S. F., Araújo1 D. A. "Production and characterization of thermophilic carboxymethyl cellulose synthesized by *Bacillus* sp. growing on sugar cane bagasse in submerged fermentation". Brazilian J. Chem. Engine., 32 (1), 35 – 42, 2015.

[6] Bajaj B. K., Sharma M., Rao R. S. "Agricultural residues for production of cellulase from *Sporotrichum thermophile* LAR5 and its application for saccharification of rice straw". J. Mater. Environ. Sci., 5 (5), 1454-1460, 2014.

[7] Holt JG., Krieg NR, Sneath PHA, Staley JT., Williams ST. In:" Bergey's manual of determinative bacteriology", Lippncott Williams and Wilkins, New York, 175-533, 2000.

[8] Khalil M. I., Hoque M. M., Basuniai M. A., Alam N., Khan M. A. "Production of cellulase by *Pleurotus ostreatus* and *Pleurotus sajor-caju* in solid state fermentation of lignocellulosic biomass". Turk. J. Agric., 35, 333-341, 2011.

[9] Lokhande S., Musaddiq M. "Isolation of cellulolytic bacterial strains for bioconversion of municipal solid waste". Internat. J. Appl. Research., 1 (11), 902-905, 2015.

[10] Acharya A., Joshi D.R., Shrestha K., Bhatta D.R. "Isolation and screening of thermophilic cellulolytic bacteria from composting piles". Scient. World., 10 (10), 43-46, 2012.

[11] Ray AK., Abhinanda B. A., Ghosh KS., Sen SK. "Optimization of fermentation conditions for cellulase production by *Bacillus Subtilis* Cy5 and *Bacillus Circulans* Tp3 isolated from fish Gut". Acta Ichthyol Piscat, 37 (1), 47–53, 2007.

[12] Attri S., Garg G. "Isolation of microorganisms simultaneously producing xylanase, pectinase and cellulase enzymes using cost effective substrates". J. Innov. Biol., 1 (1), 45-50, 2014.

[13] Verma V., Verma A., Kushwaha A. "Isolation and production of cellulase enzyme from bacteria isolated from agricultural fields in district Hardoi, Uttar Pradesh, India" Advan. Appl. Scien. Research., 3 (1), 171-174, 2012.

[14] Patagundi B. I., Shivasharan C.T., Kaliwa B. B. "Isolation and characterization of cellulase producing bacteria from soil". *Int. J. Curr. Microbiol.* Appl. Sci., 3 (5), 59-69, 2014.

[15] Chang C., Ng C., Wang C., Shyu Y. "Activity of cellulose from Thermoactinomycetes and *Bacillus* spp. Isolated from brassica waste compost". Sci. Agric., 66 (3), .304-308, 2009.