INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 4, p. 150-163, 2017

RESEARCH PAPER OPEN ACCESS

Isolation and identification of cellulase producing *Bacillus subtilis* EG7 and its potential in composting of agricultural wastes

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Key words: *Bacillus subtilis*, Identification, Cellulase, Compost, Agricultural wastes

http://dx.doi.org/10.12692/ijb/11.4.150-163 Article published on October 21, 2017

Abstract

In the current study, some bacterial isolates capable of utilizing Rice straw as an agricultural waste were obtained. Among them, the cellulolytic activity of the isolate EG7, detected both qualitatively and quantitatively, was promising. This isolate degraded the different cellulosic substrates used; Carboxymethylcellulose, Avicel, Cotton seed linters and Rice straw. The isolate was identified by classical bacteriological examination, metabolic fingerprinting using Biolog MicroPlate and phylogenetic analysis of 16S ribosomal Ribo-Nucleic Acid (16SrRNA) gene nucleotide sequence as *Bacillus subtilis*, GenBank accession number (KX817281). Cellulase enzyme of the isolate EG7 was produced under the determined optimum conditions. Among these conditions, static incubation for three days at initial pH 7.00 and incubation temperature 40°C with cellulose concentration 10 % (w/v) were favorable. Under these conditions, the enzyme activity recorded 45.00 UmL^{-1}. Purification of the produced enzyme was performed and the results revealed that the enzyme activity recorded 64.50UmL^{-1}. Molecular weight determination revealed the presence of two distinct bands of about 43 and 37 KDa. The use of the isolate EG7 in composting of Rice straw and Banana peels waste resulted in enhancing the process and reducing time of composting into five to seven days. Our results indicated the potential of using the isolate EG7 in enhancing composting of agricultural wastes, besides, the use of its cellulase in the different industrial applications.

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Introduction

Cellulases enzymes hydrolyze the β-1,4-glycosidic bonds in polymers of cellulose into glucose units. A wide variety of bacteria are known for their production of extracellular hydrolytic cellulases. Genus *Bacillus* is well known and promising in efficient hydrolysis of cellulose both *In-vivo* and *In-vitro*. Microbial cellulases in general, some aspects of production, and potential applications have been reviewed by (Fariq, 2016).

Usually the role of bacteria such as *Bacillus* in utilizing cellulosic substrates involves study the productivity and degree of synergy between different cellulase components in hydrolysis of this polymer. Some bacteria produce exoglucanases, endoglucanases, and also intra- or extra-cellular *β*-glucosidases to cleave cellobiose into glucose units. A comprehensive review for classification of cellulases according to their substrate specificities and different mechanisms of hydrolysis could be obtained by (Annamalai *et al.*, 2016).

Agricultural byproducts like Rice straw, being consist primarily of cellulosic cell walls, have a high nutritive value. Composting of agricultural wastes and byproducts by bacteria involves to a high degree the ability of powerful cellulases production. Bacterial cellulases and their numerous biotechnological applications have been reviewed by (Menendez *et al.*, 2015).

The current study has been concerned with the isolation of bacteria capable of utilizing Rice straw as an agricultural waste of economic importance. The potential of these isolates to produce cellulase enzyme from some different cellulosic substrates was exploited. In addition, identification of the most potent cellulase producer was performed. Also, production optimization was considered. Production, extraction and purification of the produced enzyme besides partial characterization for its component were all conducted. Finally, the potential of the most potent cellulase producer was exploited in composting of a mixture of Rice straw and Banana peels waste.

Materials and methods

Isolation medium and cellulosic substrate used

The samples of soil, compost, and Rice straw used for isolation were collected aseptically from different agricultural fields in Cairo, Egypt. Classic serial dilution plate technique was used for such a purpose.The following medium was used, modified inorganic salts starch nitrate medium into which starch was replaced by the cellulose substrate under study. The medium composed of (g/L): cellulose substrate (Rice straw; as the only C source), 20; $NaNO_3$, 2.0; K_2HPO_4 (anhydrous basis), 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01 and tap water, 1000 ml. The pH was adjusted at 7.5. This medium was used as liquid or solidified by addition of 2% agar. Agar plates of this medium were inoculated and incubated at 40 °C for 3-4 days. The used Rice straw was air dried for 2 days, oven dried at 50 °C for 2 days and then grinded. Colony purity was assessed using nutrient agar plates. A preliminary test for cellulose hydrolysis by using Gram's iodine reagent according to (Kasana *et al.*, 2008) was also used to aid in selecting the potential colonies where clear zone diameters (devoid of blue color) were at least 10 mm.

Screening for cellulase activity

Cellulase activity qualitative assay

This assay was achieved by growing the bacterial isolates on the above agarized medium containing one specified substrate at a time at concentration (2 % w/v). For such a purpose soluble, Carboxymethylcellulose (CMC, sodium salt, Sigma C5678), and insoluble, Avicel (PH-101, Sigma 11365), Cotton seed linters (CSL), and Rice straw (RS) were used as only carbon sources in the used medium mentioned above. The plates were incubated at 40 °C for 3-4 days. Clear zone diameters after addition of iodine reagent were recorded. Also, the assay was done by culturing the isolates in liquid state of the same medium followed by testing the activity on CMC & Avicel agar plates in which wells measured 7 mm in diameter were created. After the incubation was terminated all liquid cultures were centrifuged, filtered, and then putted in wells (about 100 µl) and allowed to diffuse for at least 6 hours at 40°C. Also, clear zone diameters after addition of iodine reagent were recorded.

Cellulase activity quantitative assay

The cellulase activity of the same culture filtrate, after growth on crystalline cellulose liquid as above in qualitative assay, was measured by determining the amount of reducing sugars liberated by using dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction mixture composed of 0.5 ml of CMC liquid or Avicel suspension, 0.5 ml of crude enzyme (culture filtrate) and 0.5 ml of 0.05 M citrate buffer pH 4.8 were incubated for 30 minutes at 40 °C before adding 2 ml of DNS solution. The treated samples were boiled for 15 min prior to cool down in cold water bath for color stabilization. The optical density was then read at 540 nm against reagent blank by UV/VIS spectrophotometer. By using a calibration curve for glucose, results were interpreted in terms of enzyme activity in which one unit (U) of enzyme activity was expressed as 1μmol of reducing sugar released per min per ml, under the above assay conditions.

Protein determination

Extracellular protein concentrations, of the same culture filtrate, after growth on crystalline cellulose substrate as above in qualitative assay, were determined and expressed as mg/ml by using Lowry method and bovine serum albumin as a standard according to (Lowry *et al.*, 1951).

Identification of the isolate EG7

The isolate EG7 was subjected for metabolic fingerprinting (using Biolog plate 3rd generation) besides traditional identification experiments according to (Logan and De Vos, 2009). In addition, the isolate was identified by phylogenetic analysis of 16S rRNA gene using 16S ribosomal Deoxyribo-Nucleic Acid (16S rDNA) as the template.

Biolog identification

First, the isolate EG7 was grown at 30°C on (Luria-Bertani) LB agar. When sufficient growth was noted, the isolate was suspended in 0.85% saline and inoculated into Biolog MicroPlate (150 µl per well) according to the manufacturer's instructions and incubated for 24 hours at 30°C. Biolog plates were read and analyzed using semi-automated Biolog Microstation System and Biolog software. This work was supported by "Research Services O" (Cairo, Egypt).

Molecular identification of 16S rDNA gene for the isolate EG7

DNA extraction, polymerase chain reaction (PCR) and purification

The isolate EG7 was inoculated into 5 mL aliquots of LB broth and incubated at 30°C on a rotary shaker at 180 rpm for 20 h. Total genomic DNA was extracted from the isolate according to the used kit's manual (QIAamp DNA mini kit, No. 51304). The 16S rDNA region was amplified (approximately 1500 bp) by PCR using the forward primer F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and the reverse primer R1492 (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR mixtures were prepared in 50 µl volumes containing 0.5 µM of primer, 200 mM of deoxyribonucleotide triphosphate, 5 µl of the 10X PCR buffer (100 mM Tris–HCl, 15 mM MgCl2, 500 mM KCl; pH 8.3), 1 U of Taq DNA polymerase, and 1 µl of the extracted DNA. DNA amplification was performed in TProfessional Basic Thermocycler PCR system with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, elongation at 72°C for 90 s, and a final extension at 72°C for 10 min. The amplicon was identified by horizontal electrophoresis on 1% agarose gel against the used DNA size marker (UMR-100) and finally was purified using PCR purification kit. This work was supported by "Research Services O" (Cairo, Egypt).

16S rDNA gene Sequencing, data analysis and phylogeny

Sequencing was performed using an Applied Biosystems 310 sequencer (Big Dye Terminator cycle sequencing ready kit) with the same primers used for PCR. The sequence was compared with similar sequences retrieved from DNA databases by using the NCBI n-BLAST search program in the National Center for Biotechnology Information (NCBI). Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar *et al.*, 2016).

Optimization of cellulase production

Optimization for cellulase produced by the isolate EG7 under investigation was considered in this section.

When, one factor was tested, all others were kept as the initially used. The tested factors included the potential of enzyme induction by this isolate using different carbon substrates, concentration of the used substrate (RS), inoculum size of the isolate, incubation period, incubation temperature, initial pH, effect of different nitrogen sources, and different NaCl concentrations. In each case, different treatments were done in triplicates and expressed as means ± standard deviation (SD). Usually, before inoculation of the previous described medium of mineral salts plus RS as the only carbon source (RS concentration 2 %), the isolate was activated by growth on CMC broth (2 %) for only 24 h and then 100 µl inoculums were used. If otherwise stated, usually the cultures were incubated for 3-4 days at 40 ^{o}C. Then after, all cultures were extracted with 5ml water and centrifuged at 140 rpm for 20 minutes and the clear supernatants were used directly as a crude enzyme to measure the activity of the enzyme quantitatively by the DNS method using Avicel only in this case as the enzyme reaction substrate. For inoculum size standard we prepared CMC broth (2 %) for 24 h (about 10 ^{7} Colony forming units per ml; CFU/ml) then we inoculated with 1ml (1000 ul) and 750 ul, 500 ul, 250 ul, & 125 ul corresponding to about (10 ^{7}, 75 × 10 ^{5}, 5 × 10 ^{6}, 25 × 10 ^{5}& 125 × 10 ^{4} CFU).

Enzyme production, concentration and partial purification

The isolate EG7 was grown under the determined optimum conditions. Then, the culture filtrate (about 100 ml) was collected by centrifugation at 5000 rpm for 5 min at 4 ^{o}C. The crude enzyme in the supernatant was then concentrated by ethyl alcohol precipitation. Ethyl alcohol (70 %) was added to the filtrate by percentage of 3:1 (v/v), then, allowed for precipitation for one hour and then centrifuged at 5000 rpm for 5 min at 4 ^{o}C. The precipitated enzyme was dissolved in 50 ml of sodium acetate buffer (0.2 M) at pH 5.5 and was dialyzed against the same buffer overnight at 4°C for partial purification. The enzyme was then concentrated into about 25 ml against sucrose and then refrigerated at 4°C.

Partial characterization of cellulase produced by the isolate EG7

Enzyme activity at different temperatures, salinities and pH degrees:

The obtained enzyme was tested to determine the best activity at different temperatures, salinities and pH degrees. Different treatments were done in triplicates then the enzyme activity was recorded as previously described using DNS method and Avicel as the reaction substrate.

Determination of molecular weight of cellulase of the isolate EG7

The molecular weight of cellulase obtained from the isolate under study was determined by using Sodium Dodecyl Sulphate–Poly Acrylamide Gel Electrophoresis (SDS-PAGE) technique according to (Laemmli, 1970).The approximate molecular weight for the enzyme was determined from the known molecular sizes of the standard proteins used. They were Phosphorylase B (97 KDa), Bovine Serum Albumin (67 KDa), Ovalbumin (45 KDa) and Carbonic Anhydrase (30 KDa).

Use of the isolate EG7 in composting

The isolate EG7 was used to compost Rice straw at very small laboratory scale together with shredded Banana peels waste. They mixed in ratio of 1:1 and kept without sterilization. The previously mentioned medium of isolation was used as stock solution after sterilization to saturate the mixture (100 g) during a period of two weeks. The incubation temperature was maintained at 40 ^{o}C limit with regular agitation of the mixture. Two sets were prepared and putted in perforated boxes, one as mixture alone and the other was inoculated with the isolate under investigation. The inoculum volume used was 5 ml of CMC broth (2 %) in which the isolate was allowed to grow for 24 h (about 10 ^{7} CFU per ml). Composting was followed up until maturity and dryness.

Statistical analysis

Statistically significant differences between means were tested by single factor analysis of variance by using (XLSTAT 2015) ANOVA software.

The differences between means were considered statistically significant when the test yielded a value P < 0.05. The data were expressed as means ± SD (n=3) where applicable.

Results and discussion

Isolation of bacterial producers for cellulase enzyme

From the collected samples, forty discrete bacterial colonies were isolated and purified. They all tested positively for cellulose hydrolysis by using Gram's iodine reagent.

Table 1. Growth of the bacterial isolates on CMC, Avicel, & Cotton seed linters with the recorded zone diameters after addition of Iodine solution.

Isolate number	CMC (mm)	Avicel (mm)	Cotton seed linters (mm)
S1	25.00	23.00	20.00
S2	25.00	22.00	22.00
S3	30.00	25.00	20.00
S4	27.00	26.00	20.00
S5	25.00	25.00	23.00
S6	29.00	27.00	22.00
EG7	35.00	45.00	41.00
S8	24.00	22.00	20.00
S9	21.00	20.00	17.00
S10	34.00	29.00	27.00
Rs11	25.00	25.00	23.00
Rs12	23.00	20.00	16.00
Rs13	20.00	17.00	15.00
Rs14	25.00	20.00	18.00
Rs15	30.00	23.00	20.00
C16	31.00	31.00	30.00
C17	35.00	35.00	33.00
C18	30.00	25.00	25.00
C19	27.00	25.00	23.00
C20	25.00	22.00	20.00

These isolates were twenty Gram positive, rod shaped and spore former bacteria (10 from soil, 5 from Rice straw, and 5 from compost). Besides them, another twenty isolates were obtained and included ten Gram positive, rod shaped and non-spore former bacteria (4 from soil, 4 from Rice straw, and 5 from compost) and ten Gram negative rod shaped bacteria (3 from soil, 2 from Rice straw, and 2 from compost). The most potent isolate EG7 (obtained from soil) mentioned below was belonged to the Gram positive, rod shaped and spore former bacteria.

Screening for the most potent cellulase producer

The forty isolates were screened for cellulase production more specifically using soluble and insoluble crystalline cellulosic substrates to help in final selection for the most potent isolate among them.

For such a purpose CMC – Avicel – Cotton seed linters were used as only carbon sources in the used medium. Data in (Table 1) show the recorded clear zone diameters after addition of iodine reagent. Moreover, (Fig.1) shows growth of the isolate EG7 (obtained from soil) on these substrates on agar plates flooded with Gram's iodine solution. The recorded zone diameters for this isolate were CMC 35 mm, Avicel 45 mm, and RS 41 mm.

Table 2. Growth of the bacterial isolates on CMC, Avicel, Cotton seed linters, and Rice straw liquid media & the recorded zone diameters of cellulase assay on CMC & Avicel**.**

Isolate No.	Clear zone diameters (mm) of the assay media							
	CMC				Avicel			
	Of the substrate				Of the substrate			
	CMC	Avicel	CSL	RS	CMC	Avicel	CSL	RS
S1	12.00	11.00	0.00	0.00	11.00	0.00	0.00	0.00
S2	12.00	11.00	11.00	11.00	11.00	0.00	0.00	0.00
S3	12.00	13.00	13.00	14.00	12.00	11.00	11.00	12.00
S4	13.00	13.00	0.00	0.00	12.00	12.00	0.00	0.00
S5	12.00	12.00	11.00	11.00	11.00	11.00	0.00	0.00
S6	14.00	13.00	11.00	11.00	13.00	12.00	0.00	0.00
EG7	15.00	20.00	19.00	17.00	18.00	24.00	24.00	23.00
S8	12.00	11.00	0.00	0.00	11.00	0.00	0.00	0.00
S9	11.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S10	15.00	14.00	13.00	14.00	13.00	12.00	11.00	13.00
Rs11	12.00	12.00	12.00	11.00	11.00	0.00	11.00	11.00
Rs12	11.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rs13	11.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rs14	12.00	11.00	0.00	0.00	11.00	0.00	0.00	0.00
Rs15	12.00	13.00	12.00	13.00	11.00	12.00	12.00	12.00
C16	13.00	13.00	12.00	12.00	12.00	13.00	11.00	12.00
C17	16.00	14.00	13.00	15.00	15.00	13.00	12.00	13.00
C18	12.00	13.00	13.00	12.00	11.00	12.00	12.00	11.00
C19	13.00	12.00	11.00	11.00	12.00	12.00	0.00	0.00
C20	12.00	11.00	11.00	0.00	11.00	0.00	0.00	0.00

Table 3. Determination of the cellulase activity of the most potent seven isolates after growth on RS on the two assay substrates CMC and Avicel**.**

Isolate No.	Enzyme activity U mL^{-1} on	
	Substrate CMC	Substrate Avicel
S3	1.70±0.02	1.20±0.01
EG7	19.00±0.06	22.50±0.07
S10	6.90±0.03	6.85±0.03
Rs15	3.00±0.02	2.80±0.02
C16	4.40±0.02	4.00±0.02
C17	8.50±0.03	8.60±0.04
C18	2.00±0.02	1.95±0.02

All gram negative and gram positive non-spore former rods recorded lowest activities on CMC while on crystalline cellulosic substrates recorded lowest activities or failed at all to utilize them so they all excluded. The rest of the isolates recorded positive results on crystalline cellulosic substrates Avicel and Cotton seed linters with well-formed clearance zones and hence were subcultured in liquid media containing all the previous used substrates (RS, CMC, Avicel, and Cotton seed linters) separately at the same concentration (2%) and incubated for 3-4 days at 40 ^{o}C.

After the incubation was terminated all broth cultures were centrifuged and the supernatants were tested by agar well method for the cellulase enzyme activity in

two assay media, containing CMC or Avicel only, by measuring the clear zone diameter after incubating the plates for at least 6 hours and addition of Iodine solution.

Table 4. Genus identification tests of the isolate EG7.

Tests for genus characteristics	Test result
Cell shape	Normal rod shaped
Endospores produced	Central ellipsoidal
Motility	Positive
Gram's stain reaction	Positive
Aerobic or Facultative anaerobic	Positive
Strict anaerobic	Negative
H_2S production	Negative
Catalase	Positive
Acid from glucose	Positive
Nitrate reduction	Positive

Table 5. Species identification tests of the isolate EG7.

Tests for species characteristics	Test result
Voges-Proskauer test	Positive
Gas from glucose	Negative
Hydrolysis of:	
Casein	Positive
Gelatin	Positive
Starch	Positive
Utilization of citrate	Positive
Degradation of tyrosine	Negative
Egg-yolk lecithinase	Negative
Growth at pH 6.8 & 5.7	Positive
Growth in NaCl 2, 5 & 7 %	Positive
Growth at 5 & 55 ^{o}C	Negative
Growth at 30, 40 & 50 ^{o}C	Positive

Data in (Table 2) show the result of the previous test and the most potent seven isolates which showed an appropriate cellulase activity on both substrates used in the assay media CMC and Avicel were selected.

These isolates were S3, EG7, S10, Rs15, C16, C17, and C18. Apparently, the isolate EG7 recorded the highest activity reached to about 24 mm of clearance zone diameter on Avicel assay medium after growth on crystalline cellulosic substrates, Avicel and Cotton seed linters, while, the zone diameter recorded after growth on RS was 23 mm on the same assay substrate (Avicel). Data in (Table 3) and (Fig.2) show the final selectionof the most potent isolate among the selected seven isolates possessing both qualitative and quantitative action based on the enzyme activity. The used growth substrate was RS only of concentration (2 %) and the assay was carried out on the two previous substrates CMC & Avicel after incubation for 3-4 days at 40 ^{o}C.

Table 6. Metabolic fingerprint obtained from Biolog MicroPlate of the isolate EG7.

1		2	3	4	5	6	7	8	9	10	11	12
Test substrate	Negative Control	Dextrin	D-Maltose	D-Trehalose	DCellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive Control	pH 6	pH 5
Test result	_	+	+	+	+	+	+	+	+	+	+	+
Test substrate	D-Salicin	D-Raffinose	α-D-Lactose	D-Melibiose	β-Methyl-D-Glucoside	N-Acetyl-D-Glucosamine	N-Acetyl-α-D-	N-Acetyl-D-Galactosamine	N-Acetyl Neuraminic	1% NaCl	4% NaCl	8% NaCl
Test result	+	-	+	+	+	+	+	-	-	+	+	+
Test substrate	α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	3-Methyl Glucose	D-Fucose	L-Fucose	L-Rhamnose	Inosine	1% Sodium Lactate	Fusidic Acid	D-Serine
Test result	+	+	+	+	-	+	+	+	-	+	-	-
Test substrate	D-Sorbitol	D-Mannitol	D-Arabitol	myo-Inositol	Glycerol	D-Glucose-6-PO4	D-Fructose-	D-Aspartic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline
Test result	+	+	-	+	+	+	+	+	-	-	-	-
Test substrate	Gelatin	Glycyl-L-Proline	L-Alanine	L-Arginine	L-Aspartic Acid	L-Glutamic Acid	L-Histidine	L-Pyroglutamic Acid	L-Serine	Lincomycin	Guanidine HCl	Niaproof 4
Test result	+	+	+	+	+	+	+	-	+	-	-	-
Test substrate	Pectin	D-Galacturonic Acid	L-Galactonic Acid Lactone	D-Gluconic Acid	D-Glucuronic Acid	Glucuronamide	Mucic Acid	Quinic Acid	D-Saccharic Acid	Vancomycin	Tetrazolium Violet	Tetrazolium Blue
Test result	+	+	-	+	+	+	+	-	+	-	-	-
Test substrate	p-Hydroxy-Phenylacetic Acid	Methyl Pyruvate	D-Lactic Acid Methyl	L-Lactic Acid	Citric Acid	α-Keto-Glutaric Acid	D-Malic Acid	L-Malic Acid	Bromo-Succinic Acid	Nalidixic Acid	Lithium Chloride	Potassium Tellurite
Test result	-	+	-	+	+	-	-	+	+	-	+	+
Test substrate	Tween 40	γ-Amino-Butryric Acid	α-Hydroxy-Butyric Acid	β-Hydroxy-D,L-Butyric Acid	α-Keto-Butyric Acid	Acetoacetic Acid	Propionic Acid	Acetic Acid	Formic Acid	Aztreonam	Sodium Butyrate	Sodium Bromate
Test result	+	-	-	-	-	+	-	+	+	-	+	-

(+): Positive reaction, (-): Negative reaction.

From these data it can be concluded definitely that the isolate EG7 is the most potent isolate among the tested seven isolates where it recorded the highest enzyme activity on both substrates CMC and Avicel which were 19.00 and 22.50 U mL^{-1}, respectively. So, this isolate had utilized soluble and insoluble substrates both *In-vivo* (growing on substrate) and *In-vitro* (extracted crude enzyme).

Several similar studies have been conducted by others. Studies of (Lokhande and Musaddiq, 2015) and (Kulkarni and Vedamurthy, 2015) are examples for such studies.

Identification of the isolate EG7

Data in (Tables 4 and 5) showed the results of the identification tests for genus and species characteristics which carried out on the isolate EG7. This isolate was suggested to be very likely belong to the genus *Bacillus* and more likely to one of the species of *Bacillus subtilis* group species based on these characters.

Biolog identification

Data in (Table 6) showed the results of Biolog MicroPlate identification system used for confirmation of identification of the isolate EG7. The readable data were used for the purpose of identification by matching with the databases using Biolog software.

Table 7. Effect ofdifferent carbon substrates, substrate concentration and inoculum size on cellulase production by the isolate EG7.

Substrate	Enzyme activity U mL^{-1}	Substrate concentration (%)	Enzyme activity U mL^{-1}	Number of CFU	Enzyme activity U mL^{-1}
RS (2%)	19.50±0.05	2	11.00±0.03	10^7	36.50±0.06
RS (1%) + Cellobiose (1%)	11.00±0.04	4	14.00±0.03	75×10^5	39.00±0.08
RS (1%) + Glucose (1%)	0.00	6	19.50±0.04	5×10^6	42.00±0.08
Cellobiose (1%) + Glucose (1%)	0.00	8	22.50±0.05	25×10^5	33.60±0.04
Cellobiose (2%)	5.50±0.02	10	28.00±0.06	125×10^4	22.50±0.02

Table 8. Effect of incubation period and different nitrogen sources on cellulase production by the isolate EG7.

Incubation period (day)	Enzyme activity U mL^{-1}	Nitrogen source	Enzyme activity U mL^{-1}
1	25.00±0.03	$(NH_4)_2SO_4$	25.00±0.01
2	31.00±0.04	$NaNO_3$	31.00±0.03
3	39.00±0.05	KNO_3	42.00±0.06
4	36.50±0.05	Urea	22.50±0.04
5	33.60±0.03	Yeast extract	11.00±0.02

The results obtained from Biolog plate for this isolate revealed that the isolate EG7 was identified as *Bacillus subtilis* subsp. *subtilis* as a first match, or, *Bacillus subtilis* subsp. *spizizenii* as a second. Usually, these two subspecies are undistinguishable phenotypically, and hence, the isolate was identified and given the name *Bacillus subtilis* EG7.

Table 9. Effect of incubation temperature, initial pH and different salinity on cellulase production by the isolate EG7.

Incubation temperature (°C)	Enzyme activity U mL^{-1}	Initial pH	Enzyme activity U mL^{-1}	NaCl conc. %	Enzyme activity U mL^{-1}
20	11.00±0.02	4	0.00	0.50	36.50±0.07
25	17.00±0.02	5	1.00±0.01	1.00	39.00±0.08
30	19.50±0.03	6	22.50±0.02	2.00	42.00±0.08
35	31.00±0.03	7	42.00±0.04	4.00	33.60±0.05
40	45.00±0.04	8	36.50±0.04	6.00	31.00±0.04
45	39.00±0.04	9	5.50±0.06	8.00	25.00±0.03
50	25.00±0.03	10	0.00		

Table 10. The optimal conditions for cellulase production from RS by the isolate EG7.

Character	Character state
Substrate concentration (%)	10.00
Inoculum size (μl)	500.00 (about 5×10^6 CFU)
Incubation period (day)	3.00
Incubation temperature (°C)	40.00
Initial pH	7.00
Nitrogen source (0.2 %)	KNO_3
NaCl conc. (%)	2.00

Molecular identification of 16S rDNA gene of the isolate EG7

PCR of the 16S rDNA gene produced the expected fragment size of approximately 1500 (bp). The partial nucleotide sequence of isolate EG7 16S rDNA gene (403 nucleotides) was compared with similar sequences retrieved from DNA databases by using the NCBI n-BLAST search program in the National Center for Biotechnology Information (NCBI) and was multiple-aligned at the same partial sequences of seven reported *Bacillus* sequences in GenBank using ClustalW program. A phylogenetic tree was generated using the Neighbour-Joining method and bootstrap analysis of 1000 replicates (Fig.3). It was revealed that the bacterium belonged to the genus *Bacillus* and was closely clustered together with *Bacillussubtilis*. The amplified 16S rDNA gene sequence of isolate EG7 was most closely related to that of *B. subtilis* I125 (GenBank accession number, KF318820) and showed 99 % identity with it. On the other hand, it was showed 99 % identity with *B. subtilis* E8 (GenBank accession number, HF585264).

Table 11. Purification steps of cellulase produced by the isolate EG7.

Purification step	Enzyme activity U mL^{-1}	Protein concentration mg mL^{-1}	Specific activity U mg^{-1} of protein	Purification fold
Crude Enzyme	45.00	93.75	0.48	1
Ethyl alcoholPpt.	55.00	91.66	0.60	1.25
Dialysis	64.50	93.48	0.69	1.44

Table 12. Enzyme activity at different temperatures, pH and salinity.

Incubation temperature (°C)	Enzyme activity U mL^{-1}	Initial pH	Enzyme activity U mL^{-1}	NaCl conc. %	Enzyme activity U mL^{-1}
20	17.00±0.03	5	5.50±0.01	0.50	67.00±0.06
30	36.50±0.05	6	63.00±0.04	1.00	71.00±0.06
40	82.00±0.08	7	79.00±0.08	2.00	75.50±0.07
50	42.00±0.06	8	71.00±0.06	4.00	47.50±0.04
60	0.00	9	14.00±0.03	6.00	42.00±0.05

The sequence has been deposited in GenBank with accession number (KX817281). On the basis of the results of analysis of 16S rDNA gene, it concluded that the isolate is a strain of *B. subtilis* and termed *B. subtilis* EG7.

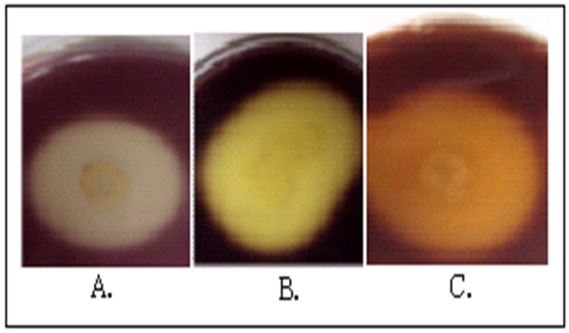

Fig. 1. Growth of the isolate EG7 on; A. CMC (about 35 mm zone diameter), B. Avicel (about 45 mm zone diameter) & C. Rice straw (about 41 mm zone diameter) agar plates flooded with Gram's iodine solution.

Usually *Bacillus subtilis* was isolated from agricultural fields as a common cellulolytic bacterium (Pokhrel *et al.*, 2014). Furthermore, (Khianngam *et al.*, 2014) have previously isolated ten cellulase producing isolates, all of them were identified as *Bacillus* except two isolates based on 16s rRNA gene analysis.

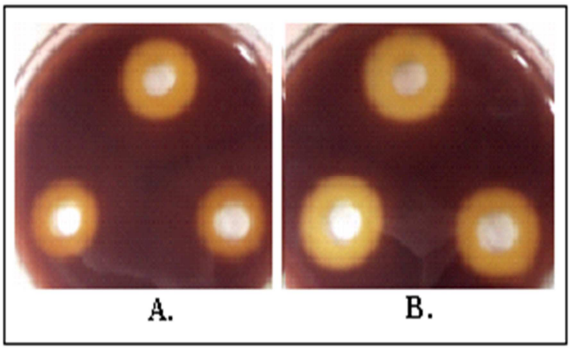

Fig. 2. Cellulase assays of the isolate EG7 on; A. CMC (about 17 mm zone diameter) & B. Avicel (about 23 mm zone diameter) agar plates flooded with Gram's iodine solution after growth on Rice straw (2 %).

Optimization for cellulase production by the isolate EG7

For optimization, the tested factors included the potential of enzyme induction by this isolate using different carbon substrates, concentration of the used substrate RS, inoculum size of the isolate, incubation period, incubation temperature, initial pH, effect of different nitrogen sources, and different NaCl concentrations.

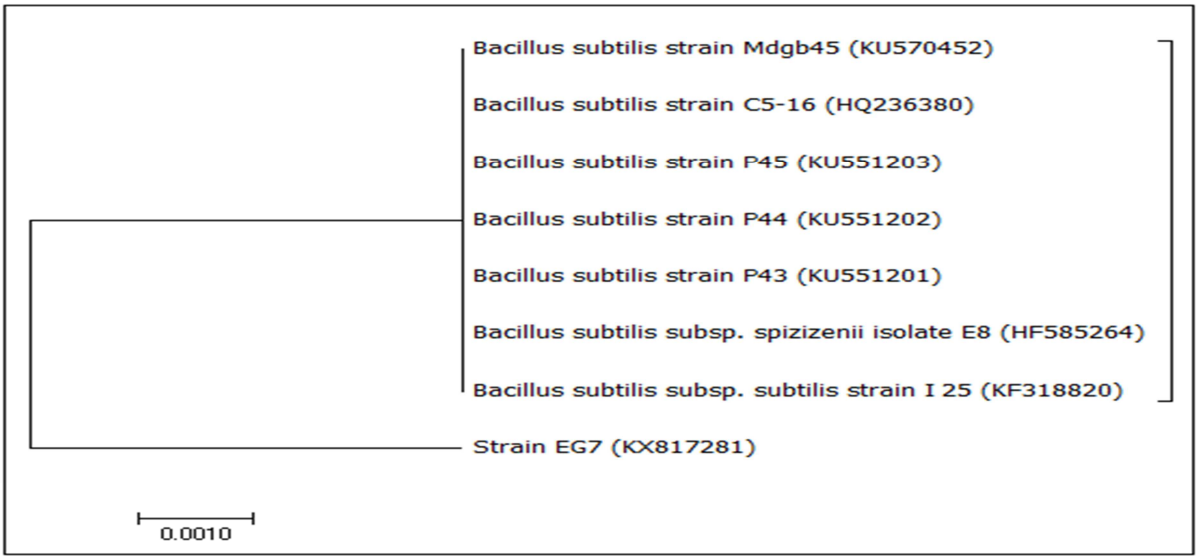

Fig. 3. Neighbor-Joining tree of 16SrDNA gene of the isolate under study; strain EG7 (accession no. KX817281) and nearest *Bacillus* sequences published in GenBank. Numbers represent bootstrap percentage values based on 1000 replicates.

First, the potential of enzyme induction by this isolate using different carbon substrates was conducted. Data in (Table 7) revealed that the cellulase enzyme produced by the isolate EG7 was highly induced by the presence of the used substrate RS. The highest cellulase activity for this isolate was recorded when grown on RS alone without any other substrates (19.50U/mL). Glucose was very inhibitory to the production of the enzyme by this isolate, since no activity was detected in case of using RS and glucose, or cellobiose and glucose, as growth substrates. Also, cellobiose was seemed to stimulate the production of the enzyme. In addition, (Table 7) shows the effect of substrate concentration on enzyme production by the isolate EG7. Data in (Table 7) revealed that the cellulase enzyme produced was generally increased with increasing substrate concentration. The highest cellulase activity recorded in this case was 28.00 U/mL. Also, (Table 7) shows the effect of inoculum size on enzyme production by the isolate EG7. Data in (Table 7) revealed that the cellulase enzyme produced was optimal with using modest size of inoculum of this isolate to inoculate RS fermentation medium. The highest cellulase enzyme activity was recorded for this isolate in this case reached to about 42.00 U/mL when using inoculum size of 500 µl corresponding to about 5×10^6 CFU.

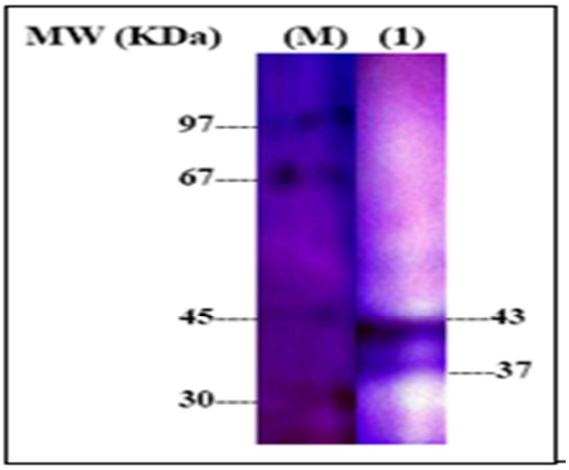

Fig. 4. SDS-PAGE of Cellulase produced by the isolate EG7 (1) & the protein marker (M); Phosphorylase B (97 KDa), Bovine Serum Albumin (67 KDa), Ovalbumin (45 KDa) & Carbonic Anhydrase (30 KDa).

The effect of incubation period and different nitrogen sources on enzyme production by the isolate EG7 was presented in (Table 8). Data in (Table 8) revealed that the cellulase enzyme produced by the isolate was gradually decreased with increasing the incubation period over three days. Within the third day of incubation the cellulase enzyme production by this isolate was optimal. The highest cellulase enzyme activity was recorded in this case reached to about 39.00 U/mL. Data in (Table 8) also revealed that the cellulase enzyme produced by the isolate was moderately affected by the nitrogen source used. Generally, the inorganic nitrogen sources used were more favorable by the isolate under study, while, organic nitrogen sources were less productive. However, the enzyme production was best with using inorganic nitrogen sources; the most productive source used was KNO_3. The highest cellulase enzyme activity was recorded in this case reached to about 42.00 U/mL.

Fig. 5. Sample of the mature compost after 5 days inoculated with the isolate EG7.

Additionally, (Table 9) shows the effect of incubation temperature, initial pH and different salinity on enzyme production by the isolate EG7. Data in (Table 9) revealed that the cellulase enzyme produced by the isolate was affected by incubation temperature. Generally, with increasing or decreasing the incubation temperature above or below 40 ^{o}C, the activity of the enzyme was decreased gradually. The highest cellulase enzyme activity was recorded for this isolate in this case reached to about 45.00 U/mL. Data in (Table 9) also revealed that the cellulase enzyme produced by the isolate was affected by pH degree, with increasing or decreasing the pH above or below 6-8, the activity of the enzyme was drastically decreased. However, the enzyme production withstands the slightly alkaline conditions, the activity was best near neutrality.

The highest cellulase enzyme activity was recorded in this case reached to about 42.00 U/mL. Lastly, data (Table 9) revealed that the cellulase enzyme produced was less affected by NaCl concentration used. Generally, the higher concentrations used were less favorable, since, less enzyme production was recorded. However, the enzyme production was best with modest NaCl concentrations; the most prolific concentration used was 2 %. The highest cellulase enzyme activity was recorded in this case too reached to 42.00 U/mL.

In our results we found that in all experiments the value of $P > 0.05$, statistically not significant differences between means, except in case of substrate concentration used where significant difference between means was recorded, a value of $P < 0.05$. Finally, (Table 10) show all concluding remarks obtained about near optimal conditions for cellulase production by the isolate EG7.

The results obtained above were in agreement with those obtained by (Lugani *et al.*, 2015) and (Nandimath *et al.*, 2016). Concerning the substrate concentration, it is usually a limiting factor in enzyme production studies and the increasing activity may be attributable to the tendency of bacteria like bacilli to increasing amounts of substrate till certain limits.

Production, extraction and purification of cellulase of the isolate EG7

The isolate EG7 was grown under the determined optimum conditions. Then, the culture filtrate was subjected to the previously mentioned extraction and purification steps. Data in (Table 11) obviously revealed that these steps have increased the enzyme activity to about 1.44 % fold. The highest activity of the partially purified cellulase was recorded 64.50 U/mL.

Usually, production optimization studies are conducted when dealing with enzymes. Previously, (Venkateswar Reddy *et al.*, 2016) has conducted similar studies and recorded similar results.

Partial characterization of cellulase produced by the isolate EG7

Enzyme activity at different temperatures, pH and salinity

The produced cellulase by the isolate under study was subjected to test its activity at different degrees of temperatures, pH and salinity. Data in (Table 12) confirmed the previous determined optimum conditions for production for the three factors. This was clearly stated since highest activities recorded for the partially purified cellulase were 82, 79, and 75 U/mL for the three factors, respectively.

In this case we found also that the value of $P > 0.05$, statistically not significant differences between means, except in case of pH degrees tested where significant difference between means was recorded while a value of P was < 0.05.

The reason in this case may be that the enzyme kinetics may differ between *In-vivo* and *In-vitro*.

These findings were in agreement with the previously reported by (Abdullah *et al.*, 2016) and (Khatiwada *et al.*, 2016).

Molecular weight determination of cellulase of the isolate EG7

The molecular weight of cellulase obtained from the isolate under study was determined by using SDS-PAGE technique. Near molecular weight for the enzyme was determined from the known protein marker. Results in (Fig.4) showed that the cellulase enzyme had separated into two distinct bands of approximate molecular weights of 43 and 37 KDa. Other studies conducted by (Kulkarni and Vedamurthy, 2015) have recorded one band only has a molecular weight of 35 KDa.

Use of the isolate EG7 in composting

The use of the isolate EG7 in composting of Rice straw and Banana peels waste resulted in reduction of the composting time into five to seven days, instead of ten to thirteen days without it, until maturity and final stabilization. Finally, (Fig.5) displays a sample of the mature compost obtained after five days inoculated with the isolate EG7. Other studies concerned with applications of bacterial cellulose decomposers as potential inoculants for process acceleration (Chandna *et al.*, 2013), and their enzymes in different industrial processes (Irfan *et al.*, 2017) have been previously conducted.

Conclusion

The results obtained from this study addressed the potential of using bacterial cellulase producers like the isolate EG7 in enhancing composting of agricultural wastes such as Rice straw and Banana peels wastes. Also, the produced enzyme may have different industrial applications.

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