



Taxonomic characterization of the chitinolytic actinomycete *Cellulomonas chitinilytica* strain HwAC11

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Abstract

Chitinases apply in several useful fields such as agriculture, food industries and environmental applications. Because it helps degradation of fungal cell walls containing chitin and thus accelerates protoplast formation. The alkaliphilic action-bacterial strain HwAC11 was isolated from compost after examine 99 different samples. This isolate exhibited good growth on medium containing chitin as sole carbon source. Macro- and micro-morphological characteristics, enzyme activities, physiological and biochemical properties of the isolate were investigated. It was concluded that the strain HwAC11 is a member of the genus *Cellulomonas*. The results were compared with the taxonomic characteristics of *Cellulomonas* members and it was found to be similar to those of *Cellulomonas chitinilytica*. The phylogenetic analysis based on 16s ribosomal RNA gene sequence confirmed the phenotypic results and the sequences were deposited in gene bank under *Cellulomonas chitinilytica* strain HwAC11 with accession number MH050787. The strain HwAC11 displayed intensive chitinase activity under alkaline conditions. It leads to apply this strain in agriculture field, especially as biological control agent for pathogenic fungi and harmful nematodes.

Keywords: Alkaliphilic action-bacteria, Taxonomy, 16S rDNA, *Cellulomonas chitinilytica*, Chitinase.

Introduction

Enzymes hydrolyze Polysaccharides as chitinases, cellulases and pectinases are widespread in nature. They can be found in all living forms, including animals, plants, algae, fungi and bacteria (Yoon *et al.*, 2008). To produce polysaccharides, micro-organisms are usually the most convenient sources and can be obtained from various natural environments Chitin, the insoluble linear -1,4-linked *N*-acetylglucosamine, is the most abundant polysaccharide in nature after cellulose, with production of 100 billion tons annually (Yu *et al.*, 1993).

Chitinases are glycoside hydrolases that degrade chitin by hydrolyzing the -1,4-glycosidic linkages. Chitinase enzyme activity has ability to degrade the cell wall of pathogens containing chitin such as insect exoskeleton, nematodes and fungi cell wall. Therefore, controlling of insects and pathogenic fungi by chitinases are powerful biological tools. In addition, the pathogens fail to develop resistance as enzymes are not persistent against them. Many studies have reported that chitinolytic bacteria inhibit the mycelial

extension of several pathogenic fungi (Chernin *et al.*, 1995; Prasanna *et al.*, 2013). Consequently, bacterial chitinases have a critical role in the breakdown of chitin in cell wall of fungi, and chitinase producing bacteria could be widely applied as ecofriendly agents against agricultural phytopathogens (Rahman *et al.*, 2016; Kamensky *et al.*, 2003; Kurzeet *et al.*, 2001). Genus *Cellulomonas* is a member of the family *cellulomonadaceae* in the phylum action-bacteria. Species of genus *Cellulomonas* have typical characteristics in that are Gram positive rods with cellulolytic activity (Stackebrandt *et al.*, 2014). Also, few members are known to have chitinolytic activity as *Cellulomonas chitinilytica*. For that, this study was concerned with isolation of alkali chitinolytic action-bacteria from Egyptian environment and taxonomic characterization of the most potent isolate depending on its enzyme activity.

Materials and Methods

Isolation and culture conditions of chitinolytic bacteria

A number of 99 samples of soil, composts and sediments were collected during 2016 from different environment sites in Egypt (Baharia Oasis, Alexandria, Alfayoum, and Wadi Alnatroun). The samples were aseptically collected in clean plastic bags at depth 15-20 cm. The collected samples were sieved to remove various contaminant materials. Then it was air-dried and mixed with CaCO₃ (1g/ 100g soil) for 24 hours at 28° C before plating to increase the numbers of action-bacteria (Tsao *et al.*, 1960). Isolation of chitinolytic action-bacteria was performed by direct inoculation of 1 g of each sample to basal agar medium (w/v, 0.5% (NH₄)₂SO₄, 0.085% KH₂PO₄, 0.015% K₂HPO₄, 0.05% MgSO₄, 0.01% NaCl, 0.01% CaCl₂, pH 8.5, 1.5% agar) containing 0.2% (w/v) colloidal chitin and incubated at 30 °C for 7 days. Selected colonies (rough, chalky) of action-bacteria were transferred onto respective agar plates and incubated for 7 days. Plates containing pure cultures were stored until further examinations.

Chitinase activity assay

Isolated chitinolytic action-bacteria were grown on basal medium containing 1% colloidal chitin for 7 days (30 °C, 150 rpm). Cells were separated by centrifugation (4000 rpm, 10 min, 4 °C) and the supernatant was assayed. The chitinase activity assay was conducted in a reaction containing one milliliter

of the extract and 1 ml of 10% (w/v) suspension of colloidal chitin, in 0.2 M potassium phosphate buffer (pH 8.5), and incubated at 30 °C for 1 h. The reaction was stopped by adding 1 ml of 1% NaOH, followed by boiling for 5 min. Tubes were then centrifuged at 7000 rpm and the reducing sugars produced were determined in the supernatants. One milliliter of supernatant and 1 ml of 1% DNS (dissolved in 30% sodium potassium tartrate in 2 M NaOH) were mixed and incubated for 5 min in a boiling water bath. Afterward, their absorbance at 535 nm was recorded. Readings were interpolated in a standard curve prepared with a series of dilutions (0– 10 µmol/ml) of N-acetyl-D-glucosamine (NAG) and DNS. The chitinase activity was defined as the amount of enzyme required to produce one µmol of NAG in 1 h (Rojas Avelizapa *et al.*, 1999). The action-bacterial isolate showing the highest chitinase activity was selected to investigate its characterizations.

Morphological characteristics

The micro-morphological and cultural properties of the most active isolate were investigated according to the international Streptomyces project (ISP) after 24, 48, 72 h and one week of cultivation. The observations and records of colors were described as in the ISP methods (Shirling & Gottlieb, 1966). The ISCC-NBS color name charts illustrated with centroid color (Kenneth and Deane, 1955) was used for this purpose. The morphology of the spore chains and sporophores was studied by direct light microscopy observation. The morphology of the spores was examined by scanning electron microscopy (Tresner *et al.*, 1961).

Physiological and biochemical properties

Growth of action-bacterial isolate HwAC11 was assessed in nutrient agar (NA) (Shirling & Gottlieb, 1966) (g/l) (Beef extract, 3.0; peptone, 5.0; sodium chloride, 5.0; Agar, 20.0; Distilled water 1000 ml) and starch-nitrate agar medium (SNA) (Tadashi, 1975) (g/l) (Soluble starch, 20.0; NaNO₃, 2.0; K₂HPO₄, 1.0; KCl, 0.5; MgSO₄.7H₂O, 0.5; CaCO₃.2H₂O, 2.0; Agar, 20.0; Distilled water up to 1000 ml) at 10, 20, 30, 40, 50 °C and pH 4.0–10.0 (at 1.0 pH unit intervals) adjusted by using HCl and NaOH (Gomori, 1955). The tolerance to NaCl was tested on starch-nitrate agar medium containing different concentrations of NaCl from 0 to 3.5 % (w/v).

Utilization of different substrates for growth

The utilization of various carbon and nitrogen sources was tested on basal mineral salts agar (ISP-9) (Shirling and Gottlieb, 1966) supplemented with different organic substrates (1 %, w/v).

Enzyme activity

Hydrolytic enzymes activity was investigated using basal mineral salts agar medium (ISP-9) supplemented with 1% (w/v) starch (Kurup, *et al.*, 1975), casein (Gordon & Smith, 1955), carboxy-methyl cellulose (CMC) (Teather & Wood, 1982), pectin (Venkata *et al.*, 2013) and tributyrin (Heravi *et al.*, 2008) for amylase, protease, cellulase, pectinase and lipase enzymes assay, respectively. Other physiological and biochemical studies were conducted according to El-Sherbiny *et al.* (2017)

Phylogenetic analysis

Genomic DNA extraction was performed by lysozyme-sodium dodecyl sulfate method of Kheiralla *et al.* (2016) as described in Sambrook and Russell (2001). DNA purity and concentration were determined by spectrophotometric analysis according to Barbas *et al.* (2007). Nucleic acids were estimated and analyzed by Agarose gel electrophoresis technique using 1-2% agarose gel (Sambrook and Russell, 2001). DNA was examined and visualized under UV trans-illuminator (Bio-Doc Analyzer, Biometra, Germany). Two sets of primers were used to amplify regions specific for almost all eubacterial 16S sequences. A region of approximately 1300bp from the 16S rRNA gene was amplified using primers F: (5' d AGAGTTTGATCCTGGCTCAG 3') and R: (5' d TACGGTTACCTTGTTACGACTT 3') (Barakat *et al.*, 2017). PCR products electrophoresed on 1% agarose gel and visualized under UV light as described previously in agarose gel electrophoresis technique.

In-silico DNA sequence analyses were carried out by Sanger sequencing technology on applied bio-systems automated DNA sequencer, model ABI 3730XL DNA Analyzer (Applied Bio-systems, USA; service provided by Macrogen Inc., South Korea). The sequence analyses and alignments were performed by NCBI-BLAST programs of the National Center for Biotechnology Information (Altschul *et al.*, 1990; Stultz *et al.*, 1993; Hobohm and Sander, 1995 and Altschul *et al.*, 1997). Multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREEVIEW program (Page, 1996).

Results

Isolation of chitinolytic action-bacteria

A total of 99 samples were collected, and the variety of samples types and ecology were taken in account to increase the diversity of the isolated microorganisms. After inoculation and incubation, 8 different isolates were selected as they exhibited good growth on chitin agar media where isolates with moderate and low growth were neglected. These isolates were re-streaked onto the same medium until pure cultures obtained and then slanted for future processes.

Selection of most potent chitinolytic isolate

The obtained isolates were cultivated into basal broth medium containing 1% colloidal chitin. After incubation, chitinase activity was investigated in the cell-free extract and results showed that isolate from compost sample at Alexandria exhibited the highest activity as shown in Fig. (1). This isolate was sympoled as HwAC11 and selected to be taxonomically described.

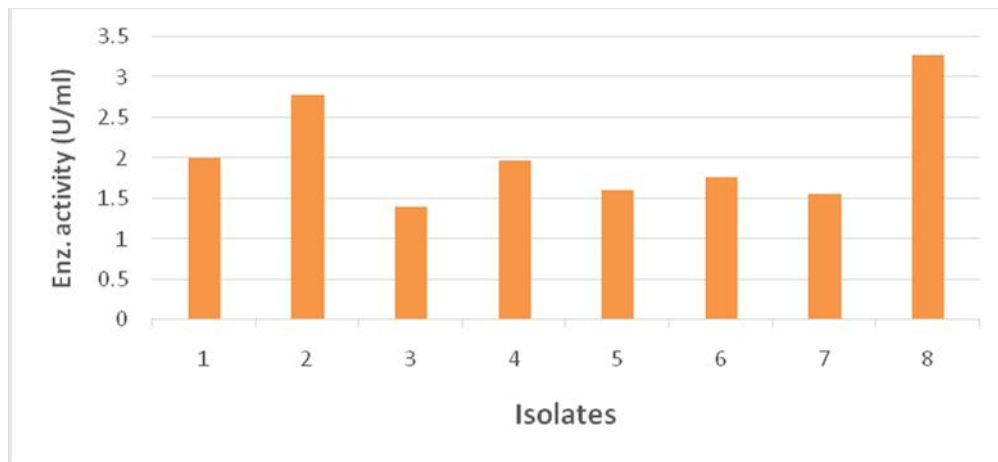


Fig 1. Chitinase activity of the selected isolates showed that isolate 8 (HwAC11) exhibited the highest activity

Description of isolate HwAC11

The isolate HwAC11 is a gram positive action-bacteria, produced substrate and aerial mycelia on SNA but no mycelium was observed when cultivated on NA or R2A media. Distinctive, circular smooth-surfaced yellow colonies with entire margins were

produced on nutrient and R2A agars at 30°C as shown in Fig. (2). Spore chains of non-motile rod-shaped spores were observed after 72h at 30°C as shown in Fig. (3). By scanning electron microscopy, the spores appeared to be rod shaped (Fig.4).



Fig.2. Yellow colonies produced on R2A medium after 72h at 30°C by isolate HwAC11

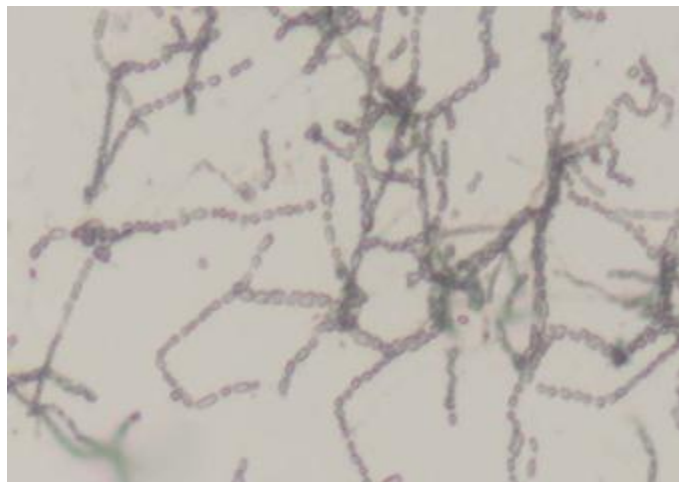


Fig. 3. Non-motile rod-shaped spores produced on SNA medium after 72h at 30°C by isolate HwAC11(400x)

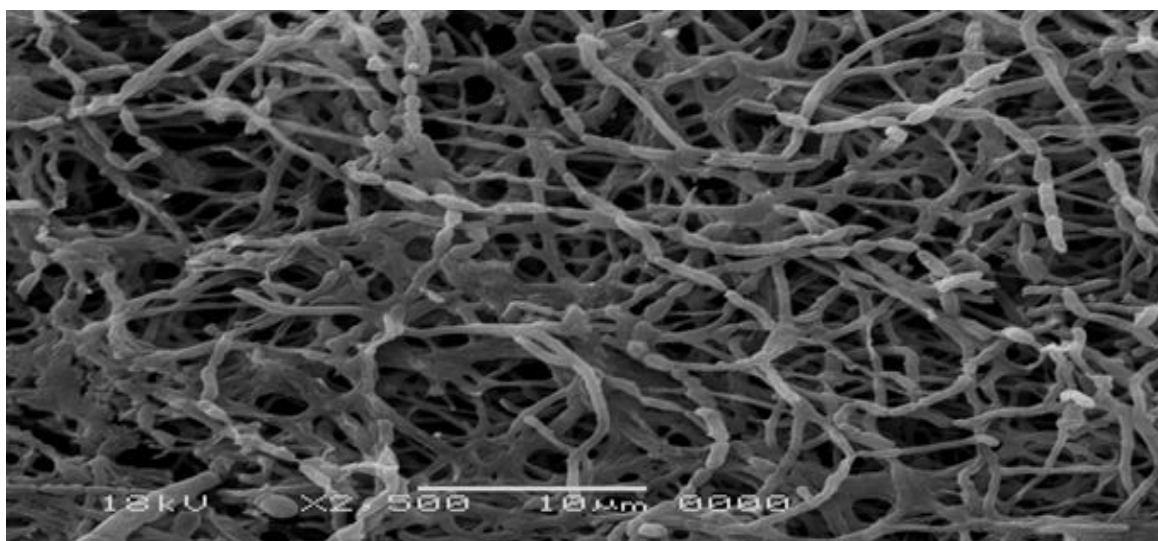


Fig. 4. Scanning Electron Microscopy image of isolate HwAC11 after 1 week of cultivation

Physiological and biochemical properties

The isolate HwAC11 was grown optimally at 30°C, pH 8.0 and without NaCl. The growth occurred between 10–40 °C, at pH 7.0–9.0 and 0–3% (w/v) NaCl (Table 1). This isolate was utilized the following substrates for the growth: D-glucose, D-fructose, D-galactose, sucrose, L-arabinose, D-mannose, D-xylose, maltose, D-lactose, cellobiose, pyruvate, glycerol, D-sorbitol, D-mannitol, L-cysteine and L-glutamate. In contrast, L-rhamnose, inositol, inulin,

lactate, L-alanine, L-arginine, L-asparagine, L-glutamine, L-histidine, L-lysine, L-proline, L-serine, L-methionine, L-valine, L-tryptophan and L-tyrosine were not utilized for growth (Table 1). For hydrolytic activity, HwAC11 isolate could hydrolyze chitin, CM-cellulose and starch but not casein and tributyrin (Table 1). Biochemical investigation indicated that HwAC11 could reduce nitrate to nitrite and exhibit catalase activity but no oxidase activity (Table 1).

Table 1. Summarization of isolate HwAC11 general characteristics

Morphology	Rods	Substrate utilization	
Motility	-	D-glucose	+
Mycelium on R2A and NA media	-	D-fructose	+
Color of colony on R2A and NA media	Yellow	D - galactose	+
		Inositol	-
		Inulin	-
		D-mannose	+
		glycerol	+
		D-mannitol	+
		L-serine	-
		L-tyrosine	-
Enzyme activity		Sucrose	+
Amylase	+	L-arabinose	+
Cellulase	+	L-alanine	-
Chitinase	+	L-arginine	-
Protease	-	asparagine	-
Lipase	-	L-glutamine	-
Cellulase	+	L-histidine	-
Nitrate reduction	+	L-lysine	-
Catalase	+	L-proline	-
Oxidase	-	L-cysteine	+
		L-glutamate	+

Phylogenetic identification

Total genomic DNA of *Cellulomonas* isolate HwAC11 was extracted and purified. A 16S rRNA gene of obtained genome was amplified using specific primers. The 16S rRNA gene fragment was sequenced resulting a sequence of 425 bps. The Blast program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to assess the DNA similarities. The search in NCBI resulting in that isolate HwAC11 was belonging to

genus *Cellulomonas*. Depending on phylogenetic and conventional identification data, isolate HwAC11 was closely related to *Cellulomonas chitinilytica* strain Xbu-b with identity of 86%. Subsequently, the partial sequence was deposited in GeneBank under *Cellulomonas chitinilytica* strain HwAC11 16S ribosomal RNA gene, partial sequence and recorded under accession number: MH050787. A phylogenetic tree was displayed using TREEVIEW program and represented in Fig. (5).

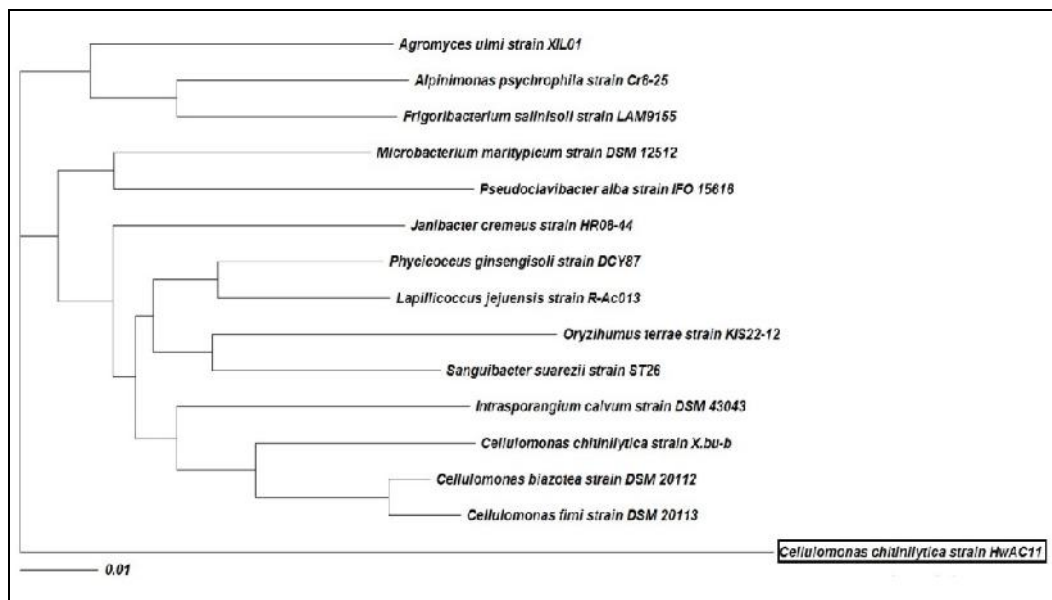


Fig. 5. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences of *Cellulomonas chitinilytica* strain HwAC11

Discussion

Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin. Chitinases have a wealth of applications, some of which have already been realized by industry. This includes bio-conversion of chitin to useful products such as fertilizer, the production of non-allergenic, non-toxic, biocompatible, and biodegradable materials and enhancement of insecticides and fungicides (Darwesh and Shalaby, 2016). The main objective of this study was to isolate chitinase producing actino-bacteria. For that, 99 different samples were collected from various environments related to harsh conditions (alkali, thermo and thermoalkali locations) in Egypt. Eight action-bacteria were isolated onto medium contained chitin as sole carbon source. The low numbers of isolates may be due to harsh conditions of sample locations as previously reported by El-Sherbiny *et al.* (2017). The isolate symbolized as HwAC11 was

selected as the most active chitinase producing action-bacterium. This isolate was subjected for characterization and identification and the result indicated that it was gram positive aerobic action-bacteria. The optimum growth conditions (30°C and pH 8) indicating that HwAC11 was Alkaliphilic (Wiegel, 1998). Depending on morphological and physiological characteristics, HwAC11 exhibit growth pattern similar to that of cellulomonadaceae that have high potential to degrade chitin and cellulose (Goodfellow & Jones, 2009). The micromorphology, cultural characteristics, physiology, and the biochemical properties data concluded that strain HwAC11 was representative of genus *Cellulomonas chitinilytica* according to Yoon *et al.*, (2008). In addition to the phenotypic characteristics, the phylogenetic data of HwAC11 confirmed its similarity to *Cellulomonas chitinilytica* strain X.bu-b with identity of 86% at the level of 16S ribosomal RNA gene.

Conclusion

It can be concluded the strain of *Cellulomonas chitinilytica* strain HwAC11 isolated from Egyptian compost sample was the most active strain in production of alkali chitinase enzyme. It can be used in many promising applications such as plant protection and fertilizer, as well as industrial applications.

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