CELLULASE PRODUCTION FROM *ACTINOMYCETES* ISOLATED FROM IRAQI SOILS: I CHARACTERIZATION OF A CELLULOLYTIC STREPTOMYCES SP. STRAIN AT7

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SUMMARY: A cellulolytic actinomycete species was isolated from Iraqi soil, described as a Streptomyces sp. Strain AT7. This strain is characterized by forming nonfragmented vegetative hyphae, spores were found on aerial mycelium in short compact chains (10-20 conidia) and whole cell hydrolysate contained L-diaminopimelic acid and the amino acids: glycine, leucine, and alanine. The strain, grew well on a mineral medium containing carboxymethylcellulose (CMC), produces C, enzyme at different temperatures; 28°, 37° and 48°C and has an acido-alkalophilic growth ability.

Key Words: Actinomycetes, cellulase production, growth conditions.

INTRODUCTION

Screening for cellulolytic microorganisms has been restricted for a long time mainly to molds, with emphasis on enzymes produced by the fungus *Trichoderma ressei*, a mesophile, grown aerobically on cellulose. Some thermoactinomycetes were found to be active cellulose decomposers and therefore useful for waste disposal and protein production (5,18). Extensive work has been done in view of obtaining new different cellulose degrading organisms from its natural habitats (1,13,17). In order to increase cellulase yields and their saccharification ability, several workers studied the nature of these enzymes and attempted to improve and optimize their cultural conditions (3,6,9).

The aim of this report is to study the characterization and identification of unknown actinomycetes isolate (AT7) isolated from Iraqi soil sample.

MATERIALS AND METHODS

Microorganisms: Srain AT7 originally isolated from a soil sample collected at Abou-Graib (near Baghdad) was grown and maintained on nutrient agar (difco Laboratories, Detroit, USA) for 2 weeks at 37°C. The slants were Kept at 4°C, a fresh subculture (10-14 days old) was used for all tests.

Cultural and Morphological Characteristics: The growth and colony characters (color of vegetative and aerial mycelium and soluble pigments) of AT7 grown on nutrient agar; yeast extract-malt extract agar, ISP no. 2 and inorganic salts-strach agar, ISP no. 4 (15) and inculated at 37°C were studied. Growth of the strain on nutrient agar at different pH value (4-11) adjusted before sterilization by either 1N NaOH or 1N HCL and various temperatures (28°, 37°, 48°, and 55°C were investigated. The shape of mycelium and spore chains of AT7 grown on ISP medium no.1 and 4 were observed by using light photomicroscope (BH2 olympus).

Analysis of whole cell hydrolysates: The isomers of diaminopimelic acids and sugar type were determined as described by methods of Kutzner (12) using diaminopimelic acids (a mixture of LL, DD and meso isomers, Sigma Chemical Co). as a standard. A mixture of amino acids (glycine, asparagine, cystein, leucine and alanine) and a mixture of sugars (glucose, galactose, mannose and xylose) were used to determine the type of amino acids and type of sugar respectively.

Biochemical Characteristics: The media and procedures used for biochemical characteristics were those described by Shirling and Gottlieb (15); Kurup *et al*, (11); Gordon *et al*, (7); Tresner *et al*, (19); Williams *et al*, (20) and Cowan and Steel's (4). The antimicrobial activity of 10 days old culture of AT7 grown on ISP no.2 at 37°C was tested using disk technique against some known Gram-positive and Gram-negative bacteria (listed in Table 2).

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Qualitative assays of cellulase production: Mineral-salt agar plate; containing 0.4% $(NH_4)_2SO_4$, 0.6% NaCl, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.01%, $CaCl_2$ (3) with 0.5% carboxymethylcellulose (CMC), both low and high viscosities (BDH Chemicals Ltd) and 1% agar (Difco) were surface innoculated in duplicates by a loop of spore suspension of strain AT7 grown on nutrient agar stant. The plates media were adjusted to various pH (range 4-10) before autoclaving and innoculated for 3 days at different temperatures (28°, 37° and 48°C). Cetyltrimethylammonium bromide solution, 1% (BDH) was used to detect cellulase activity and estimated according to Hankin and Anagnostakins method (10).

Growth and morphological characteristics of AT7 on the above mentioned medium were studied using various concentrations of CMC, low viscosity (1,2,3,4 and 5%) and different incubation temperatures (28°, 37°, 48° and 55°C) for 3-6 days.

RESULTS AND DISCUSSION

Morphological and Cultural Characteristics: The organism produced nonfragmented and nonseptated vegatative mycelium (dark brown color on ISP no.2); spores were formed on aerial mycelium (gray color in short-spiral compact chains, 10-20 conidia) and very good growth was obtained in the pH range of 4-8 (Table 1), color of the mycelium changed from gray to white (aerial mycelium) and dark brown to light yellow (substrate mycelium) in accordance with the pH values of the tested medium. Temperature range for growth was 28-55°C, and faster growth was observed on high temperatures.

Analysis of Whole Cell Hydrolysate: Results showed that whole cell hydrolysate contained LL-diaminopimelic acid with no diagnostic sugar pattern and amino acids: glycine, alanine, leucine were present, while cysteine and asparagine were absent.

Biochemical Characteristics: Table 2 shows some biochemical characteristics of AT7; melanin pigment was not produced; carbohydrates utilized were glucose, xylose, rhamnose, maltose, *arabonise, galactose*, starch and micro crystalline while sucrose and salicine were not utilized. Strain AT7 was able to hydrolyze starch, carboxymethylcellulose and casein, while negative response was observed with gelatin, esculin and tyrosine; acid coagulation of litmus milk and could tolerate up to 8% NaCl (w/v).

Table 1 : Cultural characteristics of strain AT7 on nutrient agar at different pH value.

pH	Growth	Aerial mycelium	Substrate mycelium
4	++	Gray	Dark Brown
5	++	Gray	Dark Brown
6	++	Grayish white	Yellow
7	++	Grayish white	Yellowish brown
8	++	White	Yellow
9	+	White	Light Yellow
10	+	White	Light Yellow
11	±	White	Light Yellow

++ : very good, + : good, ± : weak growth

Table 2: Biochemical characteristics of strain AT7.

Test	Reaction
Melanin production on:	
ISP no. 6	-
ISP no. 7	-
Utilization of carbon source on ISP no. 9:	
glucose	+
xylose	+
arabinose	++
rhamnose	++
galactose	+
mannitol	+
salicin	-
sucrose	-
starch	+
cellulose microcrystalline	+
Hydrolysis of:	
casein	++
tyrosine	-
esculin	-
gelatin	-
starch (ISP no. 4)	++
carboxymethylcellulose	++
Litmus milk	acid + coagulation
Sensitivity to antibiotic disk (*):	
chlorotetracyclin (30 mcg)	resistant
penicillin G (10 units)	resistant
nystatin (100 units)	resistant
novobiocin (30 mcg)	inhibition
neomycin (10 mcg)	inhibition
streptomycin (10 mcg)	inhibition
gentamycin (10 mcg)	inhibition
erythromycin (15 mcg)	inhibition
tetracycline (30 mcg)	inhibition
Maximum tolerance to NaCl (% w/v)	8

++ : very good, + : good, –: negative, *: concentration of antibiotic per dish, Difco

The strain did not show any activity against: *E. coli* 357; *E. coli* 455; *B. subtilus* 402D; *B. pumilus* 31D and *P. aeroginosa* NCTC 6750. However it was sensitive to the antibiotic discs: chromophenicol (30 mcg), nemoycin (30 mcg), novobiocin (30 mcg), steptomycin (10 mcg), gentamycin (10 mcg), erythromycin (15 mcg) and tetracycline (30 mcg), while resistant to chlorotetracycline (30 mcg), penicillin G (10 units) and nystatin (100 units).

Identity of strain AT7: Strain AT7 was classified as a species of the genus *Streptomyces*; on the basis of chemical composition of whole cell hydrolysates (i.e. presence of LL-diaminipimelic acid and sugar pattern (8) and the morphology of spore chains and hypae (14). Strain AT7 as compared with the known species of *Streptomyces* described in Shirling and Gottlieb (16) and Pridham and Treshner (19) was found to be similar to *Streptomyces thermodiastaticus* with respect to negative melanin production, form gray aerial mycelium, brown to yellowish brown vegetative hyphae, spiral chain of spores and pattern of carbohydrate utilization, however since this type of *St. thermodiasticus* does not exist any longer. Further required studies has to be made on spore surface to confirm the above mentioned name. For the time being, the strain is designed as *Streptomyces* sp. Strain AT7.

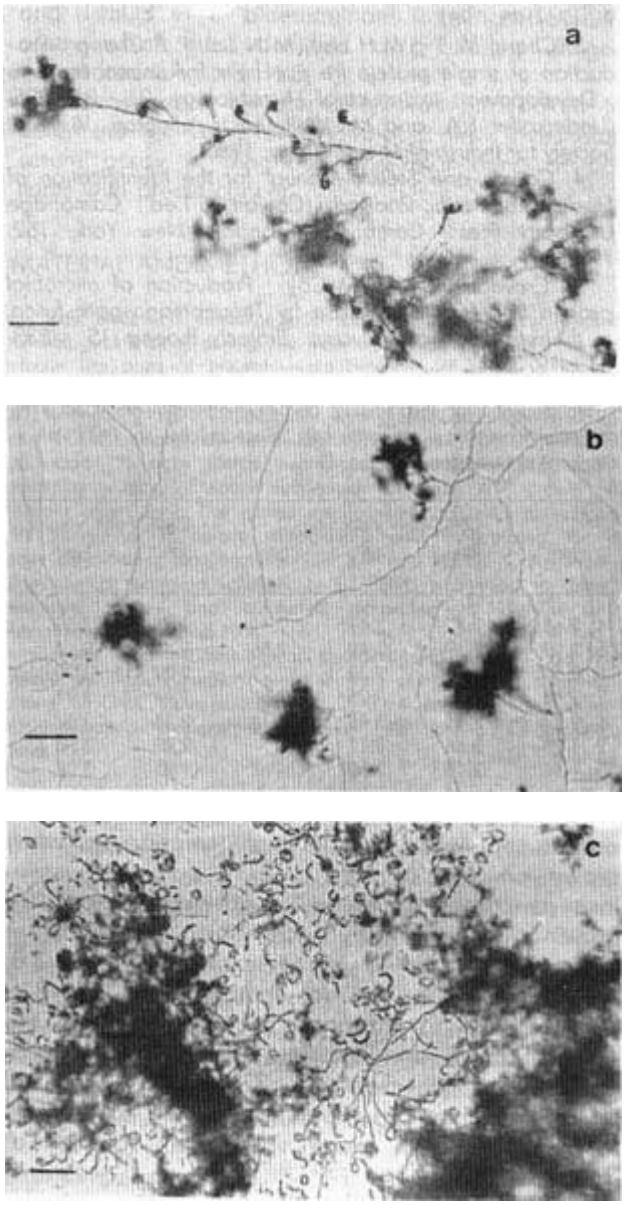
Measurement of cellulase production: In a preliminary test, the organism was found to be capable of hydrolyzing cellulose, in order to optimize culture medium conditions, a set of experiments was performed under different temperature and pH, by using CMC. As shown in Table 3 AT7 exhibited high cellulose activity measured as the ratio of size of CMC hydrolysis on colony diameter after 3 days of incubation at the three different temperatures used (28°, 37° and 48°C).

When AT7 grew at 28°C on a medium with a pH value ranges from 4-8 it gave clear and measurable zones of CMC hydrolysis. Largest zone was observed at pH 5, while no hydrolysis of CMC was observed at pH 10. At 37°C, CMC degratation occurred at all pH values tested (acidic and basic), however, the ratio of size of CMC hydrolysis to colony diameter was increased when strain AT7 was grown in alkaline medium. Other workers (10) reported on two strains out of six *Streptomyces* spp. that could grow well and produce cellulase, on media adjusted when AT7 was grown on CMC (high viscosity) under the same conditions, except at 28°C no degratation was noticed in growth medium with high alkaline pH.

Table 3: Ratio of the size of carboxymethylellulose (CMC) hydrolysis to colony diameter of Streptomyces sp. AT7 at different pH and temperatuce, °C.

	CMC-Low viscosity			CMC-High viscosity	
28°	37°	48°	28°	37°	48°
4 2.61	2.72	2.49	2.44	2.75	2.69
5 2.99	2.69	2.49	2.86	2.42	2.52
6 2.50	2.66	3.33	2.27	2.17	3.00
7 2.44	2.75	3.25	2.38	2.14	2.91
8 2.73	2.47	3.48	2.14	2.16	3.67
9 1.93	2.66	3.11	-	2.83	3.50
10 -	2.10	3.50	-	1.81	3.08

Figure 1: Streptomyces sp. strain AT7 grown on a mineral salts medium containing carboxymethylcellulose (4% w/v) for 3 days at: 28°C (a), 37°C (b) and 48°C (c), bar= 12 μm.



The above results indicate that strain AT7 was able to grow well and produce active cellulase enzyme (C_x) on media with both acid and alkaline pH and at various growth temperatures (28-48°C).

The effect of temperature on cultural and morphological characteristics: To our our knowledge no data are available on the temperature effect on cultural and morphological characteristics of *Streptomyces* spp. which tolerate high temperatures when grown on mineral salts medium containing cellulose as carbon source. Our observations on AT7 growing on this medium containing different concentrations of CMC, low viscosity (1.0, 2.0, 3.0, 4.0, and 5.0 % w/v) at temperatures 28°, 37°, 48°, and 55°C indicated that no change in the color of aerial and substrate mycelium; was observed good growth at 28°, 37° and 48°C on all CMC concentrations; less growth at 55°C and faster growth with heavy sporulation occurred at 37° and 48°C.

In studying the effect of temperature on the morphology of the organism, Figure 1a shows that at 28°C, short chain of spores (10-20 conidia), with spiral-crooked, compact at the tip of long to medium sporophores with non-fragmented hyphae, while at 37°C the shape of spore chains on short sporophores (Figure 1b). Heavy sporulation, short spore chains (5-10 conodia) fragmented to single and pairs of spore occurred at 48° and 55°C (Figure 1c). Further studies on cell growth and cellulase activity of AT7 will be described in a following report.

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