

TAXONOMIC CHARACTERIZATION OF THE THERMOPHILIC ACTINOMYCETE STRAIN 21E – PRODUCER OF THERMOSTABLE COLLAGENASE

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Summary

The thermophilic actinomycete strain 21E isolated from saline Bulgarian soils produced a highly thermostable collagenase. Macro- and micromorphological characteristics of the strain were tested on fourteen media. Some physiological and biochemical properties, enzyme activities, as well as the antibiotic activity and sensitivity were examined. It was concluded that the strain 21E was a typical member of the genus Thermoactinomyces. The comparative references to Thermoactinomyces species with similar taxonomic characteristics identified the strain as Thermoactinomyces sacchari. T. sacchari strain 21E was resistant to the antibiotic novobiocine, and displayed protease, amylase, collagenase and lipase activities.

Key words: taxonomy, thermophilic actinomycetes, *Thermoactinomyces sacchari*, soil, collagenase

Introduction

The study of microorganisms capable to grow in extreme conditions is an important field of microbiology and biotechnology. Thermophilic representatives of the family of actinomycetes are becoming more and more important in solving certain problems of ecology, industrial microbiology, hygiene and other fields. Recently, they have been subjected to intensive investigations in search for producers of new thermostable enzymes.

It was already shown that the thermophilic actinomycetes can produce amylases, xylanases and cellulose digesting enzymes which retain their activity at high temperatures (50-65 °C) [17, 22, 27]. It is commonly accepted nowadays that such enzymes will find wider application in the industrial practice. Extracellular proteolytic activity was detected in the cultural supernatant of a number of ther-

mophilic actinomycete strains [9, 11, 20, 29].

The collagenases are specific proteases capable to hydrolyze the triple helix of the native collagen, which is remarkably resistant to other proteases. It should be noted however that the collagenases described so far are isolated from mesophilic microorganisms and these enzymes are not stable at elevated temperatures [10, 15, 23, 25].

A collagen-degrading thermophilic actinomycete strain, designated as 21E, was obtained while searching for collagenolytic thermophilic microorganisms of soil origin [15]. These microorganisms are harmless for humans, and develop quickly in culture - for about 2-3 days. The potential applicability for the production of a collagenolytic enzyme preparation requires strain 21E to be determined taxonomically.

Materials and Methods

The thermophilic actinomycete strain 21E was afforded for taxonomic study by A. Gu-shterova from the Institute of Microbiology at the Bulgarian Academy of Science. It was isolated from saline soil [15] and produced a highly thermostable collagenase [24].

The general morphological, cultural, physiological, and biochemical properties of the actinomycetes were employed for the taxonomic characterization of strain 21E, and they were determined by the methods and in the media of the International Streptomyces Project (ISP) [26]. Additional nutrient media and characteristic substances were also applied in the investigation.

Micromorphological and cultural properties. The following media were used: peptone-maze extract agar (PM) [14], yeast-malt extract agar (ISP-2), oatmeal agar (ISP-3), starch agar with mineral salts (ISP-4), glycerol-asparagine agar (ISP-5), tyrosine agar (ISP-7) [26], nutrient agar with glucose [8], yeast extract agar [6], glucose-yeast extract agar [8], potato-carrot agar (PC) [7], potato-glucose agar [2], milk agar [8], and chitin agar [20].

The morphology of the sporophores was studied by direct light microscope observation. The morphology of the spores was examined by a scanning microscope. The micromorphological and cultural properties of the strain were investigated after 24, 48, and 72 hours of cultivation. Color descriptions were made after the Bondartsev's color scale [5].

Physiological and biochemical properties. Melanin production was studied on peptone-yeast extract iron agar (ISP-6) and on tyrosine agar (ISP-7) [26]. The utilization of various carbon sources was tested on basic mineral salts agar (ISP-9) [26] supplemented with the following sugars (1 %, w/v): D-glucose, L-arabinose, sucrose, D-xylose, l-inositol, D-mannitol, D-fructose, rhamnose, raffinose, D-maltose, cellulose, and lactose.

Growth temperature optimum. The optimal growth temperature was determined on PM agar in the range from 25 to 75 °C.

Resistance to heating at 100 °C. Spores of the thermophilic actinomycete 21E were suspended in distilled water, heated in boiling water for 10, 30, 60, and 120 min, and then transferred onto PM agar.

Resistance to sodium chloride. The tolerance to NaCl was tested on PM agar containing different concentrations of NaCl

from 1 to 15 % (w/v).

Decomposition of gelatin. PM agar supplemented with 0.4 % (w/v) gelatin was used. A clear zone around the colony indicated the gelatin hydrolysis [19].

Hydrolysis of starch. Ten grams of potato starch suspended in 100 ml of cold distilled water were added as a single carbon source to 900 ml of PM agar. The plates were inoculated and incubated at 55 °C, and then were flooded with iodide solution. A clear zone around the colony indicated the starch hydrolysis [19].

Decomposition of casein. The capability to decompose casein was determined as described by Gordon and Smith [12].

Hydrolysis of esculin and arbutin. Esculin or arbutin (0.1 %, w/v) were added to PM agar with 0.05 % ferric citrate (w/v). The blackening of the medium around the colony was an evidence for the hydrolysis of each of the substrates [4, 18].

Decomposition of tyrosine, xanthine, hypoxanthine, and adenine. The media and methods applied were as described by Gordon et al. [11] and Kurup and Schmitt [18], except for that the basic medium was PM. The clearing of the medium around and underneath the colonies indicated tyrosine, xanthine, hypoxanthine, or adenine decomposition.

Sensitivity to antibiotics. The agar diffusion method was applied. Disks impregnated with ampicillin, penicillin, chloramphenicol, gentamycin, erythromycin, and streptomycin were placed on the inoculated agar plates. The zones of growth inhibition were measured.

Novobiocin was added to PM liquid medium to a final concentration of 25 µg/ml and the resistance the strain to the antibiotic was recognized when apparently similar growth in both the test and the control tubes was observed [19].

Antagonistic properties. The streak test on PM agar plates was performed on *Bacillus subtilis* and *Escherichia coli* as test microorganisms [14].

Hydrolysis of tributirin. Hydrolysis of tributirin was tested by the method described by Kurup and Schmitt [18].

Decomposition of native collagen. The decomposition of native collagen was assessed according to the size of the hydrolyzed zone [28].

The genus reference, species descriptions, and species identification strain 21E were determined after Bergey's manual [3] and Agre's manual [1].

Results and Discussion

Micromorphological and cultural properties

Strain 21E produced well-developed aerial mycelium that displayed a tendency to fragment with aging of the culture. It was white and cream-white, but there were also pale-beige and ash color variants. The aerial mycelium was transient, usually disappearing within 3 to 4 days through autolysis of the hyphae. The hyphae of the aerial mycelium were short, sparse or tufted (Fig. 1, A).

The hyphae of the substrate mycelium were branching, penetrating into the agar medium, forming fast-growing, spreading colonies. Several colors of the vegetative mycelium were observed depending on the medium and the age of the culture. The young colonies were beige or cream to dark beige or yellow-brown. The old colonies were dark brown in color (Table1).

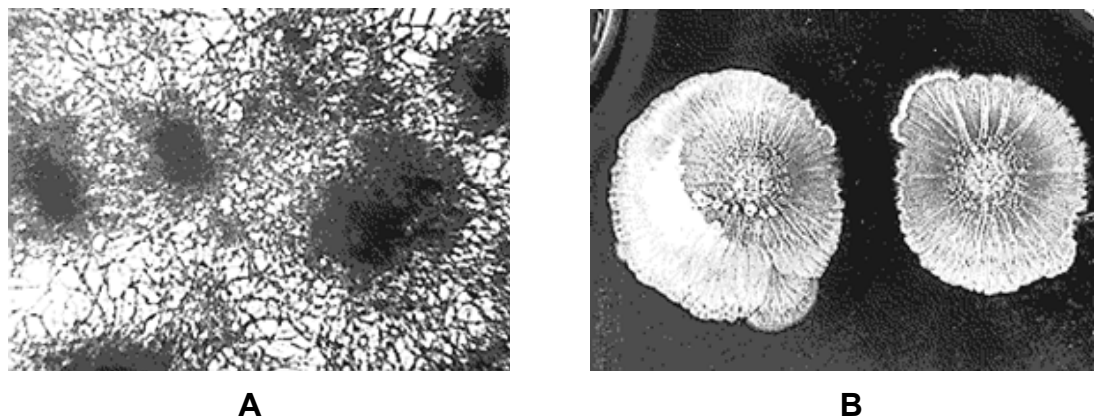


Fig. 1. Hyphae structure (A) and form of the colonies (B) of strain 21E (PM agar, 48 hour).

Table 1. Cultural properties of strain 21E.

Nutrient medium	Growth	Color of the aerial mycelium	Color of the substrate mycelium	Soluble pigment
Yeast-malt extract agar (ISP-2)	abundant	cream-white	beige	no
Oatmeal agar (ISP-3)	moderate	white	cream	no
Starch agar with inorganic ions (ISP-4)	moderate	white or cream-white	dark beige	no
Glycerol-asparagine agar (ISP-5)	scanty	no	yellow-brown	no
Peptone-yeast extract iron agar (ISP-6)	abundant	cream-white	beige	no
Tyrosine agar (ISP-7)	no	no	no	no
PM agar	abundant	cream-white	dark beige	no
Nutrient agar with glucose	scanty	white	cream	no
Yeast extract agar	abundant	pale beige	dark beige	no
Glucose yeast extract agar	moderate	ash	beige	no
Milk agar	abundant	white	cream	no
PC agar	abundant	cream-white	dark beige	no
Potato-glucose agar	moderate	white	dark beige	no
Chitin agar	no	no	no	no

The colonies were in diameter from 5-6 mm on these media where the growth of the strain was scanty to 25-30 mm on the media with moderate to abundant growth. The typical colony was with a regular round form, flat, with smooth surface and gentle radial folds to the periphery (Fig. 1, B). Its central area was slightly convex. The margin of the colony was undulated. Morphological variants of colonies were also observed with irregular form, rugged surface and fibrillated edge. They appeared in certain unfavorable conditions. The colonies became slimy and bacterial in appearance as the hyphae autolysed.

Strain 21E produced abundant or moderate aerial mycelium on organic media and scanty grew on synthetic media (Table 1). It had a short growth cycle and sporulated already after 24 hours of cultivation. The strain did not produce soluble pigments.

Single spores were observed. They were situated usually on short sporophores or directly on the hyphae of both the substrate and the aerial mycelia. The spores appeared globular under the light microscope, but they looked angu-

lar in scanning electron microscopy because of a pattern of ridges on the spore surface (Fig. 2).



Fig. 2. Spore surface of strain 21E (PM agar, 48 hour, 10 000x).

The spores of strain 21E resembled in certain features the bacterial endospores. Being thermostable, they were heat-resistant at 100 °C for 60 min. They also could be colored with stains specific for bacterial endospores, e.g. malachite-green.

Physiological and biochemical properties

Strain 21E was capable to grow in the temperature range between 35 and 65 °C, and did not grow at 33 °C and 67 °C. The optimal growth temperature was from 55 to 60 °C. Good growth occurred in the presence of 3-7 % and it was weak at 10 % NaCl (Table 2).

The carbon metabolism is significant in taxonomy and the differences in the utilization of various carbon sources serve as additional cri-

teria for species differentiation. ISP considers the utilization of nine sugars [25]. The investigation of thermophilic actinomycetes requires additional carbon sources to be tested besides the specific for the rest of the actinomycetes.

The growth of strain 21E was weak on ISP-9 [25] regardless the tested carbon sources (Table 3). Only glucose, fructose, arabinose, and mannitol weakly stimulated its growth.

Table 2. Some physiological and biochemical properties of strain 21E.

Growth at temperature (°C)		Growth in NaCl (%)	
33	-	1	+
35	+	3	+
40	+	5	+
50	+	7	+
55	+	10	±
60	+	12	-
65	+	15	-
67	-		
Enzyme production			
Protease		+	
Collagenase		+	
Amylase		+	
Lipase		+	

Table 3. Comparison of strain 21E with *Thermoactinomyces sacchari* and *T. vulgaris*.

Characteristics	Strain 21E	<i>T. sacchari</i>	<i>T. vulgaris</i>
Aerial mycelium			
Abundant	-	-	+
Transient	+	+	-
Lysis	disappearing within 3 to 4 days	rapid, within 3 days	not seen
Color	white or cream-white	white	white
Substrate mycelium			
Color	beige, cream to dark beige	colorless to cartridge buff	colorless to brown
Spores			
Single spores on aerial/ substrate mycelium	+	+	+
Spores – endospores, with ridged surface	+	+	+
Spores on sporophores	+	+	+
Soluble pigment			
Yellow-brown	-	-	-
Growth at			
30 °C	-	-	-
55 °C	+	+	+
NaCl (3 %)	+	+	+
NaCl (5 %)	+	+	-
Novobiocin (25 µg/ml)	+	+	+
Utilization of carbon sources			
D-fructose	+	+	+
L-arabinose	+	+	-
L-rhamnose	±	-	-
D-xylose	±	-	-
D-maltose	-	NT	NT
D-mannitol	+	+	+
D-glucose	+	+	+
D-raffinose	±	-	-
Sucrose	-	-	+
I-Inositol	±	-	-
Lactose	-	NT	NT
Degradation of			
Casein	+	+	+
Gelatin	+	+	+
Starch	+	+	-
Cellulose	-	-	-
Chitin	-	-	-
Arbutin	±	+	+
Esculine	±	+	ND
Tyrosine	-	-	+
Xanthine	-	-	-
Hypoxanthine	-	-	±
Adenine	-	-	-

Legend: not tested (NT); not defined (ND).

The strain grew scantily on media with xylose, ramnose, inositol and raffinose. It did not assimilate sucrose, lactose, maltose, and cellulose.

Strain 21E caused intensive hydrolysis of starch and casein, but did not decompose chitin. It coagulated and peptonized milk. The strain weakly decomposed esculin and arbutin, and did not hydrolyze xanthine, xyloxanthine and

adenine. It did not show tyrosinase activity either. The strain hydrolyzed gelatin on the 24th hour of cultivation. It displayed collagenase and lipase activities.

Strain 21E was sensitive to all the antibiotics used in this investigation except for novobiocin (Table 4). The strain did not exhibit any antagonistic activity against *B. subtilis* and *E. coli*.

Table 4. Sensitivity to antibiotics of strain 21E.

Antibiotic	Concentration (µg/ml)	Sterile zone (mm)
Chloramphenicol	10	6
Ampicillin	25	12
Gentamycin	10	25
Erythromycin	10	28
Penicillin	10	14
Streptomycin	10	16
Novobiocin	25	0

The hyphae growth characterized strain 21E as an actinomycete. The established segmentation of the both substrate and aerial mycelia, the optimum of growth in the interval 55-60 °C, the formation of spores with the characteristics of bacterial endospores, and the biochemical properties determined strain 21E as a

representative of the genus *Thermoactinomyces*. It was compared with other species of this genus (Table 3) and was found to be phenotypically close to *T. sacchari* and *T. vulgaris*. The presented data showed that strain 21E was much more similar to *T. sacchari* from which it differed by the weak utilization of some sugars.

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ТАКСОНОМИЧНА ХАРАКТЕРИСТИКА НА ТЕРМОФИЛНИЯ АКТИНОМИЦЕТЕН ЩАМ 21E – ПРОДУЦЕНТ НА ТЕРМОСТАБИЛНА КОЛАГЕНАЗА

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Резюме

Термофилният актиномицетен щам 21E, изолиран от солени почви в България, продуцира високо термостабилна колагеназа. Проучени са макро- и микроморфологичните характеристики на щама върху 14 среди. Изследвани са някои физиологични и биохимични свойства, ензимни активности, както и антибиотичната активност и чувствителност. Установено е, че щам 21E е типичен представител на рода *Thermoactinomyces*. Сравняването с видове *Thermoactinomyces* с близки таксономични характеристики определя щама като *Thermoactinomyces sacchari*. Щам *T. sacchari* 21E е устойчив на антибиотика новобиоцин и показва протеазна, амилазна, колагеназна и липазна активности.