

CHARACTERISTICS OF BACTERIAL STRAIN, SUPEROXIDE DISMUTASE PRODUCER, ISOLATED FROM BULGARIAN THERMAL SPRING

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Summary

Twenty one bacterial strains with superoxide dismutase activity were isolated from samples (flowing spring water, water with soil particles, alga bacterial mat and mud) collected near different thermal Bulgarian springs. The strains were Gram positive, rod shaped, spore forming and moderate thermophiles. Isolate M 20 showed the highest specific total superoxide dismutase activity of 530 U/mg in shaken flasks cultivation at 55 °C for 4 hours. An activity of 1300 U/mg was achieved after selection and optimization of cultivation procedures in fermentor. The optimal enzyme activity of cell free extract was at 55 °C and pH 8.0, and the half-life was 30 min at 90 °C. The strain was catalase positive, it produced peroxidase and proteinase. As a result of morphological, cultural, physiological and biochemical characteristics the isolate M 20 was determined as a moderate thermophile belonging to the genus Bacillus.

Introduction

Superoxide dismutases (SODs; EC 1.15.1.1.) are a class of metal proteins, catalyzing the dismutation of the superoxide radicals (O₂) to oxygen and hydrogen peroxide.

In the recent years, due to the big practical interest to the microbial SODs the attention was focused to the thermostable ones produced by thermophilic microorganisms. The SOD of the anaerobe bacterial hy-

perthermophil *Aquifex pyrophilus* was with half-life 175 min at 95° C [7]. The most thermostable SOD, described recently in the literature was of archaeobacterial origin. A strain of *Sulfolobus sulfataricus* produced Fe-Mn SOD with half-life 2 hours at 100° C [9].

The present work was undertaken with a view to isolate aerobic bacterial thermophiles producers of SOD from Bulgarian thermal springs.

Materials and Methods

Isolation of strains. 0.5 ml from the samples (flowing spring water, water with soil particles, alga bacterial mat and mud) collected near 16 thermal springs in Bulgaria, were added in 100 ml Erlenmeyer flasks with 25 ml of a medium consisting: Bacto peptone - 5.0 g; yeast extract - 5.0 g; K_2HPO_4 - 5.0 g; NaCl - 1.0 g; $Mg SO_4$ - 10 mg; $FeSO_4 \times 7 H_2O$, distilled water – 1000 ml, pH – 7.5. After 24 hours cultivation at 50, 55 or 60 °C suitable diluted cultures were spread on solid medium to obtain single colonies.

The isolates were cultivated in 500 ml Erlenmeyer flasks with 75 ml nutrient medium. 1 ml of 18 hours old culture was used for inoculation. Samples for determination of strains growth and SOD activity were taken on the 2^d and 4th hour of cultivation.

Cultivation in fermentor. It was carried out in fermentor Bioflo (New Brunswick Sci.) with working volume of 1000 ml, magnetic stir with 500 rpm and constant air flow of 1.0 v/v/m (volume air per volume medium per min). 1 ml of 18 hours old culture was used for inoculation. The percentage of the dissolved oxygen (DO) was registered by sterilized oxygen electrode and DO analyzer (New Brunswick Sci. Model DO – 50). Samples for determination of the bacterial growth and enzymes activities were taken every 30 min.

Bacterial growth was determined by accounting the optical density at 660 nm.

Preparation of cell free extract. Samp-

les of cultures were centrifuged at 8000 g for 15 min at 5° C. The cells pellet was suspended in 3 ml of 0.05 M potassium – phosphate buffer with pH 7.8. 2.0 g sand were added and mechanical lysis for 15 min was applied. After centrifugation the obtained supernatant was analyzed for enzymes activities.

SOD assay. The method of Beauchamp и Fridovich was used [1]. Definition of one enzyme unit was the amount of cell free extract which inhibited 50 % the reduction of nitroblue tetrazolium to blue formazan at 55 °C and pH 7.8. The total specific enzyme activity was expressed as U/mg protein. The enzyme thermostability at 90° C was recorded.

Catalase activity assay. The catalase production was tested with 3 % H_2O_2 . After 5 min appearance of bubbles was accounted as a positive reaction.

Peroxidase activity assay. The production of purpurogalin after reaction with 33 % solution of pyrogallol and 3 % H_2O_2 was accounted as a positive peroxidase reaction.

Proteolytic activity assay. Agar diffusion method (3 % fresh milk agar) was applied, as the fresh milk was centrifuged at 6000 rpm for 10 min to remove lipids and used for the test.

Protein concentration was assayed by the method of Bradford [3].

Gram staining and **electron microscopy** of thin sections were performed according to standard procedures.

Results and Discussion

Twenty one isolates from 16 thermal Bulgarian springs were tested (Table 1). All were Gram positive aerobes, producers of intracellular SOD. Six of them: isolates M [3, 6₁, 10, 19, 20, 21] expressed relatively highest total specific enzyme activity between 210 and 530 U/mg.

The cell free extracts of these six isolates were analyzed for SOD thermostability and presence of catalase, peroxidase and proteolytic activity (Table 2). Isolate M 20 showed the highest thermostability – the half-life was 30 min at 90 °C. The isolate was catalase, peroxidase and proteinase positive. Further

Table.1. SOD activity of newly isolated thermophilic strains.

Thermal springs	Isolate	Gram staining	Temperature of the sample (°C)	pH of the sample	Temperature of cultivation (°C) *	Hours of cultivation			
						2		4	
						OD _{660nm}	SOD U/mg	OD _{660nm}	SOD U/mg
Hisar Spring 1	M1	+	46	7.98	50	0.6	134	1.36	163
Hisar Spring 2	M2	+	42	7.55	50	0.28	140	1.00	130
Hisar bath Bistrica	M3	+	52	7.82	50	0.50	250	1.10	247
Village Banja (Karlovo)	M6 ₁	+	49	7.45	50	0.57	175	0.96	210
Village Banja (Karlovo)	M6 ₂	+	49	7.45	50	0.60	158	1.00	140
Village Ovoshnic	M8	+	45	7.99	50	0.64	193	1.20	155
Village Jagoda	M9	+	42	10.14	50	0.65	219	1.40	136
Village Banja (Sliven)	M10	+	55	8.27	50	0.55	169	1.00	308
Pancharevo	M12	+	49	7.96	50	0.68	109	1.34	94
Separeva banja	C1	+	47	7.5	60	0.40	27	1.14	18
	C2	+	33	7.0	60	0.26	34	1.15	14
Haskovski mineral bath	M13	+	57	7.0	55	-	-	0.53	110
River Erma	M14	+	37	7.0	55	-	-	0.55	100
River Erma	M15	+	37	7.5	55	0.70	231	1.13	100
Bedenska banja	M16	+	52	7.85	55	0.65	35	1.15	67
Bedenska banja	M17	+	71	8.0	55	0.73	200	1.12	92
Bedenska banja	M18	+	50	8.0	55	0.60	91	1.20	155
Momin prohod	M19	+	65	7.8	55	0.74	29	1.10	283
Dolna banja	M20	+	62	8.85	55	0.50	79	1.05	533
Pchelin	M21	+	72	8.0	55	0.80	82	1.22	350
Varvara	M22	+	80	8.0	55	0.90	244	0.45	104

* pH of the medium for cultivation of all the isolates was 7.5.

Table 2. Enzyme characteristics* of new isolated thermophilic producers of SOD.

Isolate	Half-life ($t_{1/2}$) at 90 °C (min)	Proteolytic activity		Catalase activity		Peroxydase activity	
		C*	CFE	C	CFE	C	CFE**
M3	15	-	+	+	+	-	-
M6	<5	+	+	+	+	-	-
M10	5	+	+	-	-	+	+
M19	5	-	+	-	-	+	+
M20	30	+	+	+	+	+	+
M21	<5	+	+	+	+	+	+

* Cultural supernatant (C); cell free extract (CFE).

studies were carried out with isolate M20 because of its relatively high enzyme activity and thermostability.

Isolate M 20 was Gram positive aerobe, rod shaped, sporulated bacterium (Fig. 1). Electron microscopic photographs of thin sections showed typical morphology and ultra structure for the Gram positive, rod shape bacterial representatives. Some of the M20 properties are presented in Table 3. It could be described as a moderate thermophile from

the genus *Bacillus*. The strain *Bacillus* sp. M20 was deposited in the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria (No. 8389).

SOD from thermophilic strains of genus *Bacillus* was studied at the species *B. stearothermophilus*. The investigations were focused mainly on clarifying the crystal enzyme structure [8], the enzyme amino acids composition [5] and the gene cloning in *Saccharomyces cerevisiae* [2] and *Escherichia coli* [4].

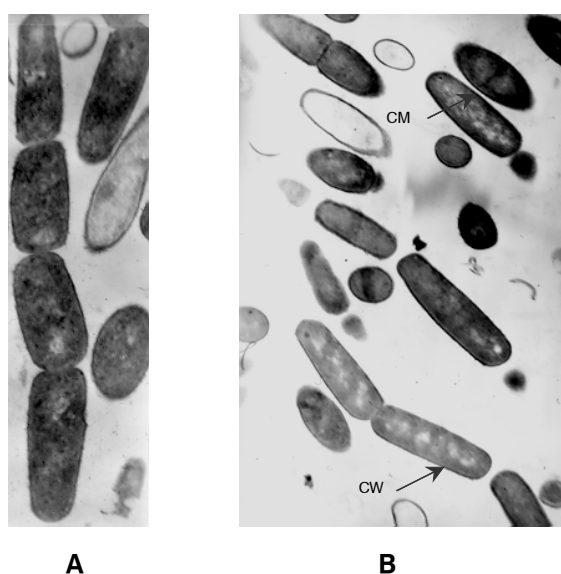


Fig. 1. Ultra thin sections of 3 hours old vegetative cells of *Bacillus* sp. isolate M 20, cultivated in shake flasks: **A.** 6 000 x; **B.** 3 250 x (CM - cell membrane, CW - cell wall).

Table 3. Morphological, physiological and cultural characteristics of isolate M20.

Characteristics		Isolate M20
Colony morphology on nutrient agar on the 18 th hour at 55 °C	size	1.0-1.5 mm
	form	circular
	color	beige
Morphology of cells on nutrient agar on the 18 th hour at 55 °C	form	rods
	size	3.0 – 7.0 x 0.8 – 1.3 µm
	motility	+
	spores	ellipsoidal, terminal
Temperatures for growth (°C)	minimal	36
	optimal	35
	maximal	65
pH ranges for growth		5.5 – 8.5
Growth in nutrient broth		turbid without sediment and pellicle
Anaerobic growth		-

There are scarce data in the literature about the optimal conditions for the cultivation of *B. stearotherophilus* strains for maximal SOD production as well as about the enzyme thermo stability in cell free extract.

Gligic et al. isolated from mineral waters a strain of *B. stearotherophilus* that produced up to 500 U/mg SOD for 8 hours cultivation on a shaker at 60° C [6]. From hot spring waters in Thailand, a strain *B. stearotherophilus* TL S33, also producing SOD, was isolated with maximal activity of 130 U/mg at 48 hours and 65° C cultivation temperature [10].

Isolate M 20 cultivated in fermentor had a maximal SOD activity of 1300 U/mg for a

relatively short period (half an hour) in the middle of the exponential phase of growth when the residual oxygen was 70 %. When the residual oxygen percentage decreased at the end of the exponential growth the SOD activity also decreased (Fig. 2).

As it can be seen from the results in Fig. 3 the optimal SOD activity of the isolate cell free extract was at temperature 55 °C and pH 8.0.

The relatively high SOD activity and thermostability of *Bacillus sp.* isolate M 20 give a reason for next investigations, related to the optimization of culture conditions for SOD production as well as to the characterization of the purified enzyme properties.

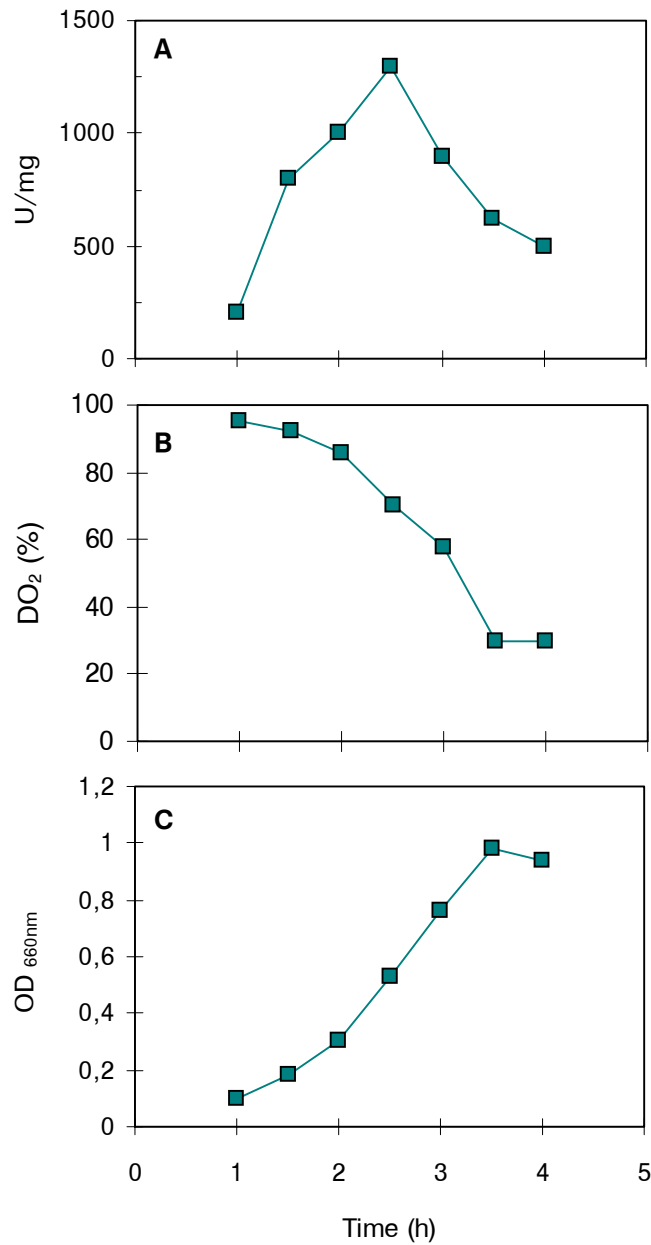


Fig. 2. Specific SOD activity (A), dissolve oxygen concentration (B) and bacterial growth (C), during cultivation of *Bacillus sp.* isolate M 20 in fermentor.

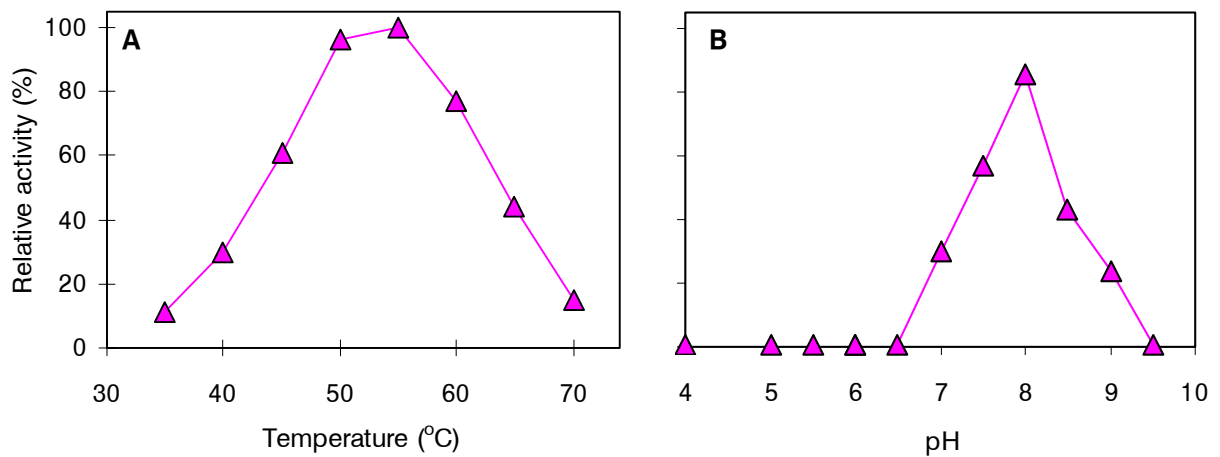


Fig. 3. Effect of the temperature (A) and pH (B) on the SOD activity of *Bacillus sp.* isolate M.

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ХАРАКТЕРИСТИКА НА БАКТЕРИАЛЕН ЩАМ, ПРОДУЦЕНТ НА СУПЕРОКСИД ДИСМУТАЗА, ИЗОЛИРАН ОТ БЪЛГАСКИ ТЕРМАЛЕН ИЗВОР

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Резюме

*Изолирани са 21 щам, продуциращи супероксид дисмутаза, от проби, събрани от различни термални извори в България (разливни води, почва, тиня). Всички са Грам положителни, спорообразуващи пръчковидни умерени термофили. При култивиране в колби на клатачен апарат при 55 °С изолат M20 показва на 4-я час от култивирането най-висока специфична тотална супероксид дисмутазна активност – 530 Е/мг. След селекция и оптимизиране на условията за култивиране в лабораторен ферментор е постигната максимална активност от 1300 Е/мг. Оптимумът на ензимната активност в безклетъчен екстракт е при температура 55 °С и рН 8.0, а полуживотът е 30 мин при 90 °С. Щамът е каталазно позитивен, продуцира пероксидаза и протеиназа. В резултат на морфолого-културалните и физиолого-биохимичните свойства изолат M20 е охарактеризиран като умерен термофил, представител на род *Bacillus*.*