BULGARIAN ACADEMY OF SCIENCE

THE STEPHAN ANGELOFF INSTITUTE OF MICROBIOLOGY

Department of Mycology

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CATALASE FROM ANTARCTIC MICROMYCETES: ROLE IN ANTIOXIDANT DEFENSE, REGULATION AND PROPERTIES

ABSTRACT

of a dissertation for awarding the educational and scientific degree PhD
in a professional direction 4.3. Biological Sciences,
Doctoral programme Microbiology

Research supervisor: Associate Professor Ekaterina Krumova, PhD

София

2024

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Research supervisor: Associate Professor Ekaterina Krumova, PhD

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София, 2024г.

ABBREVIATIONS

AFPs - antifreeze proteins

ATF - adenosine triphosphate

BA – beer agar

Bp - nucleotide base pair

CAPs - stress proteins of acclimation to low-temperature stress

CA – cold active

CAT - catalase

ChDA- Chapek-Dox agar

CSPs - low temperature stress proteins

CN - cyanide

GPx – glutathione peroxidase

DNA - desoxyribonucleic acid

DNPH - dinitrophenylhydrazine

DO - dissolved oxygen

ER - endoplasmic reticulum

FA - fatty acids

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GSH - glutathione

MDA - malondialdehyde

mRNA - matrix ribonucleic acid

NA – nucleic acids

NAD(P)H - nicotinamidadenine dinucleotide phosphate (reduced form)

OH• - hydroxyl radical

$\bullet O_2^-$ -superoxide radical

PAGE - polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

PDA – potato dextrose agar

PPP - pentose phosphate pathway

RNA - ribonucleic acid

SDS-PAGE - dodecyl phosphate polyacryl gel electrophoresis

SEM - scanning electron microscopy

SOD – superoxide dismutase

ROS – reactive oxygen species

TEM - transmission electron microscopy,

UV - ultraviolet irradiation

I. INTRODUCTION

In recent years, there has been a significant expansion of studies on the physico-chemical limits of life (temperature, pressure, drought, salt content, pH, heavy metals, etc.). Extreme environmental conditions disrupt the most important interactions that support the functions and structure of biomolecules. As a result, rapid damage to cellular integrity is observed. Therefore, the interest of many researchers turns to life in extremely cold habitats and to the organisms able to live there. These areas of the planet are dominated by bacteria, archaea, and fungi, which are the most common forms of life adapted to low temperatures in terms of species diversity and the biomass they produce. Microorganisms isolated from the depths of the oceans and from alpine and polar habitats are considered the best experimental models for studying life on Earth under extreme conditions. There is evidence that these organisms can not only survive extreme environmental conditions but in some cases are even dependent on their presence.

Representatives of the Kingdom Fungi are the most diverse group in Antarctic ecosystems. Ecological selection as well as evolutionary adaptation has ensured their survival in extreme conditions such as drought, solar radiation, low temperatures, etc. Despite intensive research in this aspect, there are still some unsolved problems related to survival mechanisms in habitats such as Antarctica. The relationship between the impact of low temperatures and the manifestations of oxidative stress attracts the interest of scientists. However, while the cellular response against cold stress has been studied in various bacteria and plants, very little is known about the adaptation of fungi to survive near subzero temperatures. The hypothesis of an increase in the level of oxidative stress at low temperatures needs more evidence by studying the level of stress biomarkers and the activity of antioxidant enzymes. The role of the cellular response against oxidative stress in the survival mechanism of microorganisms, especially fungi isolated from permanently cold habitats, has not yet been elucidated. There are no studies regarding the involvement of the antioxidant enzyme catalase (CAT) in this response. There is no data on the expression of the genes responsible for the synthesis of this enzyme at low temperatures.

Fungi isolated from habitats with extremely low temperatures show the ability to produce temperature-sensitive (cold-active, CA) enzymes, including CAT as a first line of antioxidant defense. Due to their high conformational flexibility, CA enzymes are characterized by increased turnover of catalytic action and high catalytic efficiency at low temperatures. Such

enzymes have a temperature optimum between 20 and 45ºC and maintain thermal stability at much lower temperatures. Little is known about the preparation and properties of CA CAT. In most cases, published data refer to enzymatic synthesis by bacterial species.

CA enzymes can be applied in several areas of life - in the food, textile, and pharmaceutical industries, in bioremediation technology as an indicator for the degradation of hydrocarbons in crude oil-contaminated soil, etc. They have potential as therapeutic agents in free radicals-caused diseases (eg diabetes, Alzheimer's disease, Parkinson's disease, vitiligo, and acatalasemia), as well as in the cryopreservation of human and animal gametes. It should be noted that such studies could improve the knowledge of extremophiles and extremozymes.

The studies included in the present dissertation work are focused on the indicated unsolved problems.

II. AIMS AND OBJECTIVES

The PhD thesis aims to investigate the involvement of the antioxidant enzyme catalase in the adaptative mechanisms to low-temperature stress in filamentous fungi isolated from extremely cold habitats (Antarctica).

To achieve the set goal, the following tasks are foreseen:

1. Temperature characteristics of 61 selected strains of Antarctic filamentous fungi

2. Characterization of the isolated fungi regarding the synthesis of intracellular and extracellular CAT. Selection of model strains belonging to different temperature classes.

3. Investigating the role of CAT in the cellular response of model strains to low temperatures stress.

3.1. Morphological adaptations and ultrastructural changes

3.2. Changes in oxidative stress biomarkers

3.3. Activity of the antioxidant enzyme defense

4. Isolation, sequencing, and comparison of CAT genes in the model strain

5. Study of the CAT genes expression of the model strain under low-temperature stress

6. Development of a laboratory method for CAT production from efficient Antarctic producer

7. Purification and characterization of the obtained CAT

III. MATERIALS AND METHODS

1. Characteristics of the used strains

2. Nutrient media and cultivation

3. Morphological and physiological studies

3.1. Morphological studies and electron microscopy

3.2. Temperature characteristic

3.3. Biomass determination

3.3. Preparation of cell-free extract

4. Analytical techniques

4.1. Determination of the antioxidant enzymes' specific activity.

4.2. Determination of glucose concentration and protein amount

4.3. Changes in biomarkers of oxidative stress - level of generated free oxy-radicals, content of oxidatively damaged proteins, amount of reserve carbohydrates, lipid peroxidation.

5. Genetic methods

5.1. Isolation and sequencing of genes encoding a protein with catalase activity of the model strain

5.2. Determination of gene expression of catalase genes during cultivation of the model strain under normal physiological and low-temperature stress conditions - DNA isolation, classical PCR, Quantitative Real-Time PCR

6. Purification of temperature-sensitive CAT

6.1. Steps of CAT purification

6.2. Properties of the purified temperature-sensitive CAT determination

Statistical processing of results: Statistical comparison between replicates of experiments was performed by Student's t-test and one-way analysis (ANOVA) followed by F-test at 95% significance level (α=0.05).

IV. RESULTS

1. TEMPERATURE CHARACTERIZATION OF ANTARCTIC FUNGAL STRAINS

1.1. Investigating growth and development possibilities at different temperatures

In the studies, 61 strains of filamentous fungi isolated from Antarctic soil samples belonging to the mycological collection of SAIM were used. They were studied for growth and development at different temperatures. An optimum temperature of 10ºC was determined for 2

strains isolated at 4ºC; thirteen strains have optimal temperature of 15ºC (2 strains isolated at 4ºC, 9 strains isolated at 10ºC and 2 strains at 25ºC); 8 strains showed optimal development at the temperature of 20ºC (2 strains isolated at 10ºC and 6 strains isolated at 25ºC); optimal temperature of 25ºC for 17 strains (10 strains isolated at 10ºC and 7 strains isolated at 25ºC); and 21 strains - optimal temperature 30ºC (3 strains isolated at 10ºC and 18 strains isolated at 25ºC) (Fig. 1).

Most of the Antarctic strains obtained belong to the psychrotolerant and mesophilic fungi. This distribution was made in accordance with the definition accepted in the literature by Morita (1975) and Russell (2006).

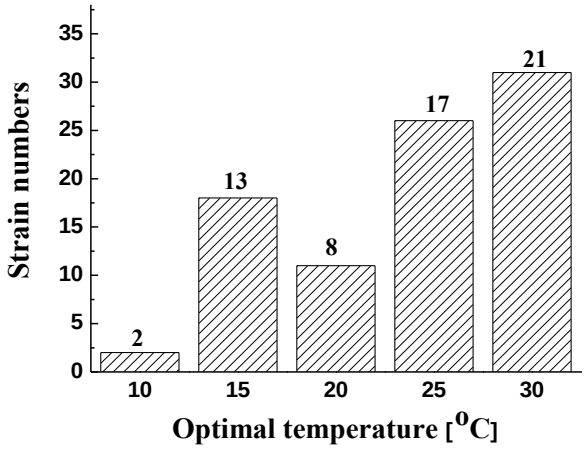

Fig. 1. Distribution of isolates according to optimum growth temperature

2. DISTRIBUTION OF CATALASES IN ANTARCTIC FUNGI

2.1. Characterization of isolated fungi in terms of synthesis of intracellular and extracellular CAT

2.1.1. Synthesis of intracellular CAT by isolated Antarctic strains at optimum growth temperatures

The isolated Antarctic fungal strains were screened for their ability to synthesize intracellular and extracellular CAT (Table 1).

Table 1. Antarctic isolates producing extracellular and intracellular CAT

N	Strain	Extracellular CAT [U/mg protein]	Intracellular [U/mg protein]	N	Strain	Extracellular CAT [U/mg protein]	Intracellular [U/mg protein]
1.	P27	2.5	22.9	32.	I-1_4	2.09	5.72
2.	P22	3.6	16,5	33.	III-2_1	1.20	8.16
3.	III-6_3	2.3	14.7	34.	III-7_1	1.33	19.54
4.	P21	0.6	11.3	35.	III-8_1	6.96	31.04
5.	P29	2.9	28.9	36.	III-8_3	1.70	26.72
6.	M12	0	16.4	37.	III-11_3	0.76	4.89
7.	P44	1.5	9.4	38.	III-2_2	0	6.32
8.	P31	0	35.7	39.	III-6_3	10.27	19.97
9.	P33	следи	5.9	40.	III-2_{21}	0	следи
10.	M5	0.9	10.4	41.	III-6_7	2.04	17.55
11.	II-6_2	2.3	10.0	42.	II-6_1	1.42	12.39
12.	II-6_5	4.0	15.3	43.	III-11_2	8.19	40.21
13.	II-6_6	1.1	14.9	44.	K1	5.36	1.61
14.	II-5_1	3.9	12.2	45.	K7	1.79	следи
15.	T35	1.6	15.8	46.	K9	1.00	5.52
16.	119	1.3	13.4	47.	15	1.93	19.97
17.	M0	4.4	6.8	48.	16	0.86	23.94
18.	I-2_1	0	9.5	49.	19	1.34	9.75
19.	I-6_1	1.3	5.7	50.	22	0	10.18
20.	I-8_2	1.12	3.8	51.	I-S	2.43	5.29
21.	I-10_1	0.89	11.9	52.	Б3_2	1.64	12.09
22.	I-13	4.25	8.7	53.	II-4_3	1.81	17.23
23.	I-1_{12}	2.27	4.7	54.	I-7_3	1.77	4.38
24.	I-11_1	3.5	33.9	55.	I-10	1.6	21.0
25.	I-14_1	7.3	15.4	56.	I-1_9	1.3	22.0
26.	I-1_1	1.7	6.8	57.	I-7_2	3.4	36.9
27.	I-BH	1.6	15.0	58.	I-9_2	2.5	6.02
28.	I-1_5	2.2	31.1	59.	I-2_1	4.8	7.1
29.	I-8_2	2.7	33.2	60.	I-7_1	2.7	27.4
30.	I-9	3.9	39.2	61.	I-11_2	2.6	17.5
31.	I-8	0.8	18.9				

After 72 h of cultivation, all strains tested demonstrated CAT activity. As can be seen from Table 5, they all produced intracellular CAT. The enzyme activity ranged widely between 0.8 and 40.2 U/mg protein.

Of the 61 strains studied, 28 appeared to be good producers with activity above 10 U/mg protein. Strains P29 (28.9 U/mg), III-11_2 (40.2 U/mg), P27 (35.8 U/mg), I-9 (39.2 U/mg), III-8_1

(31.0 U/mg), I-8_2 (33.2 U/mg), I-1_5 (31.1 U/mg), etc. demonstrated synthesis of CAT with high activity.

Analysis of the results did not reveal a clear relationship between catalase biosynthesis and strain membership in the temperature classes (psychrophilic, psychrotolerant, and mesophilic). High enzyme activity was found in both psychrotolerant and mesophilic representatives isolated from Antarctic soil samples and adapted to the living conditions there. In the low temperatures of Antarctica, mesophiles are preserved as spores and only germinate during the short summer or when they find favorable conditions.

2.1.2. Distribution of extracellular CAT in strains of different taxonomic groups

In terms of application, extracellular enzymes have many advantages over intracellular ones. They are economically more advantageous and easier to obtain in purified form. As is well known, CAT is localized in the cytoplasm and peroxisomes and is very rarely found outside the cells. Reports of extracellular CAT production by microorganisms are extremely rare. The Antarctic strains we studied showed the synthesis of extracellular CAT, but its activity was lower than intracellular CAT (Table 5). Strains showing relatively good production ability are emerging. Twenty-seven strains showed the presence of extracellular CAT, as the strains II-65, M0, and I-13 showed the highest levels (above 4 E/mg). Scientists reported 255 fungal strains of the genera *Penicillium, Talaromyces* and *Aspergillus* synthesized extracellular CAT (Kurakov et al., 2001). The apical and subapical regions of the mycelial pellet are hypothesized to be the sites of extracellular CAT production and secretion in submerged cultivation, which is influenced by the hyphae morphology in the outermost layer of the pellet.

2.2. Selection of model strains belonging to different temperature classes

2.2.1. Comparative analysis of CAT synthesis data in different strains

Changes in fungal growth were observed under submerged cultivation at different temperatures (15 and 25°C) (Fig. 2). The temperature significantly affected biomass formation depending on the strain. From the total isolated strains, nineteen with higher catalase activity were used to select model strains. Six strains out of the tested nineteen strains classified as psychrotolerant exhibited a higher amount of biomass at 15°C than at 25°C. Furthermore, 11 strains growing better at 25°C than at 15°C can be classified as mesophilic. Strain I-BH, which shows no temperature-dependent growth, can also be included in this thermal class. Our results

confirm published data about a low number of isolated psychrophilic fungi. As noted above, the Antarctic mycoflora consists mainly of mesophilic and psychrotolerant strains due to the significant temperature variations in the region.

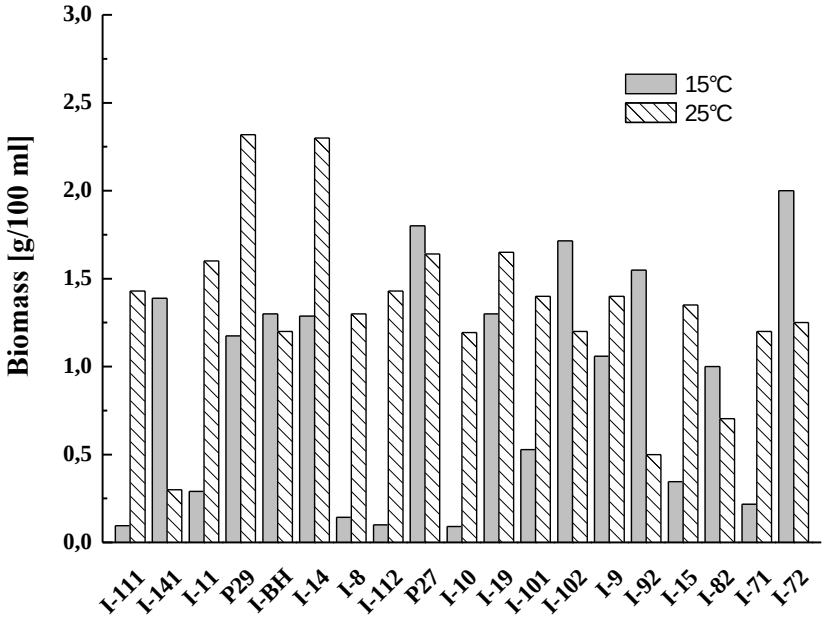

Figure 2. Relationship between cultivation temperature and biomass accumulation

Selected Antarctic strains were cultivated for 72 h at 15 and 25°C. Intracellular catalase activity was determined and presented in Figure 3A. All strains produced intracellular catalase at both temperatures tested. Among them, strains I-BH and I-14_1 showed temperature-independent synthesis of the enzyme. In contrast, sixteen catalase-producing strains achieved maximal enzyme activity at 25°C, which was significantly reduced at 15°C. As can be seen in Fig. 9 and Fig. 10A, the temperature optimum for catalase activity coincides with the optimum growth temperature of most of the strains tested (25°C). Interestingly, strains I- $1\text{-}4_1$, I-1_6, I-10_2, and I-7_2 showed a difference in their preference for the optimum temperature. Furthermore, isolate I-9_2 was found to produce 5.5-fold higher catalase activity at 15°C than at 25°C.

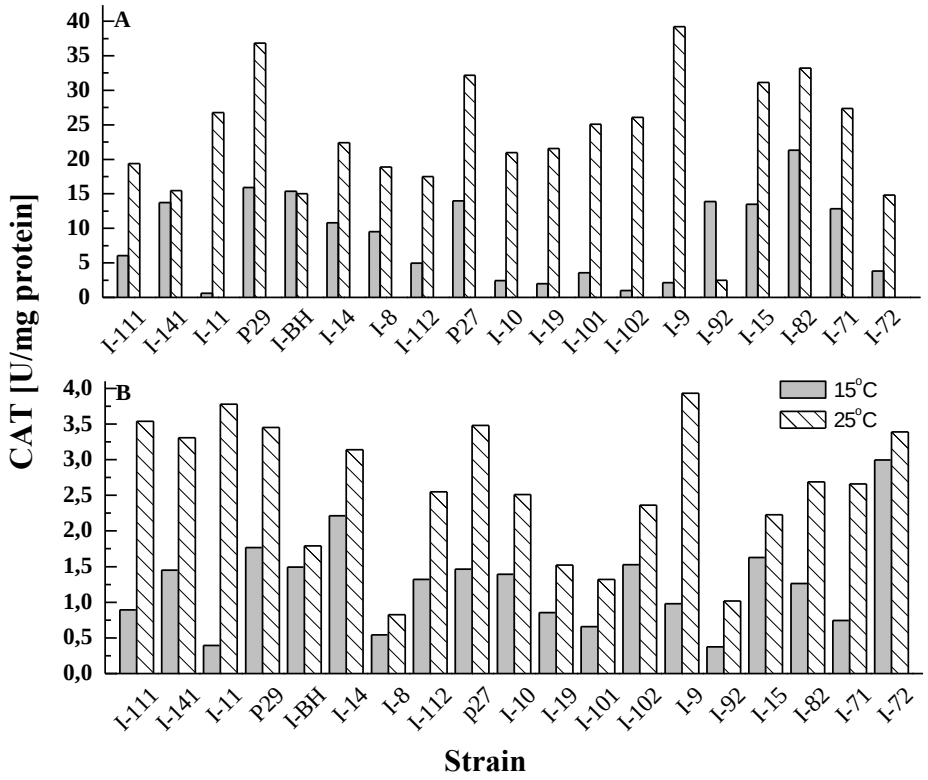

Figure 3. Synthesis of CAT from Antarctic strains cultured at different temperatures. (A)-intracellular activity; (B)-extracellular activity

The ability of the strains tested to synthesize the extracellular enzyme protecting cells from exogenous oxidative damage is presented in Fig. 3B. Almost all Antarctic isolates demonstrated extracellular CAT synthesis under submerged cultivation. Higher activity was found at 25°C than at 15°C. Our results showed that good synthesis of extracellular catalase is found in eight strains, - I-11_1, I-14_1, I-11, P29, I-14, I-16, I-9, and I-7_2. Intracellular enzyme activity was about 9-fold higher compared to the extracellular fraction of the enzyme, confirming our previous studies (Krumova et al., 2012). Fiedurek et al. (2003) found that *P. cyclopium* isolated from Arctic tundra synthesized both enzymes and the ratio of intracellular catalase to extracellular catalase has been found to be 1:3. Similar results were reported by Kacem-Chaouche et al. (2005).

2.2.2. Selection of CAT-synthesizing strains belonging to different thermal classes

After classifying the isolates into the respective thermal classes, two model strains were selected with high CAT activity: *Penicillium griseofulvum* P29 - a representative of

psychrotolerant Antarctic fungi, and *Penicillium chrysogenum* P27 - a representative of mesophilic Antarctic fungi. Both strains not only showed high enzymatic activity but also kept it stable over many fermentation processes, including large-scale transition from flasks to fermenter.

P. griseofulvum P29 *P. chrysogenum* P27

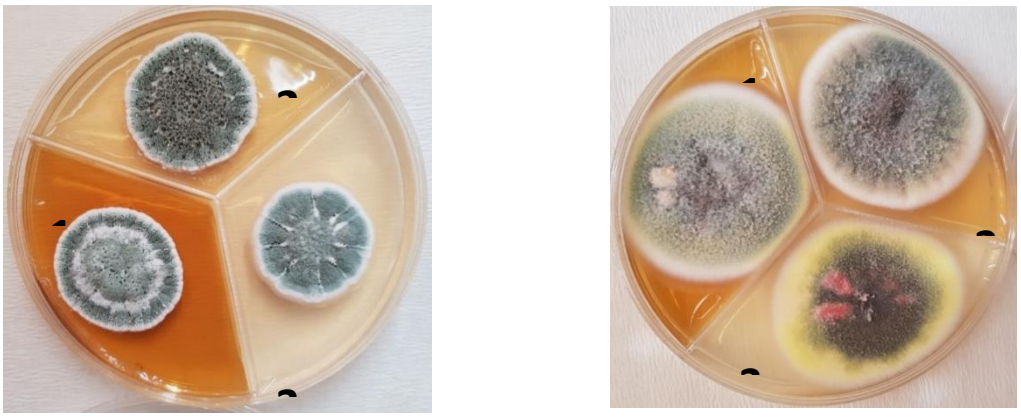

Figure 4. Macroscopic images of the selected model strains cultured on different agar media: 1- malt agar; 2- potato-glucose agar; 3- Chapek-Dox agar**.**

The model strains *P. griseofulvum* P29 and *P. chrysogenum* P27 were cultured on different agar media (Fig. 4). They showed good growth with maximum colony diameter achieved on the 5^{th} day. Different media led to changes in the morphology of the strains. Differences in conidia coloration are observed as well: *P. griseofulvum* P29 produced the darkest-coloured conidia on ChDA and *P. chrysogenum* P27 on the same media. It is very typical for *P. griseofulvum* to form white radial zones on MA, and for *P. chrysogenum* species to secrete a red pigment on ChDA.

Both strains were studied for their development at different temperatures in 3 L bioreactors.

As can be seen from the results presented in Fig. 5 A, although the optimum temperature for strain P. griseofulvum P29 appears to be 25ºC, it grows very well at 10 as well as 15 and 20ºC, confirming its psychrotolerant nature.

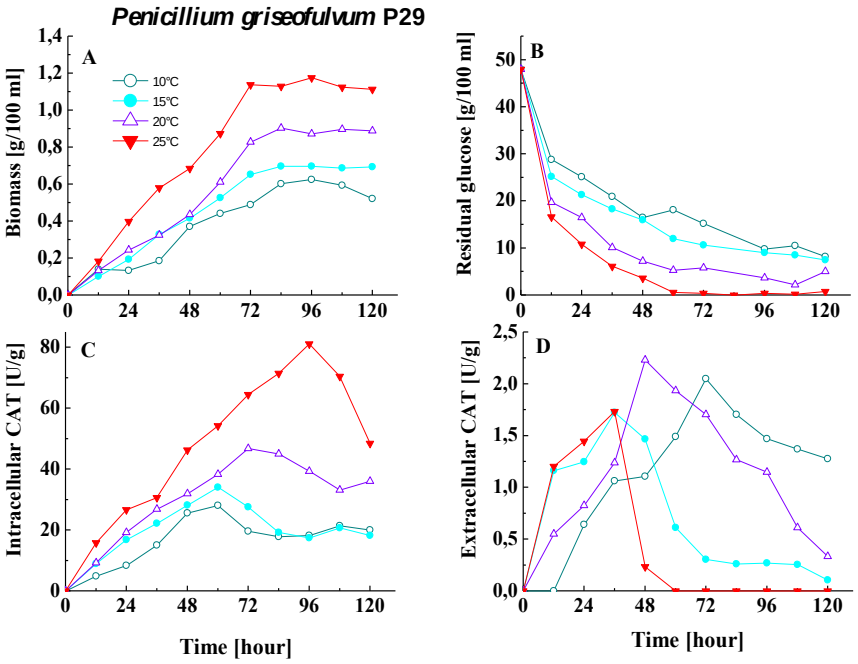

Figure 5. Dynamics of growth (A), glucose uptake (B), and synthesis of intracellular (C) and extracellular (D) CAT in *P. griseofulvum* strain P29 cultured in 3 L bioreactor at 10, 15, 20 and 25ºC, respectively.

Such strains are much more common than the psychrophilic strains, regardless of the site of isolation. More than 50% of the isolates are facultative, not obligatory, psychrophiles that can grow at temperatures ranging from 0 to 25ºC, even in extremely cold environments.

Meanwhile, *P. chrysogenum* P27 (Fig. 6A) showed growth typical of mesophilic organisms: it does not develop at 10 and 15ºC, showing accelerated growth when the temperature increases to 20 and 25ºC.

The temperature of cultivation also affected glucose consumption (Figs. 5B and 6B). The temperature lowering to 15 and 10°C caused inhibition of the process. The psychrotolerant strain demonstrated delayed glucose consumption compared to the mesophilic strain. This is probably one of the adaptive mechanisms to low temperatures.

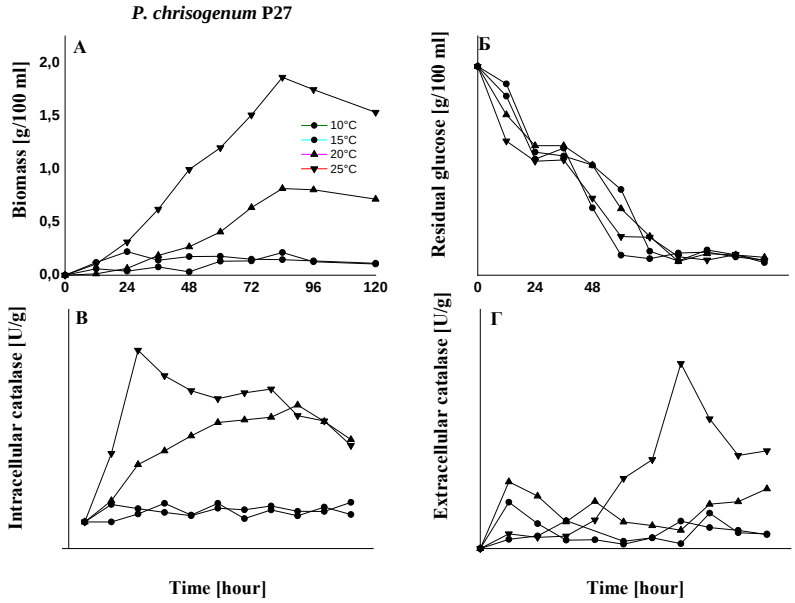

Figure 6. Dynamics of growth (A), glucose uptake (B), and synthesis of intracellular (C) and extracellular (D) CAT in *P. chrysogenum* strain P27 cultured in 3 L bioreactor at 10, 15, 20 and 25°C, respectively**.**

Both the model strains are efficient producers of intracellular CAT (Figs. 5B and 6B). Maximum synthesis occurs at 25ºC, but strain *P. griseofulvum* P29 shows 2-fold higher enzyme activity compared to *P. chrysogenum* P27. The higher level of CAT in the psychrotolerant strain at 10ºC is a clear indication of the involvement of this antioxidant enzyme in adaptation to low temperatures. The presence of extracellular CAT in both model strains is a rare phenomenon in fungi. The mesophilic *P. chrysogenum* P27 showed a notable acceleration of extracellular CAT synthesis at the end of the stationary phase (84 h) at 25°C, while the psychrotolerant strain *P. griseofulvum* P29 showed no effect of culture temperature on this synthesis (Fig. 5D and Fig. 6D).

3. ROLE OF CATALASES IN THE CELLULAR RESPONSE OF MODEL STRAINS TO LOW-TEMPERATURE STRESS

3.1. Morphological adaptations at low temperatures

Monitoring the effects of low temperatures on filamentous fungus' growth, development, and sporulation

The effect of different temperatures (6, 10, 15, and 25°C) on conidia outgrowth, growth rate, hyphae length and diameter, and conidia formation of the model strains *P. chrysogenum* P27 and *P. griseofulvum* P29 was studied (Table 2). Macroscopic studies support the hypothesis that

the isolated Antarctic strains possess different mechanisms for successful adaptation to low temperatures. Accelerated growth of the substrate mycelium and, to a lesser extent, the aerial mycelium was observed. Cultivation at low temperatures results in circular colony expansion in the form of multicellular meristem clusters.

Table 2: Colony development of the model strains at different culture temperatures

$T^{O}C$	*P. chrysogenum* P27				*P. griseofulvum* P29			
	colony diameter [mm]/ time of cultivation [days]				colony diameter [mm]/ time of cultivation [days]			
	3	5	7	9	3	5	7	9
6	-	-	-	0.6	0.1	0.9	1.2	1.6
10	-	-	0.5	0.9	0.2	0.9	1.5	1.8
25	3.8	6.8	9.0	9.0	3.1	6.7	8.4	8.4
30	2.7	5.9	7.3	7.3	2.2	4.9	6.1	6.1

The elongation rate of hyphae is also temperature-dependent (Table 3). Both strains showed better development at 15 and 25ºC. At low temperatures, *P. griseofulvum* P29 showed faster growth and a higher elongation rate compared to *P. chrysogenum* P27.

Table 3. The growth rate of hyphae of strain *P. griseofulvum* P29 and *P. chrysogenum* P27 at different temperatures

T°C	Growth rate of hyphae (mm/day)	
	P27	P29
6	.00*	0.00***
10	0.09***	0.23***
15	2.87**	1.25**
25	5.76**	4.87*

[a] Values were calculated from growth curves between days 3 and 24 and are averages of measurements at 18 - 36 hyphae. The significance of the correlation coefficient is as follows: *($p<0.05$); **($p<0.01$) and ***($p<0.001$)

3.2. Alterations in biomarkers of oxidative stress

3.2.1. Monitoring the level of generated ROS under normal physiological conditions and under conditions of short-term cold stress

Changes in cellular biomarkers of oxidative stress in model strains placed under conditions of short-term (6 h) low-temperature stress (15ºC and 6ºC) and subsequent recovery to their optimal growth temperature were investigated.

Table 4 demonstrates the effect of low temperatures on changes in superoxide radical ($\bullet O_2^-$) and H_2O_2 levels in cells from the model strains: the psychrotolerant *P. griseofulvum* P29 and the mesophilic *P. chrysogenum* P27. In all the variants, an elevated level of radicals was determined.

The drastic temperature decrease caused an imbalance in the redox status of both strains through accelerated generation of ROS. The results demonstrated that this process is more active in:

- mitochondrial fractions than in whole cells;
- the mesophilic strain *P. chrysogenum* P27, compared to the psychrotolerant *P. griseofulvum* P29

As is well known, even cells in a normal physiological state, including those of fungi, form ROS as a result of the one-electron reduction of 2% of the oxygen consumed. Under various extreme influences, the production of these radicals is accelerated (Gómez-Toribio et al., 2009; Gocheva et al., 2009; Krumova et al., 2009; Sánchez, 2017). Results of our experiments showed significant activation of this process under low-temperature stress conditions depending on the stress degree and the temperature characteristics of the model strain.

Table 4. Increase in $\bullet O_2^-$ and H_2O_2 production in whole cells and mitochondrial fractions in *P. griseofulvum* P29 and *P. chrysogenum* subjected to low-temperature shock

Variant	$\bullet O_2^-$ (μM/mg dry weight per hour)			
	P. griseofulvum P29		*P. chrysogenum* P27	
	Whole cells	Mitochondrial fraction	Whole cells	Mitochondrial fraction
Control	3.59±0.03	5.24±0.03	4.70±0.02	0.55±0.01
15ºC	4.15±0.04	6.50±0.01	6.78±0.01	2.25±0.01
6ºC	7.05±0.10	7.47±0.05	11.06±0.07	3.63±0.02

Variant	H_2O_2 (μM/mg dry weight per hour)			
	P. griseofulvum P29		*P. chrysogenum* P27	
	Whole cells	Mitochondrial fraction	Whole cells	Mitochondrial fraction
Control	5.68±0.18	12.11±0.10	11.39±1.10	6.40±0.28
15ºC	6.22±0.40	15.00±0.37	13.72±1.09	9.79±0.33
6ºC	9.18±0.11	21.57±0.44	14.61±0.56	11.58±0.43

It is rare to find such a direct analysis of changes in ROS levels in filamentous fungi in the scientific literature. Chattopadhyay et al. (2011) reported a higher production of ROS in Antarctic *Pseudomonas fluorescens* MTCC 667 after low temperatures treatment compared to that cultivated at the optimal 22ºC. According to the authors, low-temperature stress causes a decrease in the rate of enzymatic reactions, which leads to a decrease in ATP consumption and electron accumulation in some stages of the respiratory chain. This situation is the basis for a sharp increase in the ROS levels.

3.2.2. Determination of the oxidatively damaged protein levels by the amount of formed carbonyl groups

The changes in the levels of cells oxidatively damaged proteins from the model strains placed under 6 h of low-temperature stress (15ºC and 6ºC) and in the recovery phase at the optimal developmental temperature (Fig. 7) were evaluated. The content of carbonyl groups in the control variant of both strains showed no significant changes. All the other variants, however, showed a sharp increase in this indicator immediately after the onset of stress, which persisted with time. The increase in the amount of carbonyl groups was dependent on the degree of stress and the strain type. Exposure to 15°C in both strains resulted in a continuous increase in protein damage until the end of the stress and a decrease in the subsequent phase. At 6°C, a sharp and rapid increase was observed in the first 2-4 hours, and a level of protein carbonylation was maintained in the remaining periods studied.

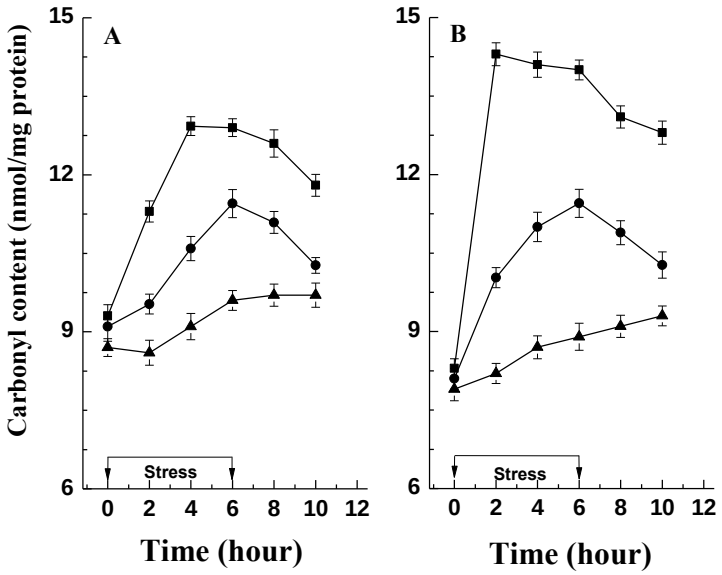

Figure 7. Production of oxidatively damaged proteins in the psychrotolerant strain *P. griseofulvum* P29 (A) and the mesophilic strain *P. chrysogenum* P27 (B) at optimal temperature (▲) and as a consequence of stress when temperature is reduced from optimal to 6°C (■) and to 15°C (●).

The psychrotolerant strain showed higher stability in terms of oxidative damage to proteins compared to the mesophilic strain. Furthermore, although a significant decrease in the amount of carbonyl groups was observed after the cessation of stress, the values at the end of the experiment remained much higher than those of the control variant. These results provide evidence for the role of ROS in changes in cellular proteins as part of adaptation to extremely low temperatures. A similar cellular response has been found in other mycelial cultures subjected to oxidative stress (Hua et al., 2017). According to Li et al. (2008), the temperature-induced carbonylation of proteins mainly affects respiratory enzymes and those localized in mitochondria, as ROS of the respiratory chain is the main source of oxidative damage found during stress.

3.2.3. Investigation of changes in the level of reserve carbohydrates - glycogen and trehalose

The bioindicators of oxidative stress include changes in the level of reserve carbohydrates - glycogen and trehalose. Fig. 8 presented the effect of lowering the temperature from the optimum to 6ºC or 15ºC on this stress biomarker.

There was a sharp increase in the content of both reserve compounds immediately after the onset of stress.

The amount of glycogen in the cells of psychrotolerant *P. griseofulvum* strain P29 increased from 1.2- to 1.8-fold above the basal level, accompanied by a 1.7-fold higher amount of

trehalose. A higher baseline level of both reserve carbohydrates was determined in the mesophilic strain *P. chrysogenum* P27 compared to *P. griseofulvum* P29. Under stress conditions (6°C or 15°C), an additional significant increase in the amount of glycogen (1.3- and 1.4-fold) and trehalose (2.2- and 1.9-fold) was observed

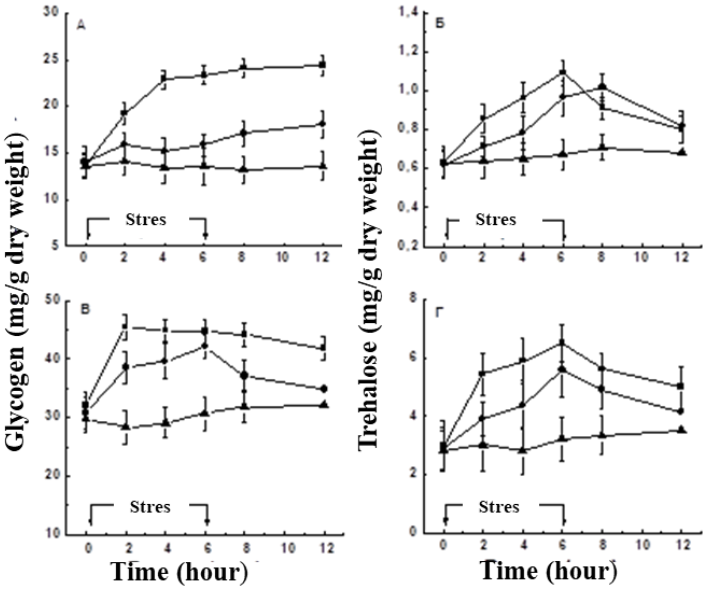

Figure 8. Glycogen (A, C) and trehalose (B, D) contents in *P. griseofulvum* P29 (A, B) and in *P. chrysogenum P27*(C, D) at optimum temperature (▲) and as a consequence of stress when the temperature is reduced to 6°C (■) or to 15°C (●).

These data clearly demonstrated the dependence of the reserve carbohydrate amount of the stress degree and the temperature characteristic of the model strain. After restoration of optimal culture conditions, the level of glycogen and trehalose showed a decreasing trend to the level of the control. The results obtained in these experiments demonstrated the essential role of trehalose and glycogen for the development of fungi from extremely cold habitats. It has been suggested that trehalose minimizes oxidative damage of ROS on proteins and lipids (da Costa et al., 2008; Jain, 2009). It slows down the rate of formation of protein aggregates and its presence in the lipid bilayer is mandatory.

3.2.4. Study of lipid peroxidation as a marker of the membrane integrity degree

As a marker of lipid peroxidation, the amount of malondialdehyde (MDA) in stressed and non-stressed cells was determined (Fig. 9).

After lowering the temperature to 6 and 15°C, a significantly higher amount of malondialdehyde (MDA) was recorded compared to the control. This raise was dose - and time-dependent.

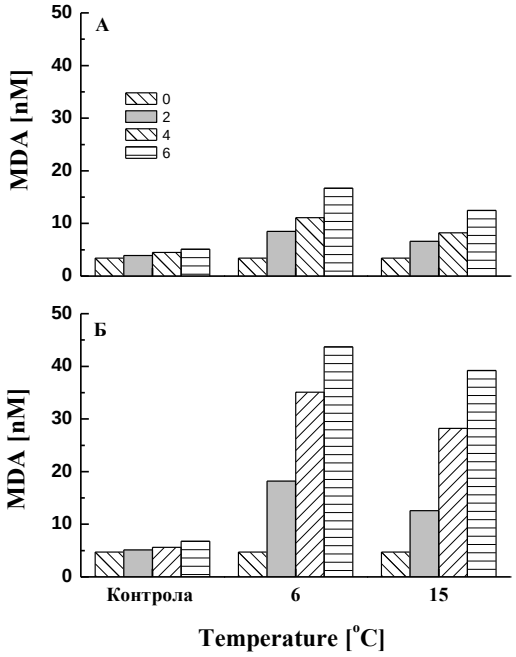

Figure 9. Lipid peroxidation level in cells of *P. griseofulvum* strain P29 (A) and *P. chrysogenum* P27 (B) cultured at optimal temperature (control) and under cold stress conditions (6 and 15°C) for 6 hours

The accelerated accumulation of damaged lipids in the mesophilic strain *P. chrysogenum* P27 is more significant than in the psychrotolerant strain *P. griseofulvum* P29, which exhibits a higher tolerance to low temperatures, probably due to its adaptation to extreme temperatures.

3.2.5. Study of the antioxidant defense enzymes activity

The cellular response against oxidative stress involves changes in antioxidant enzyme defenses. In the experiments conducted, the activity of the antioxidant enzymes SOD and CAT was examined under conditions of short low-temperature stress (Fig. 10). Exposure to temperatures of 6 and 15 °C resulted in activation of antioxidant enzyme protection. Changes in the activity of the first antioxidant enzyme, SOD, during stress and in the post-stress period are shown in Fig. 10A, C.

At the 12th hour, the SOD activity in mycelia of *P. griseofulvum* P29 treated with 6°C was about 6 times higher than the control. In *P. chrysogenum* P27 the change was about 3-fold. Short-term stress caused by lowering the temperature to 15°C did not lead to significant changes and the trend was more similar to the control variant. In the study of the second antioxidant enzyme, lowering the temperature to 15 and 6°C also induced the synthesis of the antioxidant

enzyme, CAT (Fig. 10 B, D). Lowering the temperature to 15°C for six hours did not lead to significant changes, and the observed trend was close to the control variant. Induction of the second antioxidant enzyme CAT activity at temperatures of 15 and 6°C also was observed (Fig. 10 B, D). The cellular response of cultures from both Antarctic strains showed a more pronounced increase in CAT activity after exposure to 6°C compared to that at 15°C. In *P. griseofulvum* P29, the two maxima of its activity were observed.

SOD activity increased noticeably with the temperature downing to 6°C. At the 6^{-th} hour from the onset of stress, this increase was 2 or 3-fold time more compared to controls, for *P. griseofulvum* P29 and *P. chrysogenum* P27, respectively. This trend persisted for hours after cessation of the stress.

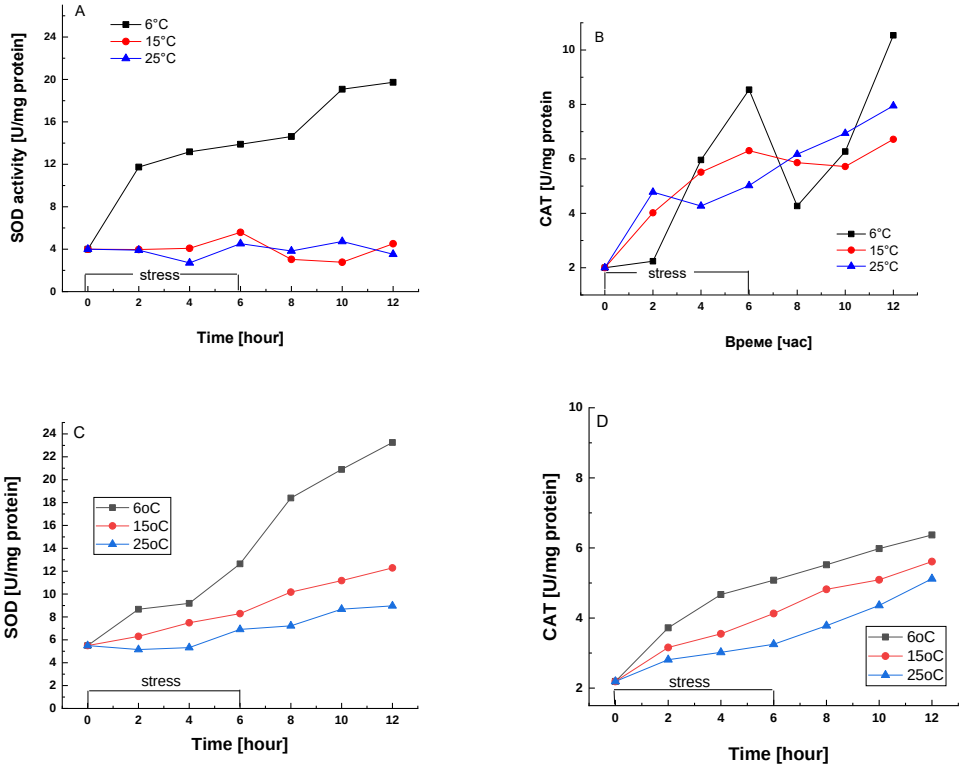

Figure 10. Changes in the activity of the antioxidant enzymes SOD (A, C) and CAT (B, D) in *P. griseofulvum* strain P29 (A, B) and *P. chrysogenum* P27 (C, D) cultured at optimum temperature (control) and under cold stress conditions (6 and 15°C) for 6 hours

The results outlined a more pronounced increase in SOD for the mesophilic strain, whereas the activation of antioxidant enzyme defense in the psychrotolerant strain was more due to increased CAT activity. One possible explanation is the higher level of H_2O_2 in cultures of *P.*

griseofulvum P29. For SOD disposal, *P. chrysogenum* P27 induces SOD synthesis to a greater extent, whereas *P. griseofulvum* P29 increases the level of CAT enzyme more substantially.

All this confirms the fine balance between oxidants and antioxidant protection upon temperature stress. The levels of different types of ROS act as stress signals that control the expression of the corresponding genes encoding antioxidant enzymes.

Based on the results obtained, it was found that strain *P. griseofulfum* P29 is a better producer of the catalase enzyme. As a psychrotolerant strain, it is a potential producer of cold active enzymes and further experiments are being continued with it.

Ultrastructural changes in the model strain cultured under normal physiological conditions and under low-temperature stress

Some basic parameters of fungal morphology of *P. griseofulvum* P29 were also studied. The results reported in Fig. 11 showed that temperatures of 6 and 15ºC are not the most favorable for the development of the model strain. In these cases, a shorter length of the main hyphae (Fig. 11A) and of the branching units (Fig. 11B) were observed, as well as the lowest number of growth units (Fig. 11D).

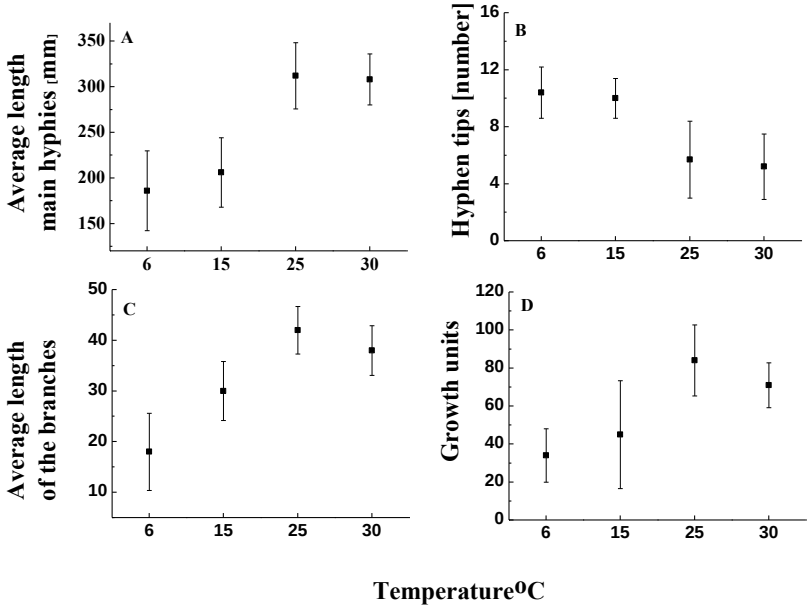

Figure 11. The average values of the main hyphae length (A); number of apices (B); branching length (C) and number of growth units (D) in the mycelia of *P. griseofulvum* P29 cultured deeply at 6, 15, 25, and 30°C

Much longer hyphae with fewer branches are reported at temperatures of 25 and 30ºC,. Under these conditions, the number of growth units also decreases. Such morphological changes are typical for the development of filamentous fungi under stress conditions. Kreiner et al. (2003) and Islam et al. (2017) demonstrated similar phenomena in *A. niger* and *A. nidulans* cultivated in the presence of different stress factors. The strain cellular response, in addition to reduced growth rate, included the formation of pellets with short and highly branched hyphae.

Determination of ultrastructural changes in cells subjected to cold stress

Using electron microscopy, the ultrastructural changes in *P. griseofulvum* P29 cells placed under low-temperature stress conditions were determined and their electron density, cytoplasmic homogeneity, free ribosome content, accumulation of fat-droplets near the cytoplasmic membrane, vacuole formation, and mitochondrial state in hyphae were monitored. In the sample cultured at the optimum temperature of 25℃, an alteration of the surface relief of hyphae was found, similar to that we observed in other species of filamentous fungi. We interpret this variation by the presence of hyphae at different degrees of maturity.

Cells cultured at the optimal growth temperature of 25℃ were taken as the "control" in the present experiment. Samples were collected at hour "0" and hour "6 stress".

Scanning electron microscopy (SEM)

Younger hyphae are thin, fine, and smooth-surfaced. The more mature ones are characterized by the presence of areas of undulating relief and show indentations - areas from which young hyphae have broken off. The sample stressed for 6 hours at 15ºC contains smooth fine hyphae similar to those found in the control sample. The more mature hyphae, however, have a damaged relief - a rough, uneven surface. The sample stressed for 6 h at 6ºC was dominated by hyphae with a wrinkled surface and rough relief, regardless of their thickness (Fig. 12).

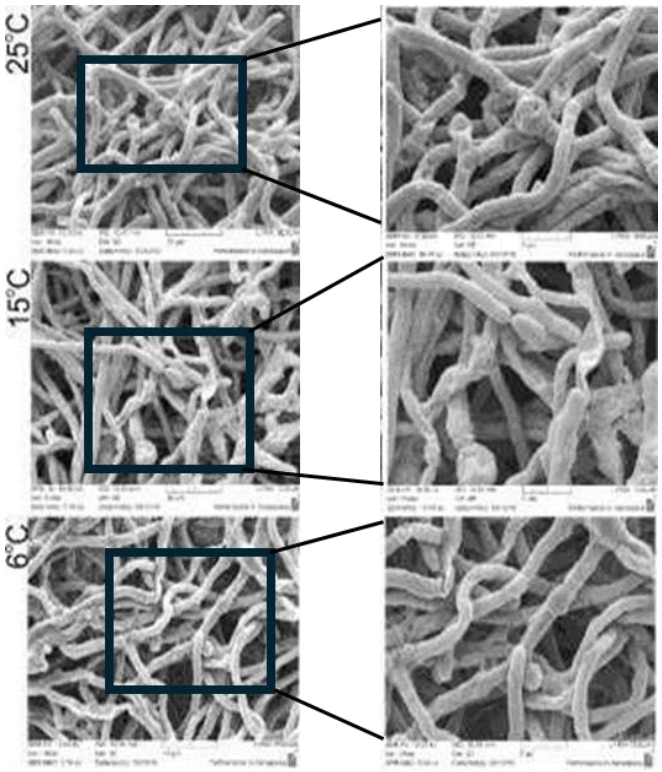

Figure 12. Comparative scanning electron microscopic study on the effect of temperature stress on the surface relief of *P. griseofulvum* P29 hyphae/

Transmission electron microscopy (TEM)

The ultrastructural characteristics of the two samples do not differ and are described in general. In Fig. 20, the samples are arranged according to their degree of development. Young hyphae have relatively dense cytoplasm. The cell wall contains up to three layers of differing electron density and fine filamentous elements are detectable on its outer side. The cell membrane is finely wavy, with more or less distinct eisosomes in some areas. Mitochondria have moderate electron density and well-defined cristae. In the course of differentiation, vacuoles are formed in the hyphae. They are characterized by the presence of electron-dense elements - initially single grains, which may subsequently aggregate into larger inclusions, and in other cases, the vacuole contents form an electron-dense network.

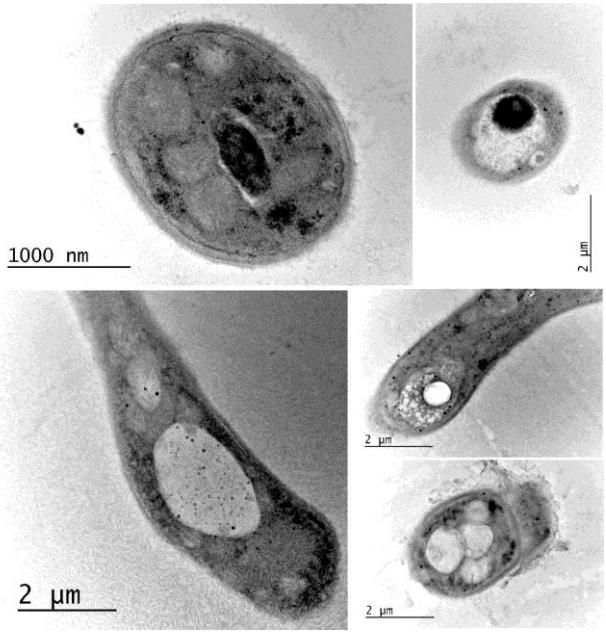

Figure 13. Transmission electron microscopy of mycelia of *P. griseofulvum* P29 cultured at a temperature of 25℃

Further stages of hyphal differentiation are characterized by the development of autolysis processes. The mitochondria are disintegrated, with a translucent matrix of single cristae. On the cell wall in such areas, an accumulation of coarse filamentous material is found, probably corresponding to the rough relief observed on the SEM.

After 6 h of temperature stress at 15ºC (Fig. 14), the main damage to the ultrastructure of the cells was to the mitochondria and cell wall.

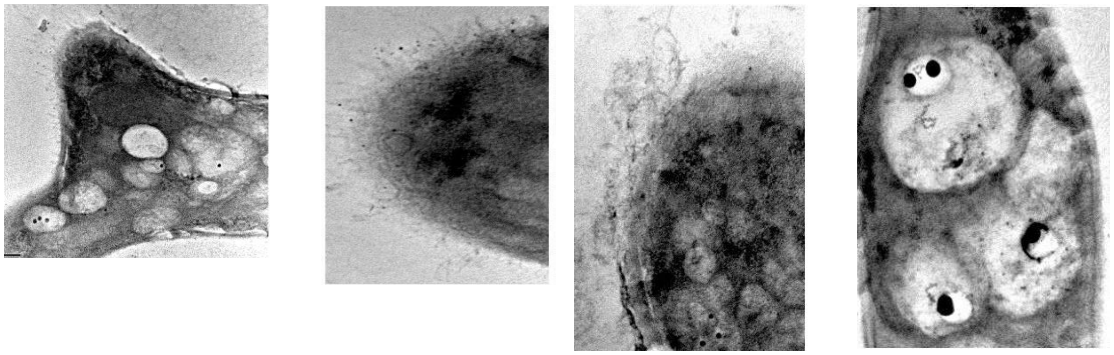

Figure 14. Cells of *P. griseofulvum* P29 after 6 hours of temperature stress at 15^{o}C

The number of distended mitochondria with transparent matrix and reduced number of cristae increased significantly. The cell walls of hyphae are often irregular in structure. Where extracellular filamentous formations similar to those observed in the control are found, the

presence of electron-dense fine granules is additionally characteristic. Cell wall disruption loci and disturbed cell wall density are observed in the more severely disrupted locations.

Compared to the sample stressed at 15ºC, 6 h of temperature stress at 6ºC (Fig. 15) resulted in a substantial extension of the range of alterations described above.

Degenerative disorders in the structure of mitochondria are typical. The cell walls are irregular, with areas of thinning and delamination and detachment, and there is also a superficial accumulation of detrital material. As a significant difference from the samples described above, a substantial alteration in processes related to cell membrane dynamics is reported here.

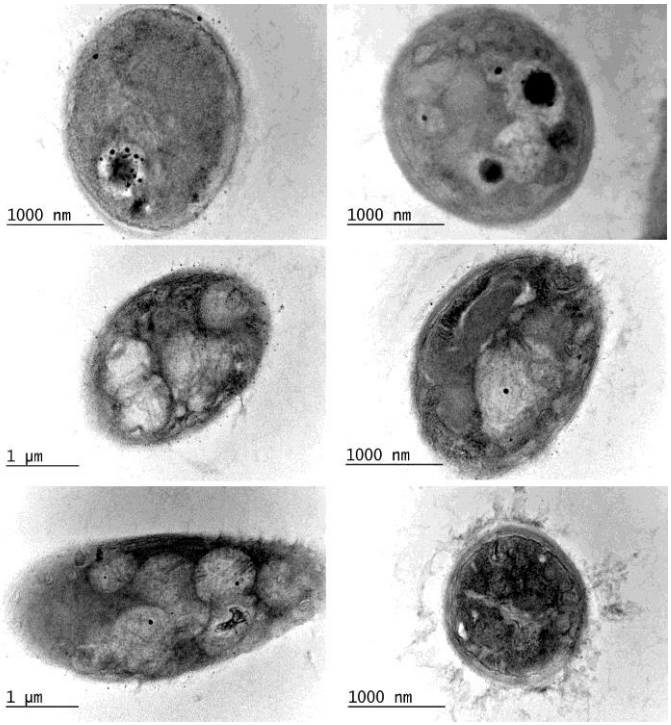

Figure 15. Cells of *P. griseofulvum* P29 after 6 hours of temperature stress at 6°C

Eisosome-like structures ubiquitously penetrate deep into the cytoplasm of hyphae. In a working interpretation, it can be assumed that low-temperature stress disrupts mitochondrial function and the renewal processes of the surface components of hyphae. A comparison of the data acquired using the two-electron microscopic techniques showed that the hyphal relief had become rougher, most likely as a result of the accumulation of cell wall components.

Temperature stress leads to changes in cell organelles. Mitochondria are the most sensitive, as the damage to their ultrastructure is typical and ubiquitous in both samples subjected to low-temperature stress. Cells at 6ºC were characterized by a significant expansion of

eisosomes, indicating impaired export and import of macromolecules or disturbances in cell membrane renewal processes.

4. CATALASE GENES

4.1. Identification and sequencing of genes encoding proteins with catalase activity in the model strain

4.1.1. Identification of genes encoding a protein with CAT activity

Molecular genetic experiments have resulted in the identification and sequencing of catalase genes in Antarctic strain *P. griseofulvum* P29. The analysis of the obtained sequences (nucleotide sequences) revealed different types of catalase genes in isolates with different activities and different temperature optima. The obtained data are an excellent basis for further studies.

By gradient PCR (BioRad apparatus) the conditions for PCR reactions with the different primer pairs were optimized. DNA Purification Kit (Himedia), QB-96 Satellite Gradient thermal PCR cycler (LKB Vertriebs GmbH, Vienna, Austria), PCR master mix (GenetBio, South Korea), GeneJET PCR Purification Kit (Thermo Fisher Scientific Inc.) were used in molecular genetic experiments. PCR products were visualized by 1% gel electrophoresis.

4.1.2. Selection, purification, and sequencing of the resulting gene fragments

Most fungi have several monofunctional heme-containing catalases. They exhibit great diversity in their structure, location, and function and vary by genus. For example, the cat A gene of *A. nidulans* is expressed specifically in spores, while others are associated with metabolism. In *Podospora anserina*, catalases are strictly required for efficient utilization of more complex biomasses such as sawdust, allowing growth in the presence of lignin (Bourdais et al. 2012). There is evidence for the presence of three catalase genes in *Neurospora crassa*, in *A. nidulans* two catalase genes have been described, with catalase genes from yeast and filamentous fungi placed in different gene families.

Based on the results obtained from the identification of the Antarctic isolate P29 as *Penicillium griseofulvum*, the complete genome sequences of this species available in public databases were examined. The complete sequence of *P. griseofulvum*, annotated as GenBank accession number GCA_001561935.1, identified 5 catalase genes (Banani et al. 2016). To prove

the presence of these genes also in *P. griseofulvum*P29, 5 pairs of specific primers were designed. Sequencing analyses resulted in products (about 550-600 bp) and these were found to be part of the 5 CAT genes mentioned above. As a next step, multiple new primers (Table 2) were developed to obtain the complete sequence of the genes.

Catalase genes in Antarctic strain *P. griseofulvum* P-29 - fully sequenced:

1. cat1 - encodes a bifunctional catalase-peroxidase enzyme, consists of 2444 bp, and encodes a protein of 744 amino acids. The closest match of this sequence (MW618002) is to the catalase-peroxidase gene katG (PGRI_023940) from *P. griseofulvum* PG3 (GenBank GCA_001561935.1; 99.72% similarity) and to *P. rubens* Wisconsin 54-1255 (GenBank AM920427.1; 87.44% similarity) (Van Den Berg et al., 2008; Banani et al., 2016).

The estimated molecular weight of the enzyme is ~82 kDa (Uniprot). A 718 bp fragment of the gene examined has 99% similarity to the bacterial catalase gene katG from Bradyrhizobium sp. BTAi1 (GenBank: CP000494.1, Giraud et al. 2007).

2. cat2 - encodes a monofunctional catalase enzyme, consists of 2445 bp, and encodes a protein of 730 amino acids. Based on a mega blast search of the NCBI nucleotide sequence database, the closest match to our sequence (MW618003) has a catalase gene PGRI_045000 from *P. griseofulvum* PG3 (GenBank GCA_001561935.1; 99.84% identity) and *P. rubens* Wisconsin 54-1255 (GenBank AM920431.1; 86.41% identity) (Banani et al. 2016; Van Den Berg et al. 2008). The estimated molecular weight of the enzyme is ~80 kDa (Uniprot).

3. cat3 – consists of 1673 bp and encodes a protein of 501 amino acids. For our sequence MW618004, the catalase gene PGRI_072960 from *P. griseofulvum* PG3 (GenBank GCA_001561935; 99.94% identity) and *P. rubens* Wisconsin 54-1255 (GenBank AM920437.1; 91.24% identity) are the closest hits. The estimated molecular weight of the enzyme was ~57 kDa (Uniprot).

4. *cat4* - encodes a monofunctional catalase enzyme. It consists of 1786 bp and encodes a protein of 490 amino acids. The closest match to our sequence is that of the catalase gene PGRI_034520 from *P. griseofulvum* PG3 (GenBank GCA_001561935; 99.94% identity). Based on a mega blast search of a nucleotide sequence database, the greatest similarity is with *P. rubens* Wisconsin 54-1255 (AM920431.1; 82.19% identity). The estimated molecular weight of the enzyme is ~54 kDa (Uniprot).

5. *cat5* - encodes a monofunctional catalase enzyme, consists of 1501 bp, and encodes a protein of 482 amino acids. The closest match of our sequence MW618006 is the catalase gene PGRI_025320 from *P. griseofulvum* PG3 (GenBank GCA_001561935; 99.73% identity). The greatest similarity is with the catalase gene (cnsD) in *Penicillium* sp. YT-2016 (KU932038.1; 95.28% identity) (Lin et al., 2016). The estimated molecular weight of the enzyme is ~54 kDa (Uniprot).

The identification of five catalase genes in the Antarctic strain *P. griseofulvum* P29 is not surprising. According to the literature, fungi have more than one catalase enzyme (Hansberg et al., 2012). The fungal CATs form a group of enzymes that show diversity in terms of their structure, location, and function. According to phylogenetic analyses, the number of catalase enzymes varies among species. The presence of at least four differentially regulated catalases is a criterion for the great ability of filamentous fungi to degrade H_2O_2. In *Pleurotus ostreatus,* two distinct catalases have been shown to play different roles during development or under conditions of temperature stress (Wang et al., 2017). For *Sclerotinia sclerotiorum*, two catalases related to antioxidant response, sensitivity to QoI fungicide, and pathogenicity have been reported (Huang et al., 2021). Five catalases have been reported in the literature on the filamentous fungus *Podospora anserina*, some of them playing a major role during lignocellulose degradation (Bourdais et al., 2012). The presence of secretory catalase 2, which is involved in the catabolism of phenolic substances, was detected in *Chaetomium thermophilum* var. *disitum* (Chovanová et al., 2019). (Kawasaki and Aguirre, 2001).

The *cat1* gene in *P. griseofulvum* strain P29, encoding a bifunctional enzyme catalase-peroxidase, belongs to the peroxidase family, peroxidase/catalase subfamily. Similar proteins have been described in *P. coprophilum, P. nordicum, P. polonicum, P. flavigenum and P. crustosum*. According to CDD (NCBI) and Expasy, the putative enzyme is a dimer with two identical subunits, each subunit consisting of two structurally homologous domains with a topology similar to that of a class I peroxidase. The active site is in the N-terminal domain. The heme-binding motif is present only in the N-terminal domain. The function of the C-terminal domain is not yet clear (Bertrand et al., 2004). This enzyme belongs to a subgroup of heme-dependent peroxidases and catalyzes a multistep oxidation reaction involving hydrogen peroxide as an electron acceptor. Catalase peroxidases can exhibit both catalase and broad-spectrum peroxidase activity, depending on the concentration of hydrogen peroxide. A fragment of the cat1

gene with 718 nucleotides has 99% similarity to the bacterial catalase gene katG from *Bradyrhizobium* sp. BTAi1 (GenBank: CP000494.1, Giraud et al., 2007). Similar data have been reported by other authors (Klotz and Loewen, 2003; Marcet-Houben and Gabaldón, 2010). This is probably part of the mechanism of adaptation to the environment.

The genes *cat2, cat3, cat4,* and *cat5,* encoding monofunctional, heme-containing catalases, belong to the catalase family. The *cat3* and *cat4* genes encode fungal catalases similar to yeast A and T. This CAT family has a relatively small subunit size forming tetramers and uses hydrogen peroxide to oxidize various substrates such as alcohol or phenols.

4.2. Expression study of cat genes found in the model strain

The expression of the five catalase genes from *P. griseofulvum* P29 cultured at different temperatures: 10°C and 25°C was investigated. Specific RT-PCR primers for the five catalase genes and three reference genes (housekeeping genes) were designed for *P. griseofulvum*. For all primer pairs, we obtained a single product of about 120bp. The purity of the RNA was demonstrated after DNase I purification. The performance of the primers was evaluated with cDNA and SYBR® Green Supermix with the 8 primer pairs in 2 replicates. Products of ~120 bp were obtained in all 16 reactions and two negative controls were identified.

Selection of a reference gene (housekeeping gene). Three pairs of primers for reference genes have been created: 18S, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and Actin. After analyzing the results 18S and GAPDH genes were selected to be used as a reference, they showed relatively equal expression under both temperature regimes.

Basic experiment to study the expression of 5 catalase genes in a strain cultured under normal physiological conditions and under conditions of low-temperature stress.

The expression levels of the detected catalase genes were examined at 10 and 25°C. A 96-well plate (Multiplate™ 96-Well PCR Plates, low profile, unskirted, clear) was used for the CFX96 Touch Deep Well Real-Time PCR Detection System. Data were analyzed by relative quantification with threshold cycle method (ΔΔCt) and CFX Maestro Software (Bio-Rad). The genes *cat1, cat2, cat3,* and *cat4* were found to have a statistically significant increased expression at 10°C, with the largest difference observed in the cat1 gene (Fig. 16). It is likely that the function of the *cat1* catalase-peroxidase gene is related to the antioxidant response and ROS elimination in mycelial cells at low temperatures. According to Pongpom et al. (2005), the

induced expression of the catalase-peroxidase gene of a pathogenic *P. marneffei* strain at elevated temperature is associated with culture survival in host cells.

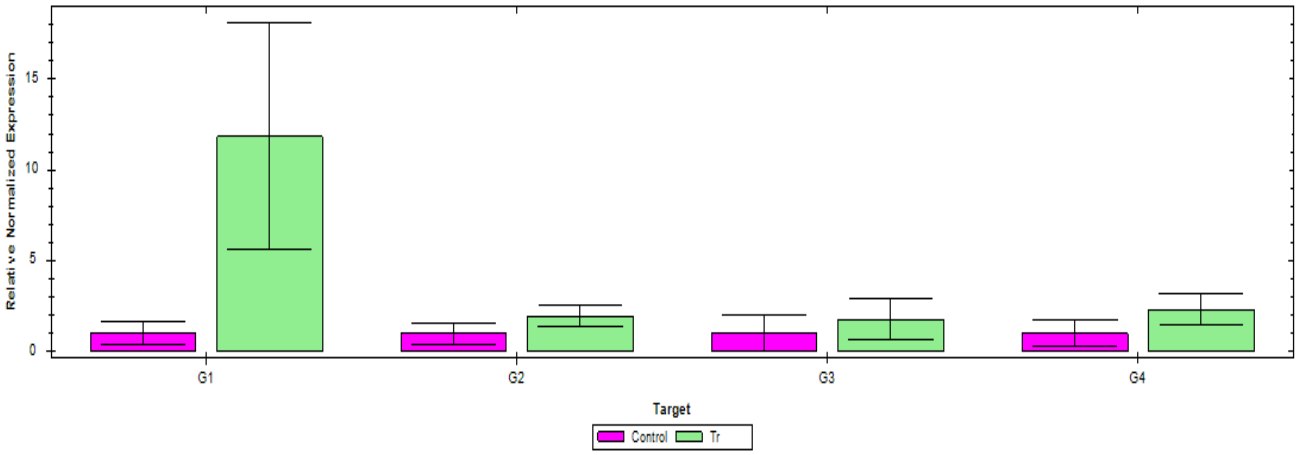

Figure 16. Relative mRNA expression determined by the $_{\Delta\Delta}$Cq method for the genes: *cat1*, *cat2*, *cat3*, and *cat4* by Reverse Transcription real-time PCR. 18S and GAPDH were applied as reference genes. Each sample was analyzed in triplicate from three independent experiments. Legend: G2-*cat2* gen, G3-*cat3* gen and G4-*cat4* gene, control - 25°C, t - 10°C.

There is evidence in the literature for the induction of catalase genes as a result of temperature stress for some fungal species, such as *S. cerevisiae*, *Rhodotorula mucilaginosa*, *A. nidulans*, *P. marneffei* and *P. brasiliensis* (Wang et al., 2017b). In our study, four catalase genes reacted at 10°C. They demonstrated induction during low-temperature stress and represented an opportunity for the mycelium to synthesize more of an enzymatic protein required for H_2O_2 degradation during a sharp decrease in temperature. Furthermore, the presence of 4 catalase genes suggests a better ability to regulate H_2O_2 degradation in the strain studied (Kawasaki and Aguirre, 2001).

As a result of our studies, we obtained the complete sequences of 5 catalase genes in *P. griseofulvum* P29 and monitored the effect of low temperature on the expression of these genes by quantitative reverse transcription polymerase chain reaction (RT-qPCR). At a temperature of 10°C, expression of the *cat1* gene encoding the enzyme catalase-peroxidase was most significantly increased. The present evidence suggests that *cat1* is one of the genes allowing the growth of *P. griseofulvum* P29 under conditions of oxidative stress induced by low temperature.

4.3. Deposit the results obtained in the form of gene sequences of the investigated enzymes and the producer strains in international databases (NCBI, kegg, Brenda) and microbiological collections (DSMZ, CBS)

All sequences obtained have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), the largest open-access public database, and are presented in Table 5.

Table 5. Nucleotide sequences deposited in GenBank open access database

Strain	sequencing analysis	GenBank accession number
Penicillium griseofulvum P29	ITS region	MT722118
Penicillium chrysogenum P27	ITS region	MT758190
Penicillium griseofulvum P29	Catalase-peroxidase *cat1* gene, complete sequence	MW618002
Penicillium griseofulvum P29	Catalase *cat2* gene, complete sequence	MW618003
Penicillium griseofulvum P29	Catalase *cat3* gene, complete sequence	MW618004
Penicillium griseofulvum P29	Catalase *cat4* gene, complete sequence	MW618005
Penicillium griseofulvum P29	Catalase *cat5* gene, complete sequence	MW618006

5. OPTIMIZING THE CULTIVATION CONDITIONS OF *P. griseofulvum* P29 TO INCREASE ENZYME CAT SYNTHESIS

The culture conditions of the model strain were optimized to increase the yield of the CAT enzyme by varying the concentration of the carbon source in the fermentation medium, the inoculum content, the temperature, and the amount of dissolved oxygen (DO) in the medium.

5.1. Effect of glucose on growth and synthesis of CAT from *P. griseofulvum* p29

Different glucose concentrations (from 1 to 10%) were tested to determine the relationship between glucose level and enzyme biosynthesis. Figure 17 shows a significant effect of glucose concentration on biomass formation, carbohydrate source uptake, and CAT activity.

Biomass content increased with increasing glucose concentration up to 5% and was maintained almost constant up to 8%. Subsequent variants (9 and 10%) showed a significant slowing of growth, but in each case, the biomass is above that recorded at 1 and 2%. Furthermore, while the glucose content in the range of 1 - 5% was completely consumed, in

cultures above 5%, significant levels of glucose were still present in the cultural liquid after 96 hours of cultivation.

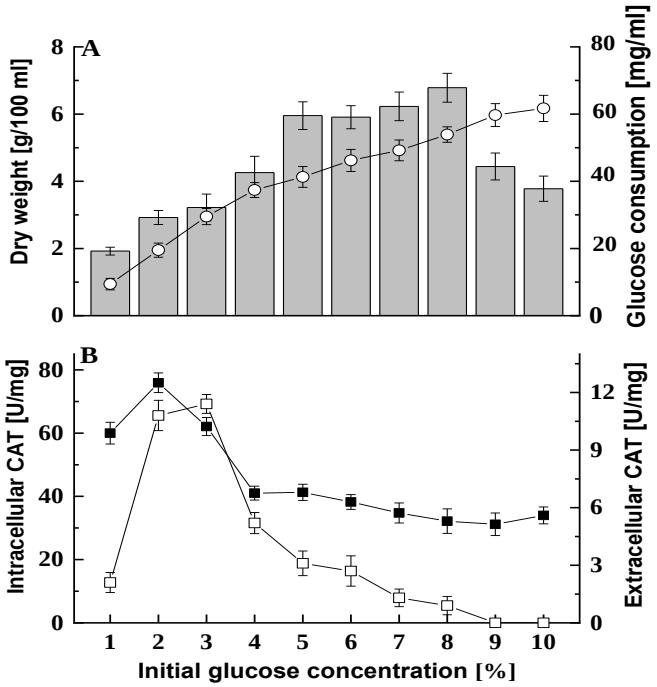

Figure 17. Glucose concentration significantly influences the producer development and enzyme synthesis. (A) - biomass (gray bars) and glucose consumption (o); (B) - activity of intracellular (■) and extracellular (□) CAT

Intracellular CAT synthesis was realized at all glucose concentrations studied. The optimal enzyme activity (77.9 U/mg) was observed at the variant 2% glucose, the next concentration used 3% resulted in a slight decrease in enzyme activity. Further increasing the initial glucose content above 3%, repressed the synthesis of the intracellular enzyme. The *P. griseofulvum* strain P29 also demonstrated extracellular CAT production, but to a much lesser extent. Based on the results obtained to date, it is evident that the *P. griseofulvum* P29 strain requires a 2% initial glucose concentration for maximal CAT activity.

5.2. Optimization of inoculum content

While there was abundant growth in all experiments with different inoculum concentrations, the highest biomass formation (about 3.5 g/100 ml) was recorded after inoculation with 10% inoculum after 60 hours cultivation (Fig. 18A). Typical fungal growth curves and rapid glucose uptake were observed. Cultures with 7.5, 10, and 12% inoculum consumed glucose in about 36 hours. As can be seen in Fig. 18B, the use of inoculum above 7.5% significantly improved enzyme synthesis, whereas at 5% seed, enzyme activity was

significantly reduced. The maximum value of CAT (56 U/mg) was observed in a variant with 10% inoculum after 84 h of cultivation.

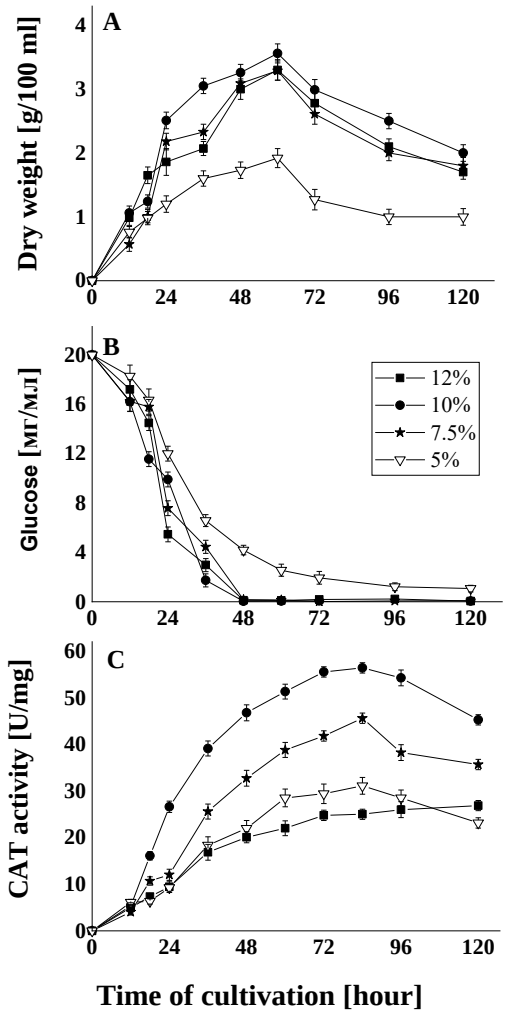

Figure 18. Amount of biomass (A), residual glucose (B), and CAT activity (C) of *P. griseofulvum* P29 as a function of inoculum amount: 5% - ▽; 7.5% - ★; 10% - ●; 12% - ■.

5.3. Strain development and biosynthesis of CAT as a function of cultivation temperature

The temperature has a substantial effect on biomass formation and synthesis of the CAT enzyme by *P. griseofulvum* P29 (Fig. 19). The strain can grow at all temperatures tested (10 to 25°C) (Fig. 19A). The temperature of 15ºC was the most favorable for biomass accumulation, with a maximum value of 2.78 g/100 ml, compared to 1.67 g/100 ml at the lowest temperature used (10°C). No significant difference in fungal strain development was observed when the temperature varied between 20 and 25ºC.

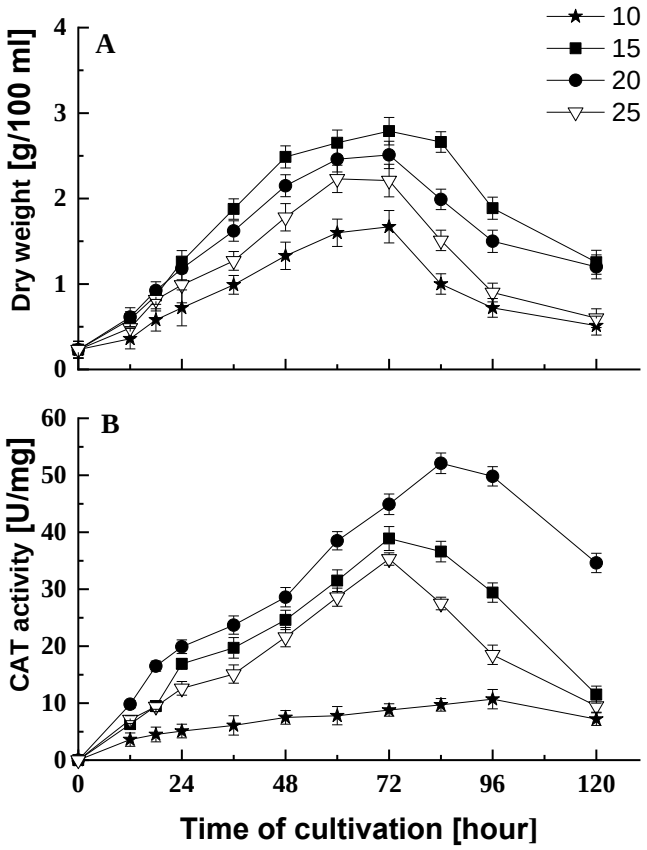

Figure 19. Biomass amount (A) and CAT activity (B) of *P. griseofulvum* P29 cultured at different temperatures: 10°C - *; 15°C - ■; 20°C - ●; 25°C - Δ**.**

In contrast to the above data, the CAT of *P. griseofulvum* P29 showed the highest activity at 20°C and its maximum (52.1 U/mg) was reached after 84 h of cultivation (Fig. 19B). Culturing at temperatures below or above 20°C resulted in a decrease in maximum activity to 38.9 and 25.3 U/mg after 72 h at 15 and 25°C, respectively.

5.4. Importance of dissolved oxygen (DO) concentration for strain development and enzyme synthesis

The results showed the strain growth significant dependence of the **DO concentration**. In cultures with controlled levels of DO of 20 and 30%, the transition from the exponential phase to the stationary phase occurred at the 84th hour, when maximum biomass accumulation (about 1.9 g/100 ml) was also observed. Increasing the DO concentration to 40 or 50% shortens the time for the stationary phase occurrence to the 36th hour. The best mycelial yield was recorded in the variant of 50% DO (3.1 g/100 ml after 60 h of cultivation). While the glucose consumption in the

30, 40, and 50% DO variants (Fig. 20B) showed an insignificant difference, the 20% DO showed a slight delay in this process.

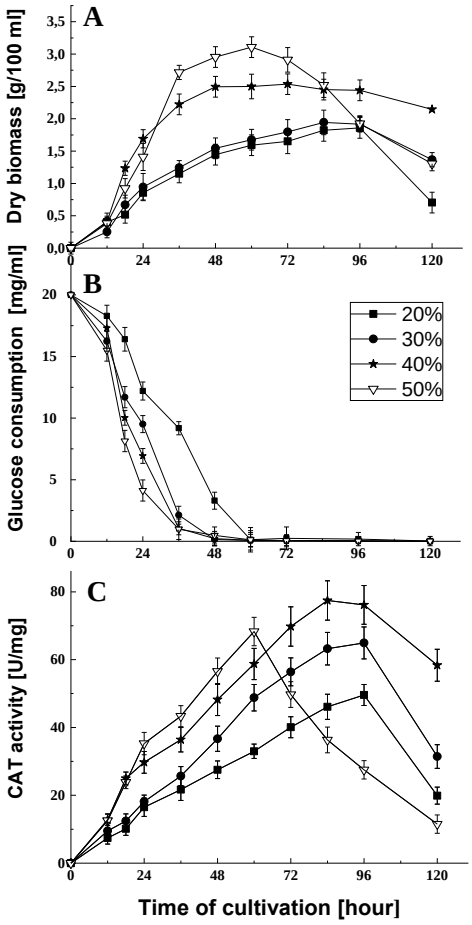

Figure 20. Dependence of the amount of biomass (A), glucose consumption (B), and CAT synthesis (C) on the level of DO: 20% -■; 30% - ●; 40% - *; 50% - Δ.

As the level of DO increased to 40%, the enzyme activity increased to the maximum of 77.5 U/mg, achieved after 84-96 h of cultivation (Fig. 20B). In cultures with 20 or 30% DO, a significant retardation of CAT synthesis was observed. Although the maximum level of CAT in the 50% DO variant was reached after 60 h of cultivation, the enzyme activity was about 15% lower compared with the 40% DO variant.

5.5. COMPARISON OF THE EFFECTIVENESS OF BIOSYNTHESIS OF CAT of *P. griseofulvum* P29 CULTIVATED UNDER DIFFERENT CONDITIONS

To develop an efficient method for obtaining the highest yield of CAT, a comparison of the enzyme production parameters in cultures at uncontrolled DO levels (Figs. 18 and 19) and

those at controlled levels (40 and 50%) was performed (Fig. 20). The data are summarized in Table 6. The results showed better growth and more accelerated synthesis of the enzyme *by P. griseofulvum* P29 was achieved under controlled DO level conditions compared with the uncontrolled DO variants. The highest value of kinetic parameters for protein yield, CAT activity, yield coefficient, and enzyme productivity was observed after 84 h of cultivation at 40% DO. Despite higher biomass content and earlier attainment of maximum enzyme activity at 50% DO, the highest value of the above parameters was observed at 40% DO (1102 versus 1085, 79 comparatively 62, 3454.1x103 versus 2164.1x103 and 41.1x103 in contrast 36.7x103).

Table 6: Comparison between productivity of CAT synthesis (at activity maxima) and DO level

Cultivation system[a]	Time of cultivation [h]	Biomass[b] [g/l]	Protein yeild[c] [mg/l]	Specific CAT activity[d] [U/mg protein]	Total CAT activity[e] [U/l] x103	Yield coefficient[f] [U/kg] $x10^3$	Enzyme productivity[g] [U/kg] hour $x10^3$
Uncontrolled level of DO	84	21.1	925.6	55	50.9	2412.7	28.7
Controlled level of DO							
40%	84	25.2	1102.0	79	87.1	3454.1	41.1
50%	60	31.1	1085.3	62	67.3	2164.1	36.7

[a] *P. griseofulvum* is cultured under uncontrolled DO or controlled DO (40 or 50%)
[b] Biomass as dry weight at the time of cessation of fermentation
[c] Protein yield (mg intracellular protein in dry biomass obtained from 1 liter of culture medium).
[d] Activity at the moment of fermentation end
[e] Total CAT activity determined for dry biomass from 1 liter of culture medium
[f] CAT yield factor determined as enzyme activity per kg dry biomass.
[g] CAT productivity determined per kg of dry biomass obtained in 1 hour

The Antarctic strain *P. griseofulvum* P29 is an efficient producer of CAT. The increased production of one of the main antioxidant enzymes by such a strain is not surprising. The harsh conditions in Antarctica, include, in addition to low temperature, high dissolved oxygen levels, increased UV radiation, strong seasonal changes, and often alternating freeze-thaw cycles resulting in enhanced production of ROS and activation of antioxidant defenses. Information on the efficient production of cold-active CAT is very scarce. Among a large number of microorganisms capable of synthesizing intracellular enzymes, including catalase, filamentous fungi are particularly interesting due to certain advantages, e.g. rapid development, abundant mycelia formation, etc. In this study, the relationship between treatment with DO concentration and enhanced antioxidant protection was exploited to develop a method for CA CAT production. The application of such a strategy allows the maintenance of a controlled and stable level of ROS

generation for optimal enzymatic synthesis without increasing the stirring rate. There is no information on the application of DO to improve CA CAT production in biotechnological processes.

6. PURIFICATION OF COLD-ACTIVE CAT

6.1. PURIFICATION SCHEME

A cell-free extract (200 ml) of 60 g fresh biomass was used to purify the enzyme.

The purification protocol included: the concentration of the protein in the cell-free extract by membrane ultrafiltration with an Amicon PM-10 or Minitan II ultrafiltration system (Millipore, USA 10 kDa), followed by one-step anion-exchange chromatography on Q-Sepharose and one-step hydrophobic interaction chromatography on phenyl-Sepharose (Table 7).

The specific activity increased more than 12-fold compared to the cell-free extract, while the total protein content decreased to 1.96 mg compared to the original content of 398.4 mg.

Table 7: Results of the steps carried out to purify CAT from *P. griseofulvum* P29

Step	Total protein content (mg)	Total catalase activity (U)	Specific enzyme activity (U/mg protein)	Yield (%)	Purification (times)
Cell-free extract	398.40	17199	43	100.0	1.00
Millipore (10 kDa)	133.75	7070	53	41.1	1.22
Q-Sepharose	71.75	5940	83	34.5	1.93
Phenyl-Sepharose	1.96	1055	538	6.1	12.51

According to the molecular standard, the resulting CAT has a molecular mass of 45.5 kDa for one subunit or about 182 kDa for the molecular weight of the whole enzyme. It should be emphasized that on SDS-PAGE, only a major band appears, which after staining for activity proves the presence of CAT enzyme (Fig. 21B).

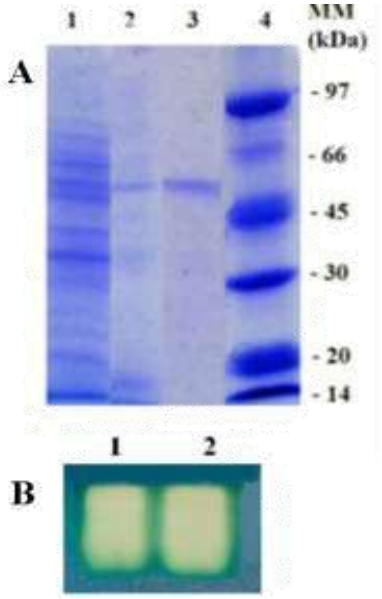

Figure 21. PAGE analyses. (A) SDS-PAGE analysis of CAT from *P. griseofulvum* after appropriate purification step: 1 - cell-free extract; 2 - after Q-Sepharose chromatography; 3 - after phenyl-Sepharose column; 4 - protein standard; (B) native PAGE of purified enzyme; 1-CAT standard from *Aspergillus niger* (Sigma-Aldrich); 2 - CAT from *P. griseofulvum* P29

Our results offer an uncomplicated and efficient procedure for CAT purification. The specific activity increased more than 12-fold compared to the cell-free extract, while the total protein content decreased to 1.96 mg compared to the initial content of 398.4 mg. Similar results on the purification rate and enzymatic yield of CAT were reported for *N. crassa* (Santoso et al., 2016), *Serratia marcescens* FZSF01 (Jia et al., 2017), *T. claveryi* (Marqués-Gálvez et al., 2019), and so on.

6.2. Effect of temperature and pH on the activity of purified novel CAT enzyme

The effect of temperature on the activity and stability of purified CAT was determined (Fig. 22A).

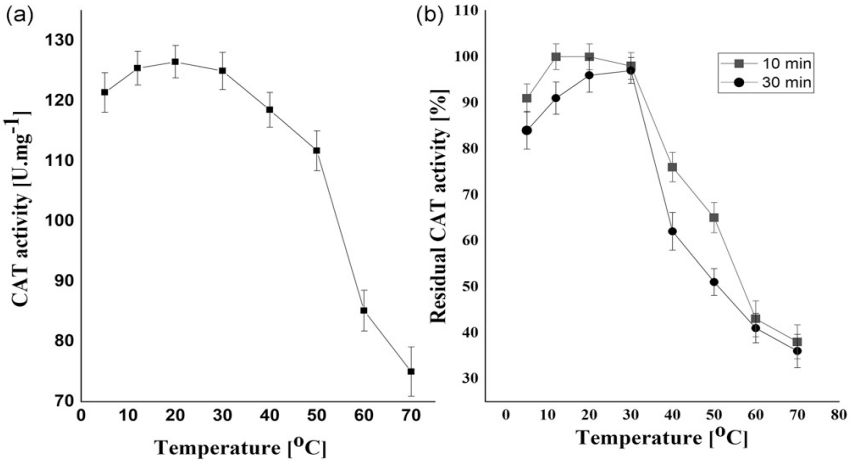

Figure 22. Effect of temperature on (A) CAT activity; (B) enzyme stability after incubation at different temperatures for 10 (■) or 30 (●) min.

The results showed a wide temperature range of the enzyme activity: from 5 to 70°C. The optimum temperature is 20°C and a high level of activity (>90%) is maintained between 5 and 40°C. At 50, 60, and 70°C the enzyme retains 88, 67, and 59% of its initial activity, respectively. The new CAT was stable up to 30°C for 10 and 30 min and maintained about 70, 65, 40, and 38% at 40, 50, 60, and 70°C, respectively (Fig. 22B).

The highest enzyme activity was found at pH values between 4.0 and 6.0 (Fig. 23A), and at pH 3.0 the enzyme was inactive. The sharp decrease in CAT activity at pH above 7.0 suggests the acidic nature of the enzyme. The enzyme pH stability was also determined at different pH values and temperatures of 20 or 30°C. Incubation for 10 and 30 min at increasing pH at 20°C showed similar stability curves (Fig. 23B).

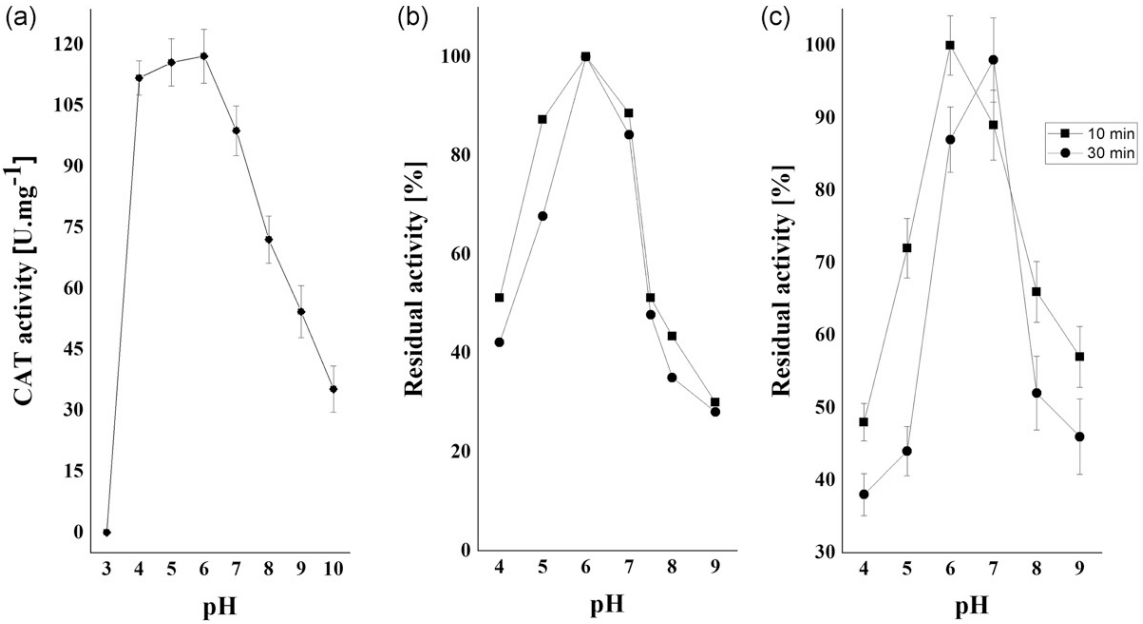

Figure 23. Effect of pH on (A) enzyme activity; pH stability at temperature 20 (B) or 30°C (C) after incubation for 10 and 30 min.

While in the acidic region (4.0 - 5.0) CAT retains 30 to 50% of the original activity, at pH 5.0 - 6.0 100% retention of activity is found. In the alkaline pH range, about 45-60% of CAT activity is retained. The experiment at 30°C also showed significant pH stability for 10 and 30 min (Fig. 23B). The enzyme appeared to be most stable at pH 6.0. About 90% of the maximum specific activity was detected at pH 5.0 and 7.0. The results showed that at 30°C pH, the stability of CAT was more pronounced in the acidic region than in the alkaline one.

DISCUSSION

The present results revealed the psychrotolerant P. griseofulvum strain P29 as an efficient producer of cold-active CAT. This strain was isolated from Antarctic soil samples, a continent that is a major source of low-temperature-adapted fungi (Robinson, 2001, Wang et al., 2008). According to Wang et al. (2017), Antarctic soils contain filamentous fungi belonging mainly to the Ascomycota division and less to Basidiomycota and Zygomycota. Bridge and Spooner (2012) also draw a similar conclusion about the distribution of filamentous fungi in Antarctica. There are several reports on Antarctic fungi and CA enzyme production (Fenice et al., 1997, Duarte et al., 2018, Pasqualetti et al., 2019), but there is a lack of data on CA CAT.

Information on the efficient production of CA CAT is scarce. Among a large number of microorganisms capable of producing intracellular enzymes, including CAT, filamentous fungi are particularly interesting because of their easier cultivation and biomass accumulation. Our results demonstrate the ability of *P. griseofulvum* P29 for CAT production under submerged cultivation in flasks, as well as in a bioreactor at multiple increases in working volume. This strain synthesized intra- and extracellular CAT with prevalence of intracellular enzyme activity (8:1). The production of both enzymes (intra- and extracellular CAT) by *P. griseofulvum* P29 was negatively affected by glucose concentration above 2%-3%, probably due to the action of the regulatory mechanism catabolic repression. Similar information has been published for mesophilic CAT producers. For example, *A. niger* AM-11 needs for CAT production the optimal initial glucose concentration of 0.2% (w/v) (Fiedurek and Gromada, 2000). Petrova et al. (2002) reported the increase of CAT activity from *S. cerevisiae* after the depletion of glucose in the culture medium. Contrary arctic fungi preferred 8% glucose content in the medium (Fiedurek, 2003).

Temperature-sensitive enzymes are required to maintain the metabolism of microorganisms under low-temperature conditions (Feller, 2013). Maximum CAT activity from the model strain was recorded at 20°C and significantly decreased at 25°C, confirming the temperature-sensitive nature of the enzyme from *P. griseofulvum* P29. The requirement for growth temperature is a strain-dependent characteristic of microorganisms adapted to cold habitats. Duarte et al. (2018) note the majority of Antarctic fungal isolates for enzymes producing at low and moderate temperatures ranging from 4.0°C to 26.0°C. In the psychrophilic strain *P. cyclopium* 1, the highest CAT activity was measured on the eighth day of the fermentation

process at 15°C, and at 20°C the time to reach maximum activity was shortened to six days (Fiedurek et al., 2003). Psychrophilic yeasts belonging to the genus Yamadazyma produce CA pectinase, amylase, and protease at 15°C (Daskaya-Dikmen et al., 2018). A temperature of 15-20°C is considered optimal for CA pectinase production by the Antarctic marine fungus *Geomyces* sp. F09-T3-2 (Poveda et al., 2018).

Aeration of the medium is another factor strongly affecting the growth and production of CAT by *P. griseofulvum* P29. While a positive effect was observed for growth up to 50% dissolved oxygen level, for CAT synthesis the positive effect was up to 40%, after which the enzyme activity decreased. Similar data were presented by Bai et al. (2003) for *A. niger* and *Fusicoccum amygdali*. Moreover, high oxygen concentration (up to certain levels according to the cell capacity) accelerates the ROS generation in filamentous fungi, leading to increased antioxidant enzymes activity, including CAT (Pashova et al., 1999; Bai et al., 2003; Lushchak, 2011). Schneider et al. (2019) reported a significant increase in laccase and peroxidase activity in the fungus *Marasmiellus palmivorus* VE11 in 50% DO maintenance. Very high concentrations of DO (50%) caused excessive production of ROS and enzymatic antioxidant defense could not restore the normal oxidant-antioxidant balance in *P. griseofulvum* P29 cells. It is likely that under these conditions $O2^{\bullet-}$ is overgenerated and the SOD level is insufficient to neutralize it. Superoxide radicals escaping dismutation can inhibit CAT activity (Kono and Fridovich, 1982). A comparable effect of DO concentration on cells was also observed in *Humicola lutea* (Pashova et al., 1999). Bai et al. (2003) present another hypothesis which suggests that a high level of oxygen enrichment (50%) leads to the involvement of an alternative respiratory pathway reducing the generation of endogenous ROS and the activities of antioxidant enzymes.

In the present study, the relationship between DO concentration and activation of the antioxidant defense was used to develop a method for efficient production of CA CAT by *P. griseofulvum* P29. The application of such a strategy allows the maintenance of a controlled and stable level of ROS generation for optimal enzymatic synthesis without increasing the stirring rate. Higher stirring speed is known to cause breakage of the fungal mycelium and the yield of the target product is reduced (Ibrahim et al., 2015). The scientific literature had no information about the use of DO in a biotechnological procedure to increase the activity of thermosensitive CAT. Fiedurek and Gromada (2000) used 30% concentrations of DO for the synthesis of a mesophilic enzyme preparation containing glucose oxidase and CAT. Aeration has been

successfully used to produce a chitinolytic enzyme from the Antarctic fungus *Lecanicillium muscarium* CCFEE 5003 (Fenice et al., 2012).

It is impossible to compare the data on enzyme production efficiency between the present study and those published by other authors for several reasons. First, there is no comparative data on the synthesis parameters of CA CAT corresponding to the information in Table 6. The report on the fungal CA CAT from *P. cyclopium* 1 provides evidence only for the cultivation time required to reach maximum activity, namely 8 or 6 days (Fiedurek et al., 2003). In the present study, maximum activity was achieved after 84 h and this shorter cultivation time to obtain the enzyme is a significant advantage. Second, data on CA CAT from filamentous fungi are extremely scarce. Third, the research design and methods for analyzing CAT activity in similar studies are quite different. While our study presents enzyme activity as units per milligram of protein (U/mg), other published results define it as units per milliliter (U/mL).

The purified and characterized CAT enzyme from *P. griseofulvum* P29 exhibits activity and thermostability over wide temperature ranges that differ significantly from those of mesophilic enzymes. Experiments showed the *P. griseofulvum* P29 CAT retention of 40% of its initial activity after incubation at 60°C for 30 min, suggesting that it is similar in thermostability to the temperature-sensitive CAT from *P. cyclopium* 1 (Fiedurek et al., 2003). Because this is the only report that notes the thermostability of fungal CA CAT, we compared the present results with data for bacterial temperature-sensitive enzymes. While CAT from *Vibrio salmonicida* LFI123 showed similar thermal stability at 60°C (50%, 5 min), enzymes from *Bacillus* sp. N2a (12%, 15 min) and *V. rumoiensis* S-1T (0%,15 min) (Lorentzen et al., 2006; Wang et al., 2008) demonstrated lower thermal stability compared to CAT from *P. griseofulvum* P29. On the other hand, this enzyme exhibits more significant thermolabile activity than mesophilic analogs (Vatsyayan and Goswami, 2016). Its lability along with its high catalytic efficiency near 5°C clearly indicates that it is a temperature-sensitive enzyme. The relationship between increased catalytic activity at low temperatures and reduced thermostability facilitates their rapid inactivation by moderate temperature increase, making them particularly suitable for application in various industrial processes and for basic research. For example, CA CAT is an important alternative to neutralize residual bleaching chemicals in the textile industry, which could save water, time, and energy as well as reduce pollution significantly. This enzyme can provide a protective effect against H_2O_2, which is responsible for the deterioration of food products and

packaging materials. Furthermore, such CAT is used in cheese production to remove hydrogen peroxide from milk after cold pasteurization. There is also interest in this enzyme as a potential ingredient in pharmacological formulations for the treatment of many diseases (diabetes, Alzheimer's, Parkinson's, etc.). Exogenous addition of CA CAT can be applied with great success in the cryopreservation of gametes.

In conclusion, CA CAT plays a major role in the survival strategy of Antarctic filamentous fungi under low-temperature stress. The present studies led to the establishment of an efficient laboratory technology for enzyme production in bioreactors that provides conditions for achieving high enzyme yield in short cultivation times, which is a prerequisite for scaling up its production. The resulting purified enzyme has a temperature optimum of 20°C and an optimum pH of the enzymatic reaction of 6.0. It maintains its thermal stability in the range 5°C to 40°C and pH stability from 5.0 to 8.0. All these characterize the CAT of *P. griseofulvum* P29 as a cold-active enzyme with potential applications for medical purposes as well as for biotechnological or industrial processes.

V. FINDINGS

1. The 61 Antarctic strains studied belong to the psychrotolerant (38%) and mesophilic (62%) representatives of fungi.

2. All the strains possess the ability to synthesize one of the major antioxidant enzymes intracellular CAT, and most of them (88%) also produce extracellular CAT.

3. Low-temperature exposure induced the oxidative stress expression in the cells of the model strains by increasing the levels of oxidative stress biomarkers.

4. Model strains respond to stress by activating their antioxidant defenses, which increases the activity of the first-line defense enzymes SOD and CAT.

5. Low-temperature stress leads to ultrastructural changes in cells of the psychrotolerant *P. griseofulvum* P29. The most sensitive cell organelles are mitochondria.

6. Five catalase genes have been identified in the psychrotolerant *P. griseofulvum* P29, one of which expresses the enzyme catalase-peroxidase and the others encode monofunctional, heme-containing catalases.

7. A sharp decrease in temperature to 10°C in the cellular response of the model strain involved induced expression of 4 of the catalase genes, most notably *cat*1, the catalase-peroxidase gene.

8. The developed laboratory method for the production of CAT from the Antarctic strain *P. griseofulvum* P29 based on changes in the DO value allows an increase in total enzyme activity, enzyme yield, and enzyme productivity.

9. The resulting new enzyme is a cold-active CAT with a temperature optimum of 20°C and a pH optimum of 6.

VI. CONTRIBUTIONS

Based on a comparative analysis of the relationship between low-temperature exposure and oxidative stress of model strains isolated from extremely cold habitats, the following contributions can be derived:

1. New evidence at the molecular level has been obtained for the involvement of the enzyme CAT in the adaptation of Antarctic filamentous fungi to low-temperature stress .
2. The distribution of intracellular CAT in Antarctic fungi of different thermal classes was demonstrated. The presence of extracellular CAT, which is a rare phenomenon, has been established.
3. For the first time, an efficient laboratory method for the production and purification of cold-active CAT was developed. The new enzyme isolated from *Penicillium griseofulvum* P29 has a temperature optimum of 20°C and a pH optimum of 6.0.
4. Five nucleotide sequences corresponding to the five genes encoding CAT synthesis in *Penicillium griseofulvum* P29 have been deposited in a database.

Application

Some of the results obtained from the conducted experiments are reflected in articles published in journals with impact factors, namely:

1. Krumova, E., Abrashev, R., **Dishliyska, V**., Stoyancheva, G., Kostadinova, N., Miteva-Staleva, J.,...& Angelova, M. (2021). Cold active catalase from the psychrotolerant fungus *Penicillium griseofulvum*. *Journal of Basic Microbiology*, 61(9), 782-794. IF 2.532; Q3.

2. **Dishliyska, V.,** Stoyancheva, G., Abrashev, R., Miteva-Staleva, J., Spasova, B., Angelova, M., & Krumova, E. (2023). Catalase from the Antarctic Fungus *Aspergillus fumigatus* I-9– Biosynthesis and Gene Characterization. *Indian Journal of Microbiology*, *63*(4), 541-548. IF 3; Q3.

Total IF of articles 3,532

Presentations on the dissertation topic at scientific events:

- Report at the XV Congress of Microbiologists in Bulgaria with International Participation (5-6 October 2022, Koprivshtitsa) on: Cell response to cold stress in Antarctic fungus *Penicillium griseofulvum* – **V. Dishliyska**, G. Stoyancheva, R. Abrashev, J. Miteva-Staleva, B. Spasova, M. Angelova, E. Krumova

- Report of the 4th Interdisciplinary Doctoral Forum with Foreign Participation (16-19 May 2023, Sandanski):

Improved production of cold-active catalase from the psychrotolerant fungus *Penicillium griseofulvum* P29 during submerged cultivation - **V. Dishliyska**, G. Stoyancheva, R. Abrashev, J. Miteva-Staleva, B. Spasova, M. Angelova, E. Krumova

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisor, Assoc. Prof. Ekaterina Krumova, PhD for her methodical guidance, valuable advice, and dedicated assistance and support throughout my studies and during the preparation of this dissertation.

I would like to thank the whole team of the Department of Mycology, especially Prof. Maria Angelova, PhD, Assoc. Prof. Abrashev, Assist. Prof. Zheni Miteva-Staleva and Boriana Spasova for their invaluable help and support.

The genetic studies of Antarctic micromycete strains, including *Penicillium griseofulvum* P29 producer of CA CAT were carried out by Assoc. Prof. Galina Stoyancheva from the Genetics Laboratory, Department of General Microbiology and Assoc. Prof. Dr. Ekaterina Krumova. Thank you for your assistance and consultation during the experiments.

Experimental studies concerning ultrastructural changes of psychrotrophic producer morphology under low-temperature stress were conducted with the kind assistance of colleagues from the Cell Microbiology Laboratory, Department of General Microbiology.

I am eternally indebted and express my heartfelt gratitude to my wonderful family for their love, patience, and unconditional support.