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Extracellular Proteases of Pathogenic Yeasts *Magnusiomyces capitatus* and *Magnusiomyces ingens*

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TITLE:

Extracellular Proteases of Pathogenic Yeasts *Magnusiomyces capitatus* and *Magnusiomyces ingens*

ANNOTATION:

Magnusiomyces capitatus and *Magnusiomyces ingens* are known as pathogenic yeasts, which cause infrequent but severe infections, especially in immunocompromised patients. We knew beforehand that these two species contain gene families potentially encoding secreted aspartic proteases. The aim of the practical section in this thesis was to examine, which proteases are secreted by *M. capitatus* and *M. ingens* under the laboratory conditions. Additional goal was to prepare sufficient amounts of one of these proteins, especially the protease encoded by the gene MIA_05579_1, and enable thus its crystallization and structural analysis.

KEY WORDS:

Yeast, *Magnusiomyces*, Protease

NÁZEV

Geny kódující extracelulární proteázy kvasinek rodu *Magnusiomyces*

ANOTACE

Magnusiomyces capitatus and *Magnusiomyces ingens* jsou známé jako patogenní kvasinky, které způsobují vzácné, ale závažné infekce, zejména u imunokompromitovaných pacientů. Věděli jsme předem, že tyto dva druhy obsahují genové rodiny potenciálně kódující sekretované aspartátové proteázy. Cílem praktické části této práce bylo prozkoumat, které proteázy sekretují *M. capitatus* a *M. ingens* za laboratorních podmínek. Dalším cílem bylo připravit dostatečné množství jednoho z těchto proteinů, kódovaného genem MIA_05579_1, a umožnit tak jeho krystalizaci a strukturální analýzu.

KLÍČOVÁ SLOVA

Kvasinka, *Magnusiomyces*, Proteáza

TABLE OF CONTENTS

LIST OF FIGURES 9

LIST OF TABLES 9

LIST OF ABBREVIATIONS 10

INTRODUCTION 11

1 THEORETICAL SECTION 12

1.1 Yeasts 12

1.1.1 History of the yeast research 12

1.1.2 Yeasts in general 13

1.1.3 The genome structure of the yeast *Saccharomyces cerevisiae* 13

1.1.4 Pathogenicity of *Candida* species 14

1.2 Family of *Magnusiomyces* 15

1.2.1 *Magnusiomyces capitatus* 16

1.2.2 Programmed translational bypassing elements in mitochondria of the yeasts *Magnusiomyces* 18

1.2.3 *Magnusiomyces ingens* 18

1.3 Endopeptidases 19

1.4 Secreted Aspartic Proteases (SAPs) 20

1.5 Genes potentially encoding secreted proteases of *Magnusiomyces* 21

2. EXPERIMENTAL SECTION 23

2.1 Preparation of cultures 23

2.2 Electrophoresis in the polyacrylamide gel with sodium dodecyl sulphate (SDS-PAGE) ... 23

2.3 Testing of proteolytic activity 25

2.3.1 Hydrolysis of chromogenic oligopeptides 25

2.3.2 Anson test with hemoglobin 25

2.4 Proteases Purification by Affinity Chromatography 26

2.5 Expression of the protein encoded by the gene *MIA_05579_1* in *Escherichia coli* 28

2.6 Trypsin cleavage of protease precursor 33

3. CONCLUSION 34

4. REFERENCES 36

LIST OF FIGURES

- **Figure 1:** Phylogenetic tree of selected yeast species was constructed by RaxML from a concatenated alignment of 824 genes……………………………………………………..15
- **Figure 2:** Colonies and cells of M. capitatus NRRL Y-17686, True hyphae……………..16
- **Figure 3:** Byp elements in *magnusiomycete* nad2 genes…………………………………18
- **Figure 4:** Colonies and cells of *M. ingens* NRRL Y-17630, pseudohyphae……………...18
- **Figure 5:** Action of endopeptidases on a polypeptide substrate and action of amino acid removing the terminal amino acid residues……………………………………………20
- **Figure 6:** Mechanism of amide bond hydrolysis by aspartic proteases…………………...20
- **Figure 7:** 15% SDS-PAGE of *M. capitatus* and *M. ingens* culture supernatants of *2 months incubation.* (pH) ………………………………………………………………….…24
- **Figure 8:** 15% SDS-PAGE of *M. capitatus* and *M. ingens* culture supernatants of *2 months incubation.* (Temperature) ………………………………………………………….24
- **Figure 9:** 15% SDS-PAGE of affinity chromatography of *M. capitatus* culture supernatant……………………………………………………………………………..27
- **Figure 10:** 15% SDS-PAGE of affinity chromatography of *M. ingens* culture supernatant……………………………………………………………………………..28
- **Figure 11:** pET-Mia vector construction…………………………………………………30
- **Figure 12:** Protease precursor……………………………………………………………31
- **Figure 13:** 15% SDS-PAGE of protein expression encoded by *gene* MIA_05579_1 (with His-Tag) in *E.coli*………………………………………………………………………32
- **Figure 14:** 15% SDS-PAGE of trypsin cleavage and protein expression encoded by *gene* MIA_05579_1 (with His-Tag) in *E.coli*…………………………………………………..33

LIST OF TABLES

- **Table 1:** Testing the proteolytic activity using Anson test. ……………………………..26

LIST OF ABBREVIATIONS

BSA - bovine serum albumin
BSL - biological safety levels
E. coli - *Escherichia coli*
EDTA - ethylenediamine tetraacetic acid
IBs - inclusion bodies
IPTG - isopropyl-β-D-galactopyranoside
kDa - kilodaltons
LB - Luria-Bertani medium
Leu - leucine
M. - *Magnusiomyces*
nt - nucleotides
OD - optical density (synonym to absorbance)
Phe - phenylalanine
RBS - ribosomal binding site, Shine-Dalgarno sequence
RT - room temperature
SAPs - secreted aspartic proteases
SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA - trichloroacetic acid
Tris - Tris(Hydroxymethyl)aminomethane
Tyr - tyrosine

INTRODUCTION

Magnusiomyces capitatus and *Magnusiomyces ingens* are opportunistically pathogenic yeasts, which cause rare but severe infections in humans. These yeasts are usually found in patients with hematological malignancies or immunosuppression as well as in human microflora. The mortality related to them is quite high. This type of biosafety level-2 (BSL-2) pathogen infects skin, mucosa of the respiratory and digestive organs of people with weakened immune system (de Hoog and Smith, 2011; Brejová *et al*., 2019). The extracellular proteolytic enzymes of pathogenic microorganisms are important as one of the virulence factors and prospective targets for the cure. The pathogens produce virulence factors, which allow them to survive in the host cells and also stay away from other microbes. In fact, secreted aspartic proteases are known to increase the ability of the yeasts to adhere to the cell surfaces and penetrate the host barriers such as skin and mucosa (Bauerová *et al.*, 2011; Brunke *et al*., 2016).

According the analysis of the both species genomes, *M. capitatus* and *M. ingens* contain genes potentially encoding secreted proteolytic enzymes. So, we cultivated these *Magnusiomyces* yeasts under a series of conditions inspired by protocols used for the pathogenic yeasts of the genus *Candida*, which have already been extensively studied. We analyzed culture supernatants, in order to examine whether these two *Magnusiomyces* species actually produce extracellular proteolytic enzymes.

1 THEORETICAL SECTION

1.1 Yeasts

1.1.1 History of the yeast research

The Dutch scientist, Antonie van Leeuwenhoek has become the father of microbiology after successfully inventing microscopy and establishing microbiology during the seventeenth-eighteenth century. He observed tiny organisms – bacteria and protozoa for the first time after creating microscopes. Later on in 1780, he became the first person who saw yeasts, but he probably did not realize that they were living organisms (Nanninga, 2016). Then, in the early nineteenth century, yeasts were first recognized as living organisms. The biologists, as well as chemists, then started to study them and the fermentation.

In 1789, the first chemist, who studied alcoholic fermentation, was Antoine Lavoisier. The study was to discover the components and final product of fermentation. He appeared to be the first person that defined the fermentation equation and the breakdown of sugar to carbon dioxide and alcohol. A French chemist Joseph Gay-Lussac in 1810 then detected the fermentation and putrefaction of grape juice. He assumed that oxygen is the primary key to the process. In 1815, he made a great premise about the exact composition of sucrose from Lavoisier's figure, and the estimation is so close to today's. From 1850–1880, yeasts became well known as microbes.

The French scientist, Louis Pasteur (1822-1895), started his marvelous research as a chemist and was known as one of the most famous microbiologists. In 1857, he discovered the ability of yeast to ferment carbohydrates to carbon dioxide and ethanol. He also confirmed in his first published paper that fermentation was not carried out by a chemical catalyst but by the living yeasts (Barnett, 2003). After that, the conclusion of Schwann and others that yeast was a living organism was admitted by other researchers and workers in the brewery. Therefore, the fermentation was again studied (Barnett, 2000).

Saccharomyces cerevisiae, or the budding yeast, is known for being one of the useful microorganisms used for baking, beer brewing, and other industries since ten thousand years ago. This kind of yeast performs fermentation very well with a high amount of sugar, which can be then made into alcohol and carbon dioxide. So, during the year 1857, Louis Pasteur began to closely study the ability of fermentation by the baker's yeast. The yeast *Saccharomyces cerevisiae* becomes

the first eukaryotic organism whose genome was sequenced and got extensively studied. More than that, aspartic proteases were also discovered on the cell surface of *Saccharomyces cerevisiae* (Liti, 2015; Brice, 2018).

1.1.2 Yeasts in general

Yeasts are unicellular eukaryotic fungi. Their cell sizes are usually about 5–10µm. They are distinguished by a clear, smooth cream color, which can be visualized on the surface of colonies. These single-celled organisms have entirely different properties, structures, and functions from prokaryotic microorganisms, bacteria. Yeasts contain antibiotics resistance and other substances against bacteria, which is very natural and cannot be transmitted to other microorganisms. They can be isolated from natural habitats such as water, soil, animals, and the environment. Plant tissues are ideal habitats for yeasts. The characterization of these fungi can be identified by using different techniques on the basis of cell morphology, molecular biology, immunology, and physiology. The significant reproduction method of yeast species is asexually by bubbling or sometimes by fission (parent cell divides into two new cells). Like other mature eukaryotic cells, yeasts also have all the crucial organelles, for example, the cytoskeleton, endoplasmic reticulum, Golgi apparatus, mitochondria, nucleus, and so many other organelles and components (Montes de Oca *et al*., 2016).

1.1.3 The genome structure of the yeast *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* was the first fully sequenced eukaryotic genome. This fungus has been intensively studied since 1996 by hundreds of researchers in the world. Its chromosomes have a single, double-stranded DNA and a few sequences encoded by rRNA (ribosomal RNA). The *Saccharomyces cerevisiae* genome contains approximately 12,156,677 base pairs and 6,275genes, which is known that only 5,800 genes are functional. It is also believed that its genes are about 31% similar to human genes and has 16 chromosomes. With its special genetic structure, *Saccharomyces cerevisiae* becomes a very helpful organism that can be used for the study research (Montes de Oca *et al*., 2016).

1.1.4 Pathogenicity of *Candida* species

There are many different yeast species with diverse range of properties, and some of the yeasts are considered to be opportunistic pathogens. Opportunistic yeast infections are diseases, which are caused by fungi that do not cause a disease in healthy individuals; instead, they live as harmless saprophytes on human skin. Candidiasis is known as the most common opportunistic yeast infection of the patients with underlying conditions (Vázquez-González *et al*., 2013).

Since the 1980s, the incidence of infection caused by *Candida* species has risen very quickly and significantly, particularly among patients with immunosuppression or having severe diseases (Sardi *et al*., 2013). Candidiasis is caused by *Candida* species, especially *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis, Candida Krusei* and *Candida glabrata.* The *Candida* genus consists of more than 150 different species and they do not produce spores. From those, around 17 different species are involved in human candidiasis (Sardi *et al*., 2013; Silva *et al*. 2012; Williams and Lewis, 2011). *Candida* species commonly colonize the skin, the mucosa of the gastrointestinal tract, also in the mouth, oesophagus, and vagina. The lung is the most severely affected organ (Sardi *et al*., 2013). About 65% of *Candida* species are not able to grow at 37ºC, which prevents them from being pathogens and therefore are not involved with human colonization (Silva *et al*., 2012).

The pathogenicity of these species is due to several virulence factors, including the ability to adhere to host cells and medical devices, to form biofilm, and to secrete hydrolytic enzymes (Silva *et al*., 2011).

The key virulence factor of human cell colonization is the adherence of *Candida* species to the host cell surfaces; however, this process is mediated by multiple cell-signaling cascades. These pathogenic fungi also have ability to adhere and form biofilm on the surfaces of various medical devices, such as prosthetic heart valves, joint replacements, and catheters. This attachment is controlled by non-specific factors such as hydrophobicity and electrostatic forces. Biofilm is known to increase resistance not only to the host but also to antifungal therapy. It has become a concern of clinical problems (Sardi *et al*., 2013; Li *et al.,* 2003).

Secreted aspartic proteases (SAPs) are considered to be an essential virulence factor for *Candida albicans*. They invade the host tissues by degrading the structure of defense protein and disrupting the host mucosal membranes (Silva *et al*., 2012). These proteases are encoded by the

family of *SAP* 1-10 genes, which encode proteins of the molecular weight between 35-50 kDa (Sardi *et al.*, 2013).

1.2 Family of *Magnusiomyces*

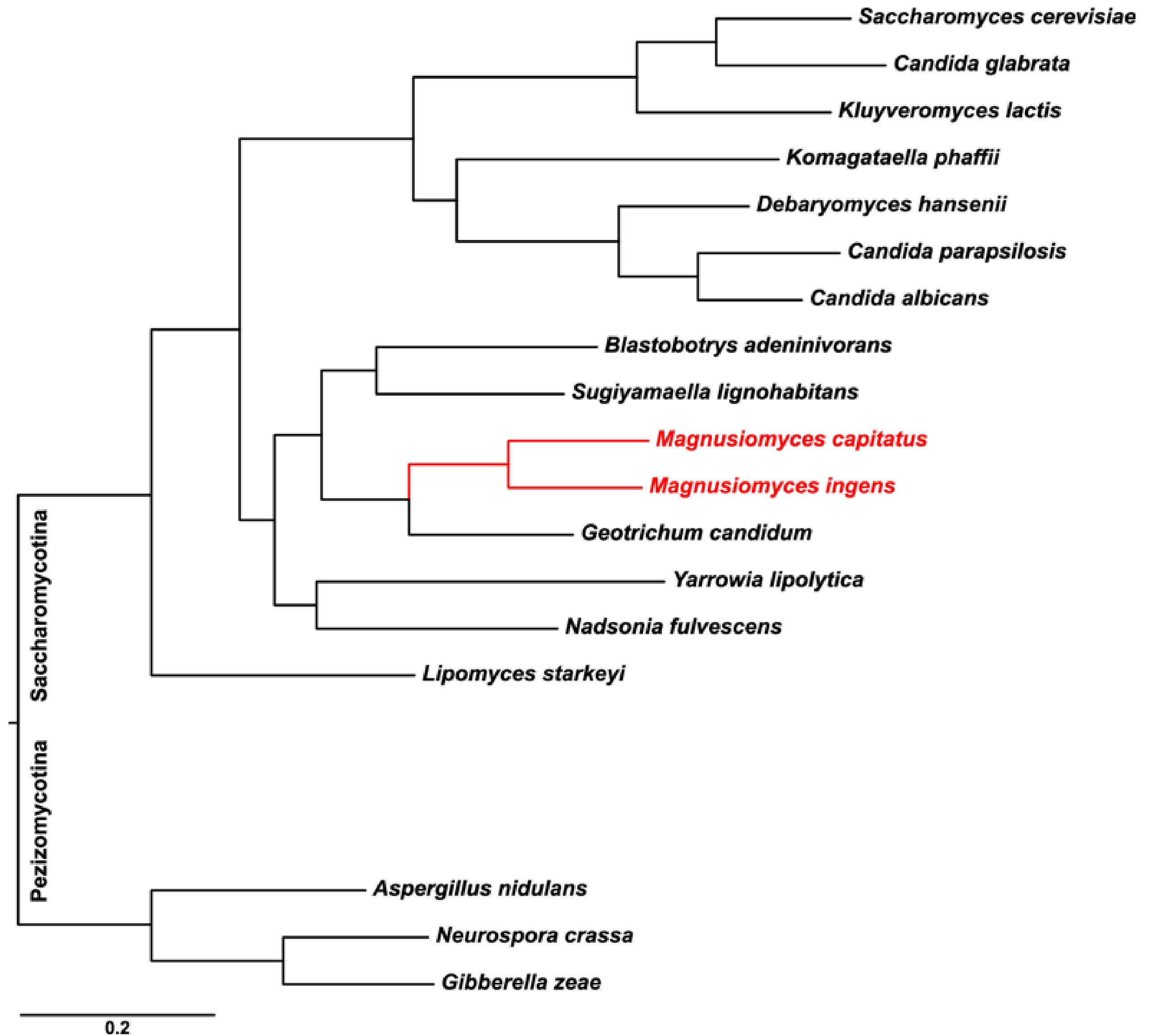

Figure 1: Phylogenetic tree of selected yeast species was constructed by RaxML from a concatenated alignment of 824 genes. The tree topology follows a previously accepted topology from the study of Shen et al. (2016) (Brejová *et al.*, 2019)

Magnusiomyces capitatus and *Magnusiomyces ingens* belong to the genus *Magnusiomyces,* which is a part of the fungal subphylum *Saccharomycotina*. Currently, the genus *Magnusiomyces* contains 14 species, and its anamorphic state is classified under the genera *Saprochaete (*Brejová *et al*., 2019).

1.2.1 *Magnusiomyces capitatus*

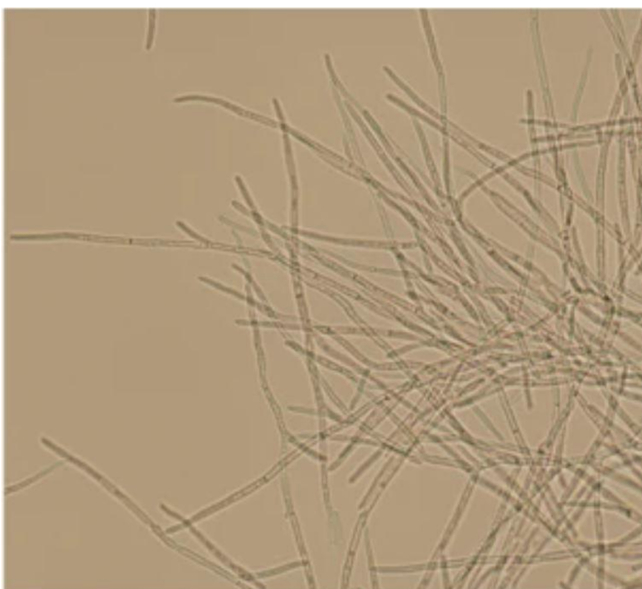

Figure 2: Colonies and cells of *M. capitatus* NRRL Y-17686, True hyphae (Brejová *et al*., 2019)

Magnusiomyces capitatus, one of the species from the family of *Saccharomycotina*, which is also known as *Blastoschizomyces capitatus, Geotrichum capitatum, Trichosporon capitatum,* and *Blastoschizomyces pseudotrichosporom*. The spreading of this emerging pathogen has recently increased and threatened a population within the geographic area in Europe and the Mediterranean. Earlier, this fungus was just known as a simple part of human microflora, which colonizes the skin and is not harmful to humans. Since 25 years ago, *M. capitatus* was considered as pathogenic yeast that can infect the skin, mucosa of respiratory and digestive organs of immunologically weakened patients. The mortality of infection is rather high, from 60% to 90% based on the patients' immunity.

Thanks to Salkin and his colleagues in 1985 (Salkin *et al*., 1985), *Magnusiomyces capitatus* was introduced as a new genus. It has smooth-thicked form with wrinkled colonies when cultivated in conventional media containing Sabouraud broth (Bouza and Muñoz, 2004).This yeast can be isolated from nature, from sputum, oral mucosa, skin, liquids for intravenous infusion, faeces and environmental samples (Sharma *et al*., 2012). Most case reports on *M. capitatus* infection (Tanabe and Patel, 2017) indicate that climatic factors may have a selective role in the epidemiology of infection. The infection is well known in restricted Mediterranean climate regions, such as Italy, Spain, and France (87%). Just a few case reports from India and other Asian countries have been released (Subramanya Supram *et al*., 2016; Brejová *et al*., 2019).

M. capitatus is known for widely spreading throughout the body and causing invasive mycoses, especially to patients with acute immunosuppression. It can also lead to localized lesions, dermatomycosis, and onychomycosis. Patients, who receive chemotherapy and patients of severe neutropenia are threatened by disseminated and invasive problem due to *Magnusiomyces*. The typical symptom is fever, and other signs that can be observed are pulmonary infiltrates, cough, pain in the chest, jaundice, spontaneous pneumothorax, and expectoration. Some reports showed that *M. capitatus* can also affect other organs, including the liver, kidney, and digestive tract. The attack of the central nervous system by this pathogen can cause not only encephalitis and meningitis but also localized lesions. Mastitis in cattle is likely to be caused by this pathogen. Many different molecular methods have been used to characterize and differentiate *M. capitatus* from other yeast (Bouza and Muñoz, 2004).

According to study report of patients with evidence of *Magnusiomyces capitatus* during 68 months in Rome (Martino *et al*., 1990), was known that there were 12 patients got infected, 4 were possibly infected and another 4 were not infected by *M. capitatus* but had this yeast colonization evidence. Two people among 12 infected patients died after not receiving any therapy; five showed no reaction to the antifungal treatment while five continued receiving amphotericin B plus 5-fluorocytosine therapy. Three patients had a successful remission of their acute leukemia and were cured, while two improved but had a significant relapse of *M. capitatus* after the recurrence of their acute leukemia (Martino *et al*., 1990).

1.2.2 Programmed translational bypassing elements in mitochondria of the yeasts *Magnusiomyces*

Programmed translational bypassing is a process that allows ribosomes to 'ignore' a particular mRNA interval of some nucleotides (nt). The study report (Lang *et al*., 2014) has recently announced the discovery of more than 80 byps (translational bypassing elements) in the pathogenic yeast *M. capitatus*. They are in the length of 27–55 nt in protein-coding mitochondrial regions, categorized into several families according to the first triplet triggering ribosome stalling. It was experimentally showed that byps are stored in mRNA but are not converted into protein (Nosek *et al*., 2015).

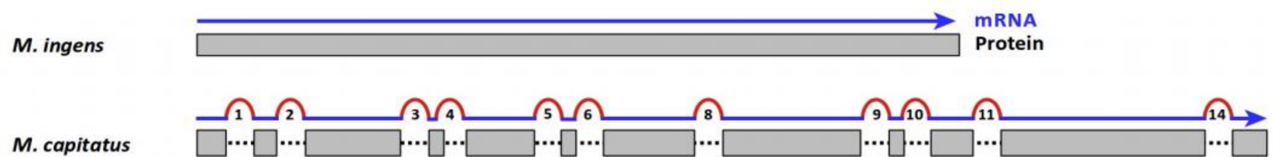

Figure 3: Byp elements in *Magnusiomycete* nad2 genes. The occurrence of byps (red arcs in mRNA; identical numbers indicate the presence of a byp in the same position) in the nad2 gene, encoding a subunit of NADH:ubiquinone dehydrogenase (Nosek *et al*., 2015)

1.2.3 *Magnusiomyces ingens*

Figure 4: Colonies and cells of *M. ingens* NRRL Y-17630, pseudohyphae (Brejová *et al*., 2019)

Magnusiomyces ingens is known as a species from the family of *Saccharomycotina* (figure 1). Through comparative genome analysis, the sequence diversity between *M. capitatus* and *M. ingens* appears to make these two species closely related. Recently, this new yeast has been studied; however, the literature on *M. ingens* is sparse. This fungus mainly grows in the form of filaments called pseudohyphae. As far as we know, not only the cells, mycelia, and the colonial morphology of *M. ingens* but also the ability to expand without vitamins in media are different from *M. capitatus*. It can utilize D-glucitol, while *M. capitatus* utilizes D-mannitol. Moreover, this yeast can live in different environments where it can be isolated from plants from water, sea, wine, basement rooms, or waste from industry. It cannot grow at a temperature higher than 37ºC, so it possibly does not infect humans.

There is no byp in the mitochondrial genes of *M. ingens* (figure 3). However, this yeast species is considered important for bioremediation and biotechnology. It's ideal for nanoparticles' biogenic synthesis and can also be used to break down azo dyes compound (Brejová *et al*., 2019).

1.3 Endopeptidases

Proteolytic enzymes, also referred to proteases or peptidase, are a group of enzymes that cleave proteins, and there are several types of them, according to the catalytic mechanism. There are two main groups of proteolytic enzymes, endopeptidases, and exopeptidases. Endopeptidases are proteolytic enzymes, which hydrolyze alpha-peptide bonds in the middle of polypeptide chains. Whereas, exopeptidase hydrolyze the peptide bonds at the substrate's *N*- or *C*- terminal ends (Mótyán *et al*., 2013) (Figure 5). Information on the known proteases and their inhibitors are deposited in Merops database (http:// merops.sanger.ac.uk).

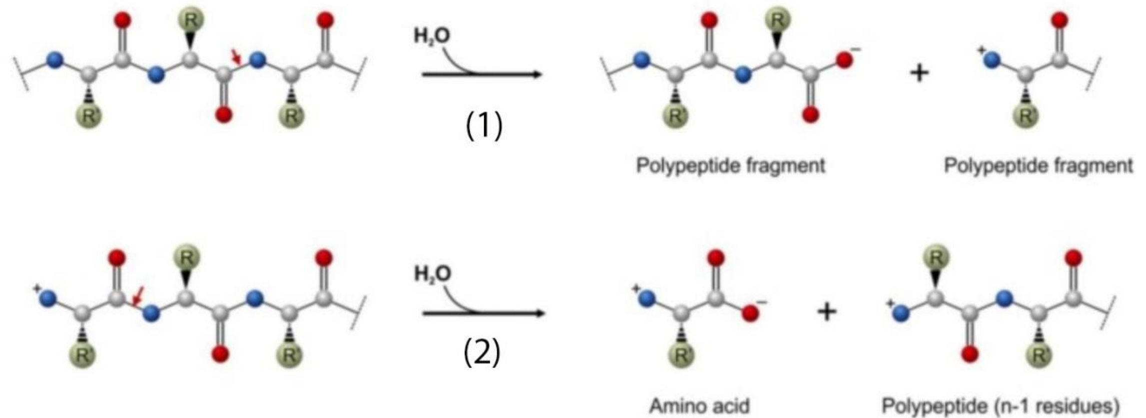

Figure 5: (1) Action of endopeptidases on a polypeptide substrate (having n residues). (2) action of aminopeptidases removing the terminal amino acid residues. Red arrows show the peptide bonds to be cleaved (Mótyán *et al*., 2013)

The most well-known examples of endopeptidases are pepsin, papain, and chymotrypsin. Some yeasts produce secreted proteases, which usually are of the aspartic type, containing two DT(S)G motifs, where the aspartic acid residues are indispensable for catalysis (Figure 6). Besides that, there are also cysteine, serine, threonine peptidases, and metalloendopeptidases (Rawlings *et al*., 2018).

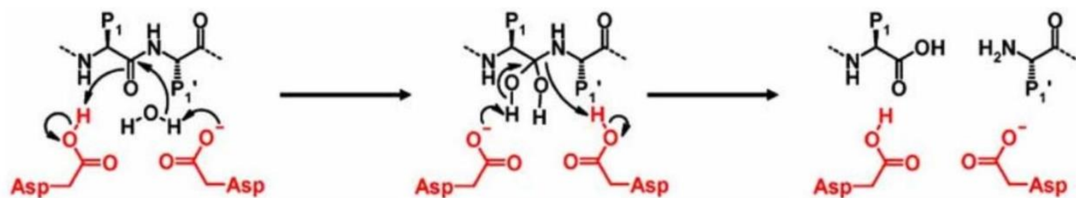

Figure 6: Mechanism of amide bond hydrolysis by aspartic proteases. Catalytic aspartic residues are shown in red. P1, substrate residue N-terminal of the scissile bond binding in the non-primed side; P1', substrate residue C-terminal of the scissile bond binding in the primed side of the protease.

1.4 Secreted Aspartic Proteases (SAPs)

Aspartic protease of pathogenic yeast (SAP) was first reported in 1965. It was called *Candida* acid protease since it was found in the yeast *Candida albicans,* and it was vigorously active in acidic pH (Silva *et al*., 2014). A molecule of water is activated by two characteristic conserved aspartic acid residues in the active site to break down peptide bonds

between amino acids with hydrophobic side-chains like Leu-Tyr, Phe-Phe, and Phe, Tyr (Guo *et al*., (2019). The molecular weights of SAPs are about 40 kDa and the optimal pH for their activity is between 3-4 (Monod and Zepelin, 2002).

Similarly as other aspartic proteases, SAPs are inhibited by pepstatin, a peptide product of *Streptomyces*. The classification of inhibitors can be made based on their molecular weight and protein natural molecule (Mandujano-González *et al.*, 2016).

There are 10 SAP genes present in the genome of *Candida Albicans*. Saps are divided into two groups based on the residues of the amino acid at the cleavage point of the peptide bond. The first group, Sap1, 6, and 8, contain a large substrate and have hydrophobic residues like Phe, Leu, and Tyr to break down the peptide bonds. Moreover, at the P1 site, there are also some positive charge residues like Lys and Arg. On the other hand, the second group, which includes Sap 7, 9, and 10, have a smaller substrate, and the peptide bonds are hydrolyzed after Arg, Met, and His. Sap7 is known to be the only candidal aspartic protease, which isn't inhibited by the inhibitor pepstatin A (Rapala-Kozik *et al*., 2018)

Yeasts contain various types of proteases, which play a wide range of roles inside the cells—some yeast species in addition secret proteases to the extracellular space. Specifically, several pathogenic species of the genus *Candida* secret aspartic proteases. These enzymes help to evade the immune system by destroying its proteins, such as antibodies, cytokines, and complement to allow the yeasts to stay away from the host defense (Staniszewska *et al.*, 2017). Secreted aspartic proteases also enable the pathogenic yeasts to penetrate the host barriers such as skin and mucosa and thus enhance the virulence. By degrading the host proteins, the secreted protease produces short peptides that can be internalized by the yeast cells and provide a source of nutrients, especially nitrogen. Since *Candida* secreted proteases have been extensively studied, we took them as an inspiration for the study of potentially secreted proteases of *Magnusiomyces* species. We knew in advance that *M. capitatus* and *M. ingens* contain genes resembling SAPs from *Candida* species.

1.5 Genes potentially encoding secreted proteases of *Magnusiomyces*

Thanks to collaboration with the laboratory of Prof. Jozef Nosek in the Comenius University, Bratislava, Slovakia, we knew the sequences encoding potential secreted aspartic proteases from two *Magnusiomyces* species. This lab sequenced the genomes of several yeast

species, including *M. capistatus and M. ingens.* The genome sequencing was followed by the computer translation of the gene sequences to theoretical protein sequences. And these theoretical proteins were further analyzed *in silico* to find specific motifs, which would suggest, what the features of the respective proteins are. Moreover, we selected the gene MIA_05579_1 from *M. ingens,* encoding potential secreted protease for a more detailed analysis.

There are theoretically several secreted aspartic proteases, but of course, only an experiment can verify whether they are really secreted, and if so, which of them are secreted under the laboratory conditions. It is the aim of this study to find the particular aspartic proteases secreted to the extracellular space of *M. capitatus* and *M. ingens*, and compare them to the gene sequences.

2. EXPERIMENTAL SECTION

2.1 Preparation of cultures

The yeast strains were kindly provided by Prof. Jozef Nosek, Comenius University, Bratislava, Slovakia. The yeasts were cultivated in a set of liquid media consisting of 1.2% (w/v) Yeast Carbon Base (Difco), 0.2% (w/v) bovine serum albumin (BSA), 20 mM sodium citrate, with three different pH values (3,4,5) and were left incubating for a week and longer at 4ºC, laboratory temperature or 37ºC.

We observed that two samples of *M. capitatus* at pH 3 and 4 began to flocculate. Before further analysis, we took an aliquot of the culture, and centrifuged it at 13 000 g.

2.2 Electrophoresis in the polyacrylamide gel with sodium dodecyl sulphate (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method for separating proteins on the basis of their molecular weight. The proteins migrate through the gel in an electrical field in the direction to anode.

20 µl of a supernatant sample were mixed with 30 µl of the loading buffer (50 mM Tris-Cl, pH 6.8, 10% glycerol, 100 mM β –mercaptoethanol, 2% SDS, 0.1% bromphenol blue) in a microtube and denatured at 100°C for 5 minutes. 10 µl of each prepared sample and 3,5 µl of molecular weight marker were loaded to 15% polyacrylamide gel. Electrophoresis was performed under a constant voltage of 200 V for approx. 50 minutes.

Result: It is obvious that the both *Magnusiomyces* species produce some proteolytic enzymes, which hydrolyze BSA (molecular weight 66 kDa). The BSA fragments occur the most at pH 3 (Figure 7) and 37°C or room temperature (Figure 8) for both *M. capitatus* or *M. ingens*.

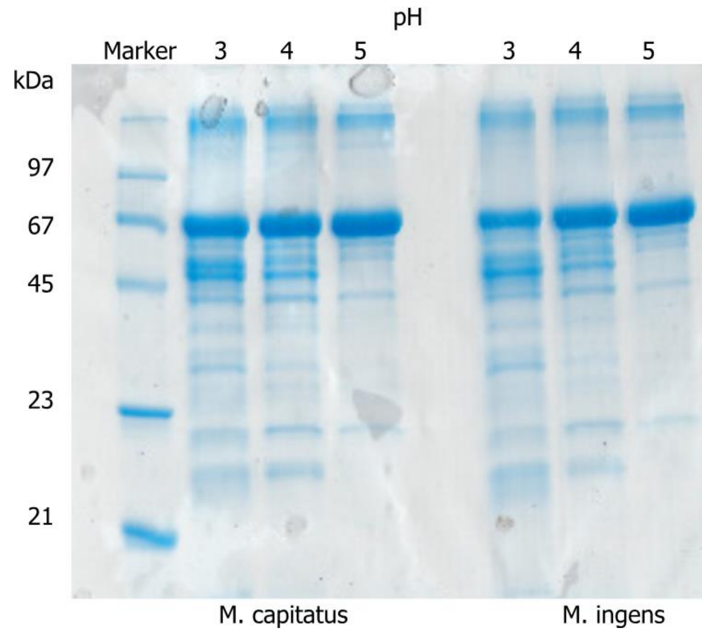

Figure 7: 15% SDS-PAGE of *M. capitatus* and *M. ingens* culture supernatants. *Coommassie Staining. "Marker" - Molecular marker; Protein - Test Mixture 6* (Serva)
2 months incubation

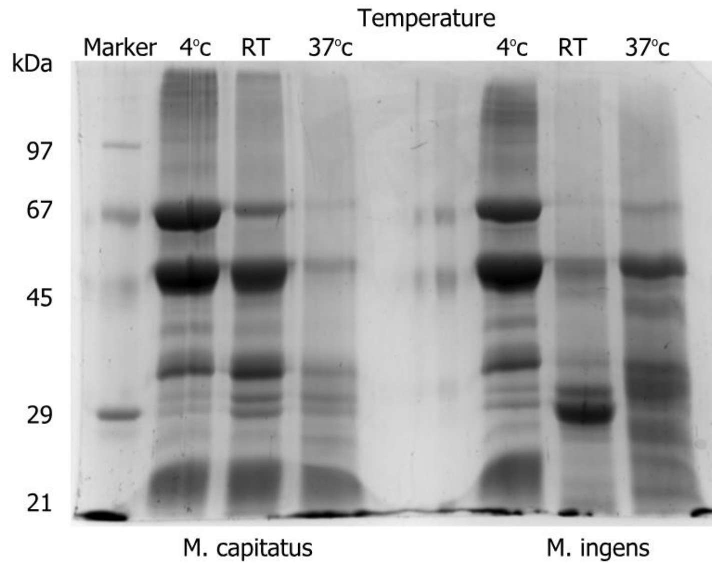

Figure 8: 15% SDS-PAGE of *M. capitatus* and *M. ingens* culture supernatants. *Coommassie Staining.*
"Marker" - Molecular marker; Protein- Test Mixture 6 (Serva).
2 months incubation

2.3 Testing of proteolytic activity

2.3.1 Hydrolysis of chromogenic oligopeptides

Chromogenic oligopeptides are useful tool for proteolytic activity testing and in this particular case the peptides contain para-nitrophenylalanine (**F**) adjacent to the expected scissile bond. The absorption maximum of para-nitrophenylalanine shifts upon cleavage, and therefore it is possible to monitor cleavage of the substrate using decrease of absorbance at 300 nm. We used this method to measure and monitor enzyme activity during the attempts to purify the proteases (Robert, 2007).

Two chromogenic peptides (KARQNle**F**EANle and KPVEF**F**RL) were used and dissolved in water, to prepare stock solutions of 5 mg/ml. 1 ml of each sample of *M. capitus* and *M. ingens* culture (4°C, Room Temperature, 37°C) was centrifuged for 3 minutes at 13 000 g. Into a microtube, 10 µl of a supernatant sample was added, 1 ml of 20 mM citrate buffer (pH 3) and 5 µl of each chromogenic substrate. After 5 minutes incubation, the enzyme kinetics of prepared samples was measured by spectrophotometry, as a decrease of absorbance at 300 nm, using 1 ml of citrate buffer as blank.

Result: This method was not successful, as there wasn't any decrease of absorbance to be observed. It may be among other reasons because we did not have good substrates, since nothing is known about the substrate specificity of *Magnusiomyces* secreted proteases, or 5 minutes of incubation were too short. We therefore began using the Anson test based on cleavage of hemoglobin, and an end-point analysis of the reaction mixture.

2.3.2 Anson test with hemoglobin

Anson test is a method for testing of proteolytic activity. Hemoglobin is used as a substrate in this method. The hemoglobin is cleaved by the protease from the tested sample, the short peptides are released, and the absorbance is measured by spectrophotometry.

We had new samples (*M. capitatus, M. ingens* pH 3), which were cultivated for 14 weeks at three different temperatures (4°C, Room Temperature, 37°C). First, 10 µl of each proteinase sample (except Blank) was mixed with 750 µl of 100 mM sodium citrate buffer at pH 3.4 and 250

µl of 4% (w/v) hemoglobin solution into seven different test tubes. All prepared samples and blank were incubated at 30°C overnight. The reaction was stopped by adding 200 µl of 10% TCA (Trichloroacetic acid) and centrifuged (15min, 13 000 g). The activity of the enzyme corresponds to the absorbance of the supernatant, which was measured at 280 nm.

Table 1: Testing the proteolytic activity using Anson test. Absorbance of the samples at 280nm.

Samples		Absorbance
M. capitatus	4°C	0.018
	RT	0.22
	37°C	-0.006
M. ingens	4°C	-0.03
	RT	0.33
	37°C	-0.04

Result: We saw the proteolytic activity in both samples from the incubation at room temperature (Table 1).

2.4 Proteases Purification by Affinity Chromatography

Affinity chromatography is a method of separating proteins or other molecules from complex mixtures based on their interaction with a specific ligand such as enzyme and inhibitor (Marjeta *et al*., 2009). Pepstatin is used in this method. It is a classical low molecular weight and potent inhibitor of aspartic proteases. It has Ki value of about 10^{-10} M for pepsin, which is very strong inhibitor (Marciniszyn *et al*., 1977).

300 µl of pepstatin was mixed with 300 µl of 100 mM citrate buffer at pH 3,4 (binding buffer) into two different microtubes. Both microtubes were quickly spun down, and the supernatant was discarded. This step was repeated two times. Into each microtube, 2 ml of a supernatant sample (*M. capitatus* and *M. ingens* pH 3, RT) was added, quickly spun down, and the supernatant was taken into new microtubes. In theory, the supernatant contains everything except aspartic proteases, which are bound to the pepstatin agarose. Then, 300 µl of binding buffer was added to wash the content of the microtubes and to remove everything that binds nonspecifically, quickly spun down and repeated this step two times. All the supernatants were taken into new

microtubes. After that, 100 µl of 100 mM sodium carbonate buffer at pH 9 was added to elute proteases, quickly spun down, repeated this step two times, and the supernatant was pooled in the new microtube. After purification of the proteases, all prepared samples from each *Magnusiomyces* species were analyzed by electrophoresis in the polyacrylamide gel (SDS-PAGE).

Result: In the lane "Elution" of the gel of *M. capitatus* version (Figure 9), we observed a faint band of a protein of approx. 45 kDa. The sequences of potential proteases contain more than 300 amino acids, which means proteins of the size bigger than approx. 30 kDa. If the protein is posttranslationally modified, its molecular weight may be higher. In contrast, in *M. ingens* version (Figure 10), we noticed that the yeast consumed all the BSA (the band of 67 kDa), but the protease responsible for the BSA cleavage remained under the detection limit.

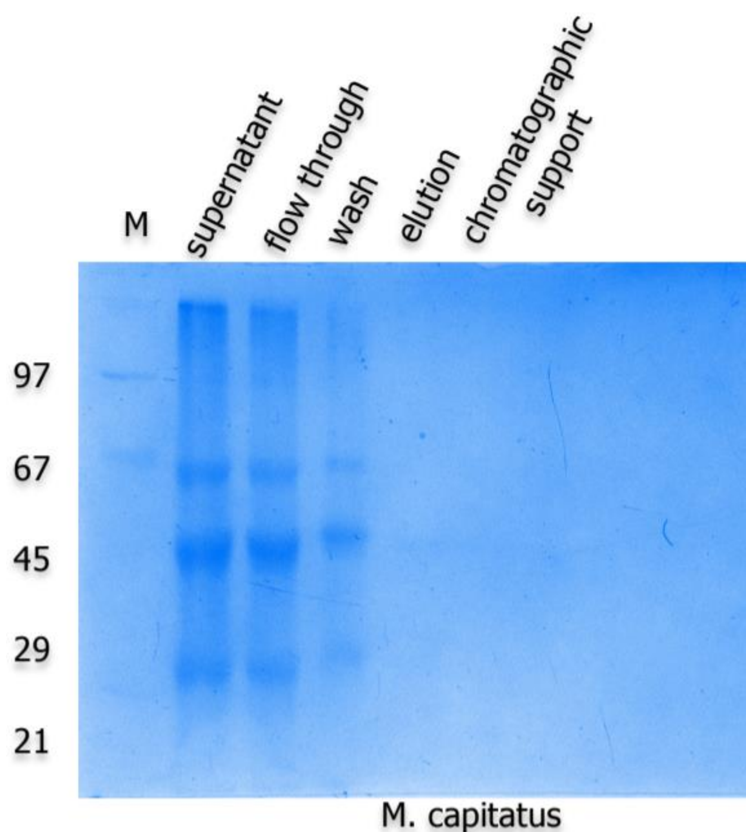

Figure 9: 15% SDS-PAGE of affinity chromatography of *M. capitatus* culture supernatant. *Coommassie Staining. "M"- Molecular marker; Protein Test - Mixture 6* (Serva)

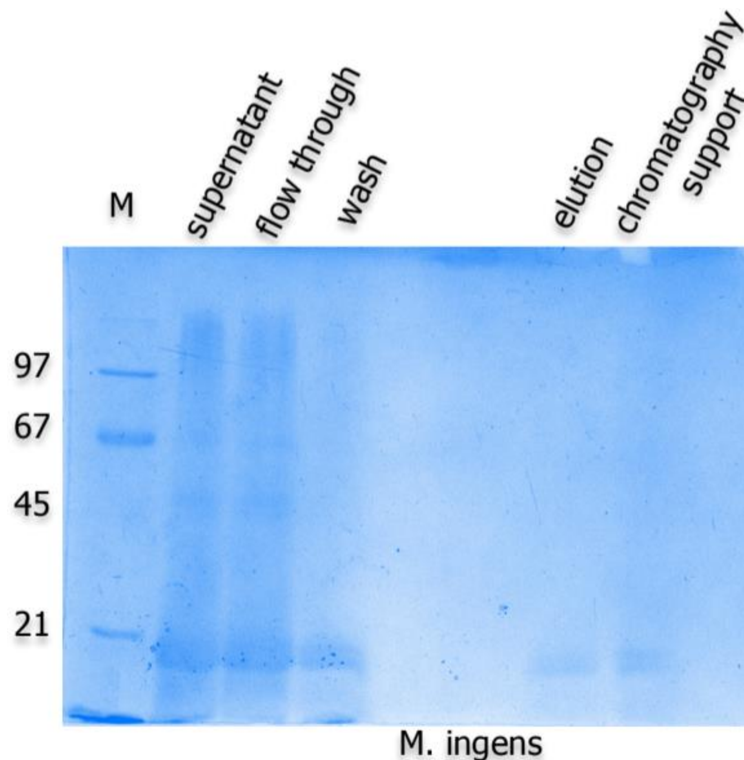

Figure 10: 15% SDS-PAGE of affinity chromatography of *M. ingens* culture supernatant. *Coommassie Staining*. *"M"- Molecular marker; Protein Test- Mixture 6* (Serva)

2.5 Expression of the protein encoded by the gene *MIA_05579_1* in *Escherichia coli*

The recombinant protein expression was first established in 1973 by Stanley Cohen and Herbert Boyer during their discovery of vitro DNA-cloning. The first product of this method was insulin in 1982. In this method, bacterium *Escherichia coli* is used as the primary host for the expression of a recombinant protein. Depending on the expression strategy, the recombinant protein can be localized intracellularly, which often leads to protein aggregates inside of the cytoplasm, forming so the called inclusion bodies (IBs) (Slouka *et al.*, 2019).

<u>Preparation of LB medium:</u> 500 ml of LB (Luria-Bertani) medium was prepared in the first day by adding 500 ml of distilled water and 10 g of LB mixture (Sigma), which consists of tryptone (pancreatic digest of casein), yeast extract and NaCl and kept stirring until it completely dissolved,

then divided into two different Erlenmeyer flasks. These two flasks of medium were left autoclaved at temperature 120ºC for 15 minutes.

Competent *E. coli* and transformation: Competent *E. coli* is very important and needed at this step because it is able to accept a vector easily. *E. coli* was treated with $CaCl_2$ and was left freezing at -80ºC. 100 µl of competent *E. coli* and vector pET28-MIA_05579_1 with His-Tag (N-term) was incubated on ice and was subjected to heat shock at 42ºC for 2 minutes to get the vector DNA into *E. coli*. This step is called "transformation". Then, the cells were regenerated at 37°C for an hour to help bacterial cells to recover from heat shock and to produce a new generation of cells, harboring the vector with the gene conferring resistance to kanamycin. After that, the culture was plated on LB agar containing kanamycin, to select for the positive transformants.

Vector for expression: pET28-MIA_05579_1 vector (Figure 11) is artificial circular DNA derived from bacterial plasmids. It was constructed so that it contains ribosomal binding site (RBS; Shine-Dalgarno sequence), Lac promoter, which is lactose induced, our gene (encoded by the gene MIA_05579_1 with His-Tag), antibiotic resistance and T7 promoter derived from T7 bacteriophage and interacts with T7 polymerase, which contains in *E.coli* BL21-(DE3) strain. T7 polymerase can use most of the nucleotides in the cells so that our gene will be transcribed, not enough nucleotides for transcription of other genes.

His-Tag or polyhistidine tag is a sequence of six histidine residues at the N-terminal of a recombinant protein. It's a part of the commercial vector. We inserted the MIA_05579_1 coding sequence to the vector in that way so that His-tag coding sequence present in the vector would cause N-terminal extension of the resulting protein by 6 histidine residues.

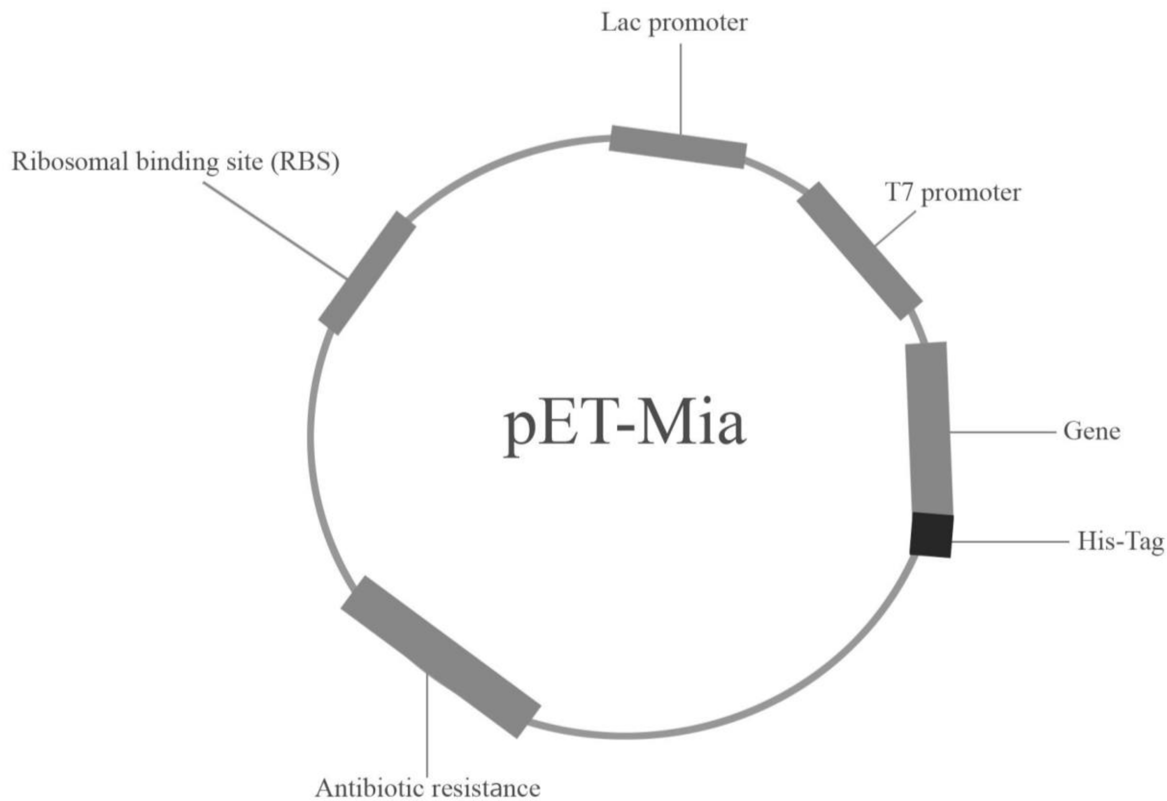

Figure 11: pET-Mia vector construction

Protease precursor (Figure 12): The N-terminal part of this protein is called signal peptide. Upon secretion, the signal peptide is removed by signal peptidase. Proteases are often expressed, both naturally and by recombinant expression, as precursors containing N-terminal extension, which has to be removed, otherwise the protease would not be enzymatically active. Removal of this N-terminal extension called propart, can proceed either autocatalytically or in trans, by the action of another protease. We first attempted to express the protease precursor, which accumulated in inclusion bodies. We isolated, solubilized, and dialyzed the inclusions. Only then we tried to cleave the precursor and to activate the MIA_05579_1 protease in trans by trypsin (Section 2.6).

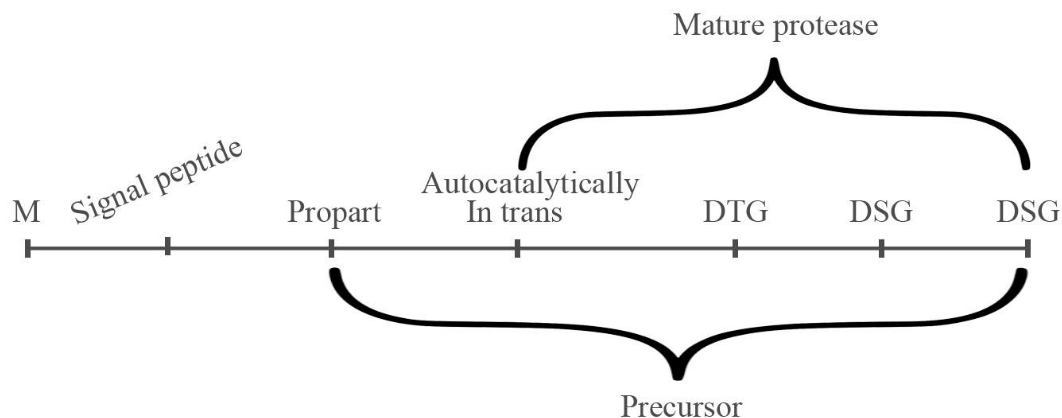

Figure 12: Protease precursor

Induction: 50 µg/ml of kanamycin (antibiotic) was added into each flask of the LB medium. The cells transformed with pET28-MIA_05579_1 vector and pET28-MIA_05579_1 with His-tag were cultivated in two different flasks of LB medium. The culture was cultivated at 37ºC in the incubator shaker until OD_{600} = 0.8. 100 µl of each culture was taken for analyzing on SDS-PAGE gels as time zero samples. Then, the expression of the recombinant protein was induced by adding 1 mmol/l of IPTG (isopropyl-β-D-galactopyranoside) – the lactose analog, which induces the lac promoter, but cannot be metabolized by *E. coli*. The cultivation proceeded for 3 hours in the incubator shaker. 100 µl of the culture was taken every hour for further analysis on SDS-PAGE gels. The cells were then harvested by centrifugation for 10 minutes at 4000 g.

Isolation of inclusion bodies: The pellets of cells were suspended by the addition of TN buffer (100 mM Tris-HCl, 150 mM NaCl at pH 7.4) and lysozyme (1mg/g of wet biomass), to lyse the cells. The mixture was left stirring for 30 minutes at a room temperature, and then it was frozen at -20ºC. After thawing the mixture, DNA was disintegrated by sonication for 1 min for three times until the mixture became homogenous. The inclusion bodies were recovered by centrifugation for 10 min at 9000 g. The cell supernatant was kept in the freezer for further analysis. At the same time, the pellets or sediments with inclusion bodies were washed twice with wash buffer (100 mM Tris-Cl, 2M urea, 5 mM EDTA, 2% triton at pH = 7.0) and centrifuged.

Dialysis of inclusion bodies: The inclusion bodies were solubilized by adding dilution buffer (50 mM Tris-HCl, 8 M urea, 1 mM glycine, 1 mM EDTA, 100 mM β-mercaptoethanol at pH 8.0) and vortexed until the pellet dissolved. The mixture was transferred into the tubing (molecular weight cut off 12-14 kDa) and left in the 500 ml buffer (20mM TRIS, 150 mM of NaCl) overnight. The next day, the tubings with mixture were transferred into another 500 ml of buffer (20 mM TRIS, 150 mM NaCl) and slowly stirred for 3 hours. After that, the mixture inside the tubings was taken for analysis.

All the prepared samples with ratio of 100 µl and 40 µl loading buffer were finally analyzed by SDS-PAGE gels.

Result: From SDS_PAGE gel (Figure 13), we observed a large amount of protein in the inclusion bodies, and this protein has approximately the molecular weight we expect the MIA_05579_1 protease precursor should have (45 kDa). After repeating many times of the same experiment, we got almost the same result and it looks like our protein is accumulated mainly in the inclusion bodies. Since the cytosol was removed, the next step is how to purify the inclusion bodies we had.

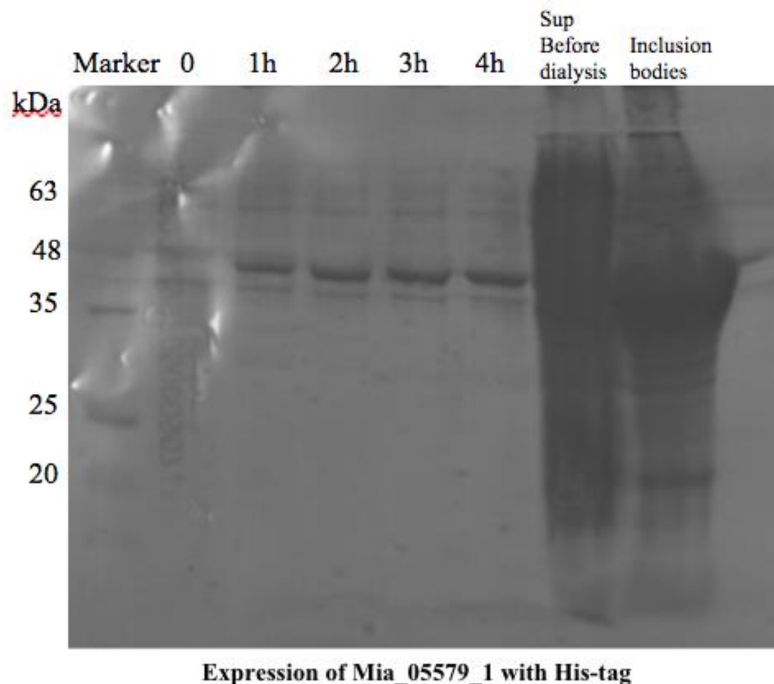

Figure 13: 15% SDS-PAGE of protein expression encoded by *gene* MIA_05579_1 (with His-Tag) in *E.coli Coommassie Staining*. *"Marker"- Molecular marker; Protein Test- Mixture 6* (Serva)

2.6 Trypsin cleavage of protease precursor

Trypsin is a serine protease from S1 endopeptidase family. It can cleave proteins at the sites with lysine and arginine amino acid residues on the C-terminal side. It is known as a proteolytic enzyme, which is important to protein digestion. Trypsin is produced within the pancreas in its inactive form, trypsinogen (http:// merops.sanger.ac.uk; Evnin *et al*. 1990; Khurana *et al*., 2019). We selected this method to attempt to activate the MIA_05579_1 protease in trans, by trypsin.

1 µl of trypsin solution (0.5 mg/ml) was added to 2 ml of dissolved and dialyzed inclusion bodies of MIA_05579_1 protease precursor with His-tag. Every 5, 10, 30, and 60 min, 40 µl aliquot of the mixture was mixed with 30 µl of SDS-PAGE loading buffer in new microtubes. To prepare sample zero, we needed only 40 µl of dissolved and dialyzed inclusion bodies of MIA_05579_1 with His-tag with 30 µl of loading buffer. All samples, including the sample zero, were boiled for 3 minutes and were analyzed by SDS-PAGE gel.

Result: The vector (pET28-MIA_05579_1) with His-tag that we used to express the proteins was constructed to contain ribosomal binding site, lac promoter, T7 promoter, antibiotic resistance gene, and our gene. We expressed the protease as a precursor. From the SDS-PAGE gel (Figure 14), we observed a trypsin cleavage on the approx. band 48 kDa from 5 min to 60 min. This means that trypsin did cleave the precursor and the most was at 60 min.

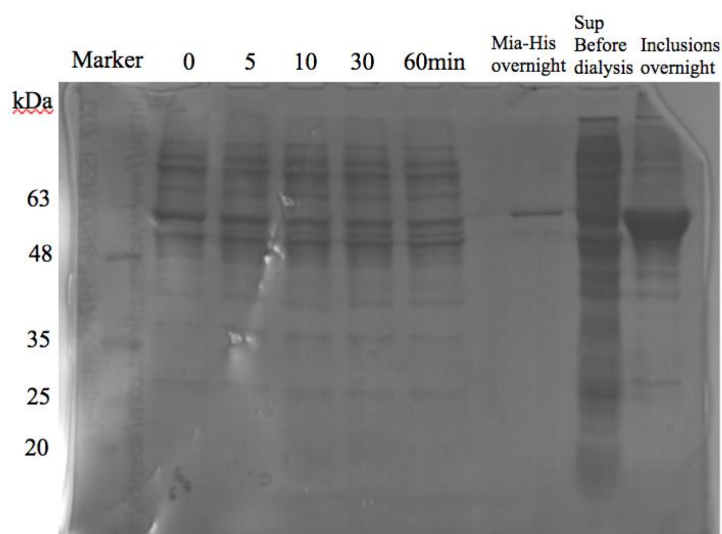

Figure 14: 15% SDS-PAGE of trypsin cleavage and protein expression encoded by *gene* MIA_05579_1 (with His-Tag) in *E.coli. Coommassie Staining*. *"Marker"- Molecular marker Protein Test- Mixture 6* (Serva)

3. CONCLUSION

The whole study has begun from the *M. capitatus* and *M. ingens* genome sequences provided thanks to the collaboration with the laboratory of Prof. Jozef Nosek in Bratislava, Slovakia. The genome sequences of the *Magnusiomyces* yeasts were then analyzed *in silico*, and it was showed that both species contain gene families potentially encoding secreted aspartic proteases. Yet, nobody has investigated them at the protein level. So, under a set of conditions inspired by protocols used for the pathogenic yeast of the genus *Candida* that have already been extensively studied, we started to cultivate both *Magnusiomyces* species that same way.

We were able to detect proteolytic activity, which degrades BSA in the culture medium in case of not having other sources of nitrogen. In general, a very small amount of enzymes is enough to catalyze biologically significant reactions. So, it's not strange at all when we fed the yeasts with BSA, and we observed the cleavage of BSA but not the proteases we were looking for. After checking the expected sequences of potential proteases, we estimated that most proteins carry more than 300 amino acids. It means that we're looking for proteins with an approximate size bigger than 30kDa.

After analyzing the two months´ incubation culture supernatant of both species, we observed that the *Magnusiomyces* yeasts produce some proteolytic enzymes, which hydrolyze BSA. In addition, the optimal condition for both species producing most secreted proteases is at room temperature from 20-23°C and at pH 3. To prove these results, we decided to examine the proteolytic activity with another method called Anson test by using hemoglobin. As expected, we observed the proteolytic activity in both samples from incubation at room temperature and pH3.

Besides, affinity chromatography on pepstatin agarose showed promising outcomes of *M. capitatus.* Since the concentration of purified protease was very low, the method has to be further optimized. Whereas for *M. ingens*, all the BSA was consumed by the yeast and the potential protease remained under the detection limit. We also tried to analyze the samples by using another technique, mass spectroscopy. Since the protease has a low concentration and the samples have a lot of hydrolytic products of BSA, we have still not been able to detect the protein we are searching for.

To step further and to get the crystals and structure analysis of proteases, we selected the potential protease of *M. ingens* encoded by the gene MIA_05579_1 with His-tag to express it in *Escherichia coli*. According to the results, we did observe a big amount of proteins with molecular weight 45kDa approximately in the inclusion bodies. So, we assumed that our protein is mainly accumulated in there. The trypsin cleavage experiment showed that the precursor was cleaved by trypsin eventually from 0 to 60 minutes. We managed to successfully express the protease precursor, but we have not managed to activate the protease as yet. However, the attempts to activate the protease continue.

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