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Influence of mineral substances on the excretion of specific enzymatic activity of fungal pathogens as important recognition factor for the identification of invasive infections

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ABSTRACT : *Minerals are crucial components of all living beings, because they are the main building material for building cell structures and tissues, they are part of enzymes and maintain protein structures. In the host organism, the availability of essential metals is limited and controlled by the host in order to control microbial growth. However, increasing the concentrations of certain essential elements in the host can promote the growth and survival of fungal pathogens, and positively affect the secretion of their various metabolic products, which are frequent factors in the virulence of these pathogens. One of the most common factors in the virulence of pathogenic fungi is the excretion of an extracellular enzyme, aspartyl proteinase, whose characterization may indirectly indicate the presence of pathogens as well as the potential danger of invasive fungal infections for the patient. Measurement of the enzyme aspartyl proteinase secreted by fungal pathogens has become very important in understanding cell metabolism and their application in medical diagnostics and the pharmaceutical industry. The aim of this study was to examine the effect of certain concentrations of alkaline and alkaline earth minerals on increasing the metabolic activity of fungal pathogens, enzymes aspartyl proteinase, which can be detected by a biosensor chip.*

KEYWORDS: *Minerals, fungi, aspartyl proteinase, biosensor*

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I. INTRODUCTION

Minerals due to their great nutritional importance in performing normal life processes of both macro and microorganisms are essential for all living things [1-3]. However, their lack can lead to dysfunction from optimal to suboptimal values. Therefore, there is an increasing use of dietary supplements, mineral supplements in order to prolong life, achieve and maintain good health, disease prevention, as well as increase strength and endurance [4]. Vitamin and mineral supplements can be contained in amounts that are ten to one hundred times higher than the reference dietary intake (DRI), and thus can cause potentially serious health problems if taken in excess for a long time [5]. In the composition of dietary supplements, important and necessary elements for living beings are allowed in certain chemical forms. Generally, the sources of macronutrients, elements of group I (sodium and potassium) and group II (magnesium and calcium) of the periodic table are essential elements for all living beings.

Elements of the IA group have very pronounced alkaline properties [6]. Alkali metals are ubiquitous and play an important role in maintaining the membrane potential of fungi and bacteria. This is achieved by the optimal concentration of alkali metals in the cellular cytoplasm, and there are numerous transport systems for the entry or removal of cations. Alkaline cations in fungi contribute to growth and virulence by regulating cell adhesion and hydrophobicity, as well as cell morphology [7]. Alkali metals, sodium and potassium, in humans, regulate water and salt metabolism within the cell, osmotic pressure, acid-base balance, and by activating

Na+/K+-ATP-ase participate in maintaining membrane potential whose strict control is important for nerve impulses, muscle contraction, especially cardiac [8].

Elements of group II A of PSE examined in the study were magnesium and calcium. These elements are alkaline [9]. In the human body, they function as co-factors of many enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis, and regulate the work of nerves and muscles, are also involved in muscle contraction and normal transmission of nerve impulses [8]. In addition to the fact that these elements also affect the growth of microorganisms, they affect the regulation of pH, namely calcium, and magnesium is necessary in the oxidation process [10-11]. These elements of groups I and II of the Periodic Table of the Elements in microorganisms may act to activate and/or induce certain enzymes [11].

Extracellular enzymes are the most common virulence factors of pathogenic organisms, the characterization of which may indirectly indicate the potential danger of infection of the patient. These enzymes primarily contribute to microbiological pathogenicity, emphasizing their adhesion, tissue damage, avoidance of the immune system, as well as their spread. Proteinases, extracellular enzymes, are considered one of the most common virulence factors of pathogenic fungi (*Candide albicans* and *Aspergillus niger*) because they contribute to the host-pathogen interaction. Proteinase enzymes break down surface proteins and impair local immunity, resulting in tissue invasion [12].The aspartyl proteinase enzyme secreted by the pathogenic fungi *Candida albicans* and *Aspergillus niger* was determinedfrom the group of proteinases. In fungi, aspartyl proteinase is synthesized as a zymogen, which is converted into an active enzyme with a change in pH. The active site of the enzyme is located between the two lobes, and the carbonyl groups of one of the two residues of aspartic acid are responsible for its activity [13]. Due to the extremely important role of minerals for human health, the main goal of this paper is to examine the effect of different concentrations of chemical forms used in dietary supplements on the excretion of extracellular enzymes secreted by fungi *Candide albicans* and *Aspergillus niger*. Molecular evolution has prompted the incorporation of selective metal binding sites to improve the activity, diversity, and/or stability of many enzymes. Following these discoveries, it has been observed that many enzymes show increased activity in the presence of metals. For many systems, selectivity for certain metals is low, and a weak increase in activity is achieved by higher cations [14].

II. MATERIAL AND METHODS

In this research, chemical forms of essential alkali and alkaline earth metals that are allowed in the production of dietary supplements were used. Concentrations of carbonate and chloride salts of sodium, potassium, magnesium and calcium used correspond to the daily intake of these elements for adults. Treated fungi of the genera *Candida*, *Candida albicans*, and *Aspergillus niger* of the genus *Aspergillus* were isolated from clinical samples of patients and inoculated under sterile conditions in 2%-Saburaud dextrose broth at a concentration of 102 CFU/mL. Prepared fungal samples were incubated at 37 °C and test metal salts were added every 24 hours, three consecutive days for *Candida albicans* and five days for *Aspergillus niger* due to slower fungal growth. Samples were aliquoted and analyzed in different time periods depending on the strain of the fungus.

2.1. Sample preparation and determination of total protein (aspartyl proteinase)

Aspartyl proteinase activity was determined spectrophotometrically after degradation of bovine serum albumin as a substrate as described by Crandall and Edwards (1987). To 0.5 mL of culture supernatant was added 2 mL of 1% bovine serum albumin BSA prepared in 0.1 M citrate buffer, with 3.5 pH value. The mixture was then incubated for 30 minutes at 37°C [15]. The reaction was then stopped by the addition of 5 mL of chilled 10% trichloroacetic acid, TCA. The precipitated protein was removed by centrifugation for 10 minutes at 1500 rpm. As a blank, 2 mL of 1% BSA was used, incubated at 37°C for 30 minutes, after which 5 mL of 10% TCA was added, and centrifugation was performed as in the supernatant culture.

The concentration of aspartyl proteinase was determined using a spectrophotometer and the absorbance of the test sample at 260 nm and 280 nm was measured (according to Warburg Christian). The final protein concentration is determined by the following equation: mg of protein/mL = $1{,}55 \times A_{280} - 0{,}76 \times A_{260}$.

Probes in which the concentration of lytic enzymes was analyzed by spectrophotometric method were also detected on biosensor chips, produced in laboratory conditions. Biosensors are printed based on K Printing Proofer (ERICHSEN) devices. For the functional efficiency of the sensor, one of the most important points is the biodegradable polymer layer, which is transferred directly to the Inconnel layer by the surface coating method via the mentioned device.

This layer is a polymer that usually consists of PLGA (poly (lactic-co-glycolic acid)) dissolved in ethyl acetate containing different amounts of desmodur. Desmodur serves to bind PLGA molecules, and affects the composition and viscosity. The polymer layer is sensitive to the analyte, has mimetic properties, shrinks and swells and must have the ability to react to various microbial and cellular lytic enzymes. Lytic enzymes secreted by fungi in contact with the polymer layer cause its degradation [16-17].

The process of degradation is irreversible, which results in a certain "signal" on the sensor in the form of irreversible color change [18]. 10 µl of the sample was pipetted to the surface of the biosensor and incubated in an oven for 16 hours at 37 °C. After that, the biosensor was washed with doubly distilled water and then dried under an intense stream of air.

III. RESULTS AND DISCUSSION

Mineral substances of alkali and alkaline earth metals as necessary components of all living organisms in certain concentrations can increase the secretion of lytic enzymes of treated pathogens. Aspartyl proteinase is an enzyme that acts as one of the key determinants of the virulence of *Candida albicans* and *Aspergillus niger* and is also involved in the spread of infections. Our aim was to examine the effect of alkali and alkaline earth metals on the secretion of aspartyl proteinase. IA group metals have small ionic radius that carry a strong positive charge without electron pairs in the valence shell. They have a low affinity for electrons and a strong tendency towards hydration. The strong charge and small ionic radius of the IA group metal give bond characteristics that are more covalent in nature. However, the interaction between the metal and the ligand is based solely on electrostatics and is not technically a "bond". Importantly, ligand and metal exchange rates are very high and allow for rapid kinetics of association and dissociation [14]. Within the IA group Periodic Table of Elements, sodium after lithium has the smallest ionic radius of 0.97 Å where the charge density is 1.05 q^2/r, therefore the free binding energy of the metal to a given ligand is more favorable than other elements of this group, and builds stronger metal-ligand bonds [14][19]. We compared the influence of chemical forms of sodium and potassium allowed in dietary supplements on the excretion of aspartyl protease of treated fungi, and determined which metal has a higher ability to secrete virulent enzyme.

Table I. Influence of alkaline minerals on aspartyl protease excretion of treated fungi

Minerals	Mineral concentrations	c (aspartyl protease) mg/mL						
		0h	8h	24h	32h	48h	56h	72h
Na_2CO_3	0.769 mg/mL	0.1094	0.0806	0.1318	0.1826	0.1449	0.0948	0.0940
K_2CO_3	0.923 mg/mL	0.1052	0.0767	0.1293	0.1451	0.1922	0.0528	-0.0416
NaCl	0.307 mg/mL	0.0622	0.1360	0.0988	0.1511	0.1662	0.0906	0.1306
KCl	0.353 mg/mL	0.0998	0.1173	0.1107	0.1645	0.1924	-0.1943	0.0835
Candida albicans	10^2 CFU/mL	0.1050	0.0830	0.0864	0.1752	0.1617	0.1741	0.0714
Minerals	Mineral concentrations	0h	24h	48h	72h	96h	120h	
Na_2CO_3	0.769 mg/mL	0.0264	0.0272	0.0424	0.1393	0.1216	0.0615	
K_2CO_3	0.476 mg/mL	0.0315	0.1122	0.0730	0.1870	0.0785	0.0599	
NaCl	0.769 mg/mL	0.0252	0.0490	0.0179	0.1540	0.0707	0.0656	
KCl	0.476 mg/mL	-0.1386	0.0218	0.0674	0.1604	0.1497	0.0559	
Aspergillus niger	10^2 CFU/mL	0.0096	0.0757	0.1058	0.1063	0.0815	0.0923	

The most significant values of the obtained results are presented in the Fig. 1.

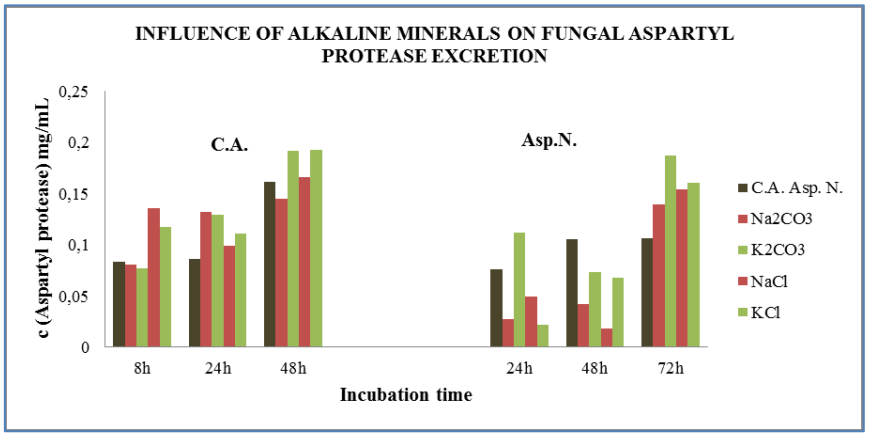

Fig.1. Comparison of alkaline minerals on aspartyl protease excretion of treated fungi

On the Fig. 1, we can see that most alkali metal minerals have a stimulating effect on the secretion of aspartyl proteinase, and that the greatest influence on the treated fungi is shown by potassium minerals. Potassium chloride at a concentration of 0.353 mg/mL and potassium carbonate at a higher test concentration of 0.923 mg/mL showed the most significant effect on the secretion of aspartyl proteinases with an increase in enzyme concentrations by 1.18 times over 48 hours in *Candida albicans* compared to the test fungi without added minerals.

Potassium minerals had a significant effect on the excretion of lytic enzyme in the fungus *Aspergillus niger*, at a concentration of 0.476 mg/mL of potassium carbonate after 72 hours of incubation at 37 °C, the concentration of enzyme increased by 1.75 times, and at the same concentration of potassium chloride slightly less stimulated the secretion of aspartyl protease by 1.5 times, which is again a higher concentration than in the test with sodium compounds. Sodium and potassium have one s-electron on the outer shell, which is why they have small ionization potentials and a fairly large size of their ions, which grow along the group.

This is explained by the fact that sodium, due to higher charge density and smaller ionic radius, has more favourable free binding energy for a certain ligand, which is why it built stronger metal-ligand bonds, however, potassium builds weaker bonds due to higher ionic radius. It then allows the binding of more ligands to compensate for these weaker metal-ligand interactions [19]. In the proteins Na^+ and K^+ they are virtually always bound to "hard" oxygen ligands rather than nitrogen or sulphur ligands (hard ligands have a more concentrated, less polarizing electron shell), oxygen atoms donated from amino acid side chains, and carbonyl oxygen atoms from the backbone of the water molecule. Negatively charged ligands Asp/Glu leucine aminopeptidase and Asp aspartyl proteinase that form the active site of these enzymes have a favourable charge and charge for a given metal ion [14].

Significant alkaline earth metals such as calcium and magnesium, and their mineral forms of dietary supplements, were used in the study. Magnesium, an element with a small ionic radius of 0.72 Å and a high charge density of 6.15 q^2/r, allows it a very favourable free binding energy for a particular ligand, as a result of which it builds strong metal-ligand bonds. The coordination number of magnesium is 2 and 4 [14]. Magnesium ion ligands are usually oxygen atoms, but not nitrogen or sulphur atoms. In proteins, the magnesium ion typically has six oxygen ligands in an octahedral arrangement [14] [20]. In addition to magnesium, the influence of calcium minerals on the excretion of extracellular enzymes of treated pathogens was also researched. Along the group, the ionic radius of calcium increases, so the value of the ionic radius for calcium is slightly higher than that of magnesium, and is 0.99 Å, and the charge density is 4.04 q^2/r, which is why it builds weaker metal-ligand bonds than magnesium. Calcium has a coordination number of 6 [14]. At the strongest sites of calcium binding on proteins, coordinating oxygen atoms (provided by carboxylates of amino acid residues Asp and Glu and carbonyl polypeptide chain) are located at the tops of the slightly distorted pentagonal bipyramid or octahedron. In the pentagonal bipyramid, five oxygen atoms are located in the equatorial plane at a distance of 2.5 Å from each other [14] [20].

The obtained results of spectrophotometric measurements in examining the influence of magnesium and calcium minerals on aspartyl proteinase excretion in treated fungi are presented in the Table II. and we compared which of these two minerals has a greater impact on the secretion of aspartyl protease by treated fungi.

Table II. Investigation of the influence of alkaline earth minerals on aspartyl proteinase secretion in samples of tested fungi

Minerals	Mineral concentrations	c (aspartyl proteinase) mg/mL						
		0h	8h	24h	32h	48h	56h	72h
$MgCO_3$	0.107 mg/mL	0.1107	0.1326	0.1327	0.1239	0.1558	0.0215	0.0832
$CaCO_3$	0.1153 mg/mL	0.1813	0.2108	0.2797	0.0209	0.2298	0.0153	0.1276
$MgCl_2$	0.107 mg/mL	0.1160	0.1367	0.1645	0.0549	0.1657	0.0321	0.0887
$CaCl_2$	0.1153 mg/mL	0.1013	0.0995	0.3364	0.1266	0.2053	0.1623	0.1241
Candida albicans	10^2 CFU/mL	0.1050	0.0830	0.0864	0.1752	0.1617	0.1741	0.0714

Minerals	Mineral concentrations	0h	24h	48h	72h	96h	120h
$MgCO_3$	0.0769 mg/mL	0.0117	0.1053	0.0392	0.1327	0.1167	0.0601
$CaCO_3$	0.0769 mg/mL	0.0021	-0.0608	0.1152	0.1759	0.0012	0.0876
$MgCl_2$	0.107 mg/mL	0.0279	0.0471	0.0299	0.1373	0.0819	0.1052
$CaCl_2$	0.1153 mg/mL	0.0707	0.0923	0.1067	0.1503	0.0815	0.0844
Aspergillus niger	10^2 CFU/mL	0.0096	0.0757	0.1058	0.1063	0.0815	0.0923

The most significant values of the researched alkaline earth minerals on the lytic enzyme excretion of the treated fungi were presented in the growth diagram.

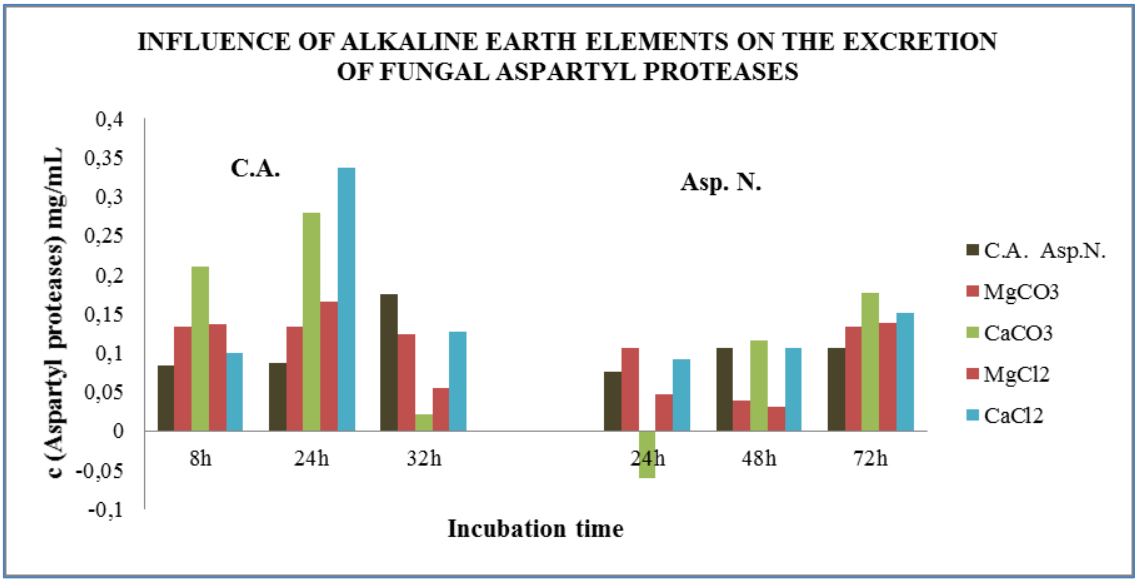

Fig.2. Comparison of the influence of alkaline earth minerals on the excretion of aspartyl protease by treated fungi

The alkaline earth minerals magnesium and calcium at the concentrations used showed a stimulating effect on the extraction of aspartyl proteinase in the treated fungi. Calcium minerals, calcium chloride concentration of 0.1153 mg/mL increased the concentration of secreted enzyme by 4.05 times in *Candida albicans*, and in experiments with *Aspergillus niger* the greatest impact on increased enzyme activity showed calcium carbonate concentration of 0.0769 mg/mL with increasing aspartyl proteinase concentrations by 1.75 times.

Magnesium and calcium are characterized by fully filled electron shells ($2s^2$ $2p^6$ i $3s^2$ $3p^6$); therefore, they have no preference for the direction of making connections. The atomic radius of Mg^{2+} is 0.65 Å, while the atomic radius of oxygen is 0.99 Å [6]. For this reason, only six oxygen atoms can be found in the octahedral geometry near Mg^{2+}. Due to the significantly higher charge density of Mg^{2+} compared to Ca^{2+}, the energy of a single Me-O bond appears to be higher for magnesium than for calcium.

Water molecules dissociate from aqueous magnesium complexes significantly more slowly than calcium. Mg^{2+} binding sites in proteins contain at least one carboxylate ligand that coordinates the Mg^{2+} ion in a monodentate manner. Because the atomic radius of Ca^{2+}, is larger, it has a tendency to bind carboxylate binding but lower affinity for water [19] [20]. The larger ionic radius of calcium, gives it a greater ability to bind more ligands to compensate for the weaker metal-ligand interactions [19].

The biggest problem today is opportunistic infection caused by the fungi *Candida albicans* and *Aspergillus niger*. Invasive fungal infections are one of the leading causes of morbidity and mortality in hospitalized patients and the immunocompromised population. Due to the constant increase in the number, severity and complications of these infections, our primary goal was to develop a biosensor chip to demonstrate the specific enzymatic activity of the fungus.

This approach is an additional fast and cost-effective screening instrument for the widespread detection of fungal pathogens in clinical trials, and thus for the identification of invasive fungal infections. The biosensor is based on the local degradation of a specific biolayer by lytic enzymes secreted by fungi, which enables sensitive optical reading. The mechanism of action is based on the fact that the fungi, after adhering to the polymer layer of the sensor, begin to secrete their extracellular enzymes that degrade the polymer layer of the sensor, and which is a prerequisite for the manifestation of the signal, that is, the change of colour on the sensor surface which is visible to the naked eye [21].

From our tests that we presented in the results, we know that the fungi *Candida albicans* and *Aspergillus niger* show the necessary enzymatic activity. The sensor that showed a very good signal for the lytic enzyme of *Candide albicans* is a polymer in composition of 23% PLGA (50:50) with 1% desmodur, 0.5 mg of starch and 2 μL of oil dissolved in ethyl acetate. Biosensors with 20% PLGA (50:50), 1% desmodur, glucose, trypton and tributyrine showed a strong signal for the fungus *Aspergillus niger*.

The obtained biosensors after application of pathogen samples treated with minerals are shown in the following Fig. 3 and Fig. 4.

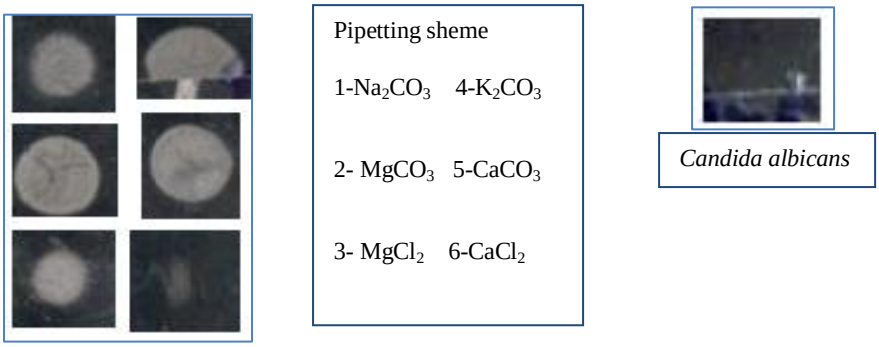

Fig.3. Signal intensity after incubation with samples of *Candida albicans* and minerals

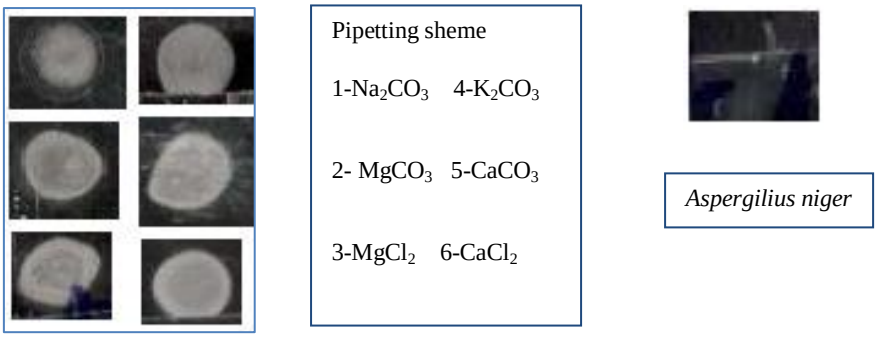

Fig.4. Signal intensity after incubation with samples of *Aspergilius niger* and minerals

Mineral probes with high enzymatic activity of aspartyl proteinase which was spectrophotometrically measured also showed a visible signal of high intensity on the biosensor chip. This finding suggests that certain chemical forms of essential macro elements of groups I and II of the periodic system of elements, which are allowed in the production of dietary supplements, can be used to stimulate metabolic activity of fungal cells and provide a strong signal for biosensor detection.

IV. CONCLUSION

Our goal was to research the influence of certain essential alkaline and alkaline earth minerals on the secretion of the lytic enzyme aspartyl proteinase, as a virulent pathogen factor of the most common invasive fungal infections. The chemical forms of minerals used in the production of dietary supplements, used for a certain period of time showed a stimulating effect on the excretion of extracellular enzymes.

Metals of larger ionic radius such as potassium from group I and calcium from group II PSE build weaker bonds compared to other tested metals, allowing more ligands to bind to compensate for these weaker metal-ligand interactions. Higher binding to ligands increases the activity of virulence factors, i.e. the excretion of the analysed extracellular enzymes.

The enzyme activity of the treated fungi was detected by biosensor chips, and a strong detection signal was visible to the naked eye, a change in the colour of the biosensor, which is the result of degradation of the polymer layer of the biosensor due to the lytic enzymes. The signal on the biosensors was product of mineral probes that also showed an increased amount of aspartyl proteinase activity which was possible to measure using spectrophotometric method. The presented biosensors enabled a fast and cheap way of detecting the tested fungal pathogens.

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