Enzyme production by soilborne fungal strains of *Aspergillus niger* isolated from different localities affected by mining

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**Abstract.** The research focused on the enzyme production/activity of sixteen *Aspergillus niger* strains isolated from different localities. The soils vary mainly in their pH value, which ranges from an ultra-acidic (< 3.5) to a very strongly alkaline (> 9.0) environment. Contamination caused by several centuries of mining activities at old mining sites persists at all the localities. The concentrations of toxic elements, such as As, Sb, Zn, Cu and Pb, very often exceed the common limit values. Presence of toxic elements in contaminated sites may affect microscopic fungi and cause changes of their physiology, including the production of different metabolites, such as enzymes. Production of esterase, cellulase, lipase and protease was investigated. Changes in physiological properties, such as the growth and enzymatic activity of the sixteen *A. niger* wild type strains, were determined. Esterase, cellulase and lipase activity was not determined in the sixteen strains tested. Considerable differences were recorded in the size of the colonies also within strains cultivated on the same nutrient medium. The control strain from locality Gabčíkovo formed the smallest colonies when tracking LA, EA and PA compared with the other strains. Lipase production was determined for several strains at different intensities and was highest in the strain isolated from the uncontaminated locality Gabčíkovo. The enzymatic activity of the other strains isolated from contaminated sites was very low. The achieved results confirmed the direct influence of environmental factors on the physiological properties of the studied strains of *Aspergillus niger*.

1. Introduction

Soil enzymes, which are primarily of microbial origin, play a key role in the transfer of energy in the soil by the decomposition of organic matter. They help stabilize the soil structure and catalyse important reactions necessary for the life processes of microorganisms. The enzymatic activity of microorganisms is closely connected to the physico-chemical and biological properties of the soil [1]. Enzymes, such as lipase, cellulase, esterase and protease, for example, have an important role. Lipase and esterase belong to the class of hydrolases, which hydrolyse fatty acid esters in soil, thus promoting the decomposition of organic matter. They occur abundantly in nature, especially in bacteria, yeasts and microscopic filamentous fungi [2]. Proteases play an important role in the nitrogen cycle. A large amount of nitrogen is found in soil in the form of proteins, which are hydrolysed by the proteolytic activity of microorganisms, resulting in the release of this element into the environment [3]. Cellulase is an enzyme that hydrolyses the polysaccharide cellulose, which occurs in the walls of plant cells. This process leads to a significant input of organic matter into the soil environment [4]. The enzyme production depends on wide range of environmental factors. The physico-chemical and biological properties of the soil are often affected by anthropic pollution which in most cases is caused by several
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centuries of mining activity. Presence of toxic compounds in contaminated sites may affect microscopic fungi and cause the genome alteration, which may result in changes of their physiology, including the production of different metabolites [5].

The aim of the study was to compare the production of the enzymes lipase, protease, esterase and cellulase by several strains of *A. niger* isolated from various localities. These localities differ from one another by the value of the soil reaction and by the varying degrees of contamination with heavy metals and potentially toxic elements; most of them are sites of old ecological burdens.

2. Material and method

2.1. Strains of the microscopic filamentous fungus *A. niger*
The localities or substrates from which the *A. niger* strains were isolated differed mainly in the value of the soil reaction from ultra-acidic (<3.5) to strongly alkaline (8.5 – 9.0) as well as in values of potentially toxic elements (Tab. 1). For monitoring the enzymatic activity, we used sixteen strains of *Aspergillus niger* (*A. niger*). We selected *A. niger* from uncontaminated alluvial forest soil in the Gabčíkovo locality as the control strain.

2.2. Enzyme activity

We observed the enzyme activity of the strains on diagnostic nutrient media according to Kraková et al. [6], specified for the determination of individual enzymes: cellulase activity (CA) on CongoRed medium, esterase activity (EA) on Tween 80 medium, lipase activity (LA) on Spirit Blue medium (HiMedia, Mumbai, India) and protease activity (PA) on Gelatine P3 medium. The production of an enzyme is visible in the form of the so-called “halo” effect (CA, LA) or as an opaque (PA) or yellow zone around the growth of the organism (EA) [7]. The growth and colony size of all the *A. niger* strains were monitored for comparison by culture on SDA agar (Sabouraud Dextrose Agar, HiMedia, Mumbai, India).

3. Results and discussion

We monitored the enzymatic activity of the microscopic filamentous fungus *A. niger* in strains isolated from environments that differed by soil pH reaction and the presence of potentially toxic elements. We classified the strains on the basis of these characteristics in table 1. The strains *An – S* (Banská Štiavnica – Šobov, an eroded area of dystric cambisol of modal var. acid form, without vegetation) and *An – N* (Nováky, coal dust) come from an ultra-acidic soil environment of pH < 3.5. An extreme acidic soil environment from 3.5 to 4.4 is characterised by the technosol from the Poproč site (*An – Pop 4*) and a very strong acidic environment from 4.5 to 5.0 by the haplic leptosol from the same locality (*An – Pop 1*). The Pezinok locality, as well as river sediment from the Blatina stream (*An – P*), river sediment from the Smolník stream (*An – Sm*) and soil from the base of the Kýčera hill in the Belianske Tatras (*An – Kn* i) are among the areas of strong acidic soil reaction from 5.1 to 5.5. The locality Laguna Ostrava (Czech Republic), which contains sludge from oil refining (*An – L18*), typifies a neutral area with an environmental pH of 6.5 – 6.8. Strains from the haplic leptosol in the Poproč locality (*An – Pop 3*), from an ash layer in the Zemianske Kostoľany locality (*An – ZK*) and from the eutric fluvisol at the Gabčíkovo locality (*An – G*), which served as the control or reference strain in our experiments, come from a slightly alkaline soil environment from 7.4 to 7.8. A medium alkaline soil environment with a pH from 7.9 to 8.4 is represented by the strain *An – KD* (strain isolated from a sample from the desert in Kuwait). A strong alkaline soil environment with a pH from 8.5 to 9.0 is represented by the strain *An – KF*, taken from soil at a farm in Kuwait, and the strain *An – SL* isolated from the technosol at the Slovinky site. A weak acidic soil environment (6.1 – 6.5) and very strong alkaline soil environment (> 9.0) are not represented. A detailed description of the strains is presented in Šimonovičová et al [8]. The *An – Aral* sample comes from the strongly saline bottom of the Aral Sea in Uzbekistan, which has a strong alkaline sediment (pH 8.6) [9].
Table 1. *A. niger* strains sorted based on the range of pH values isolated from soils and substrates and their chemical characteristics.

| Strain  | pH          | potentially toxic elements in soil/substrate | Strain    | pH          | potentially toxic elements in soil/substrate |
|---------|-------------|---------------------------------------------|-----------|-------------|-----------------------------------------------|
| 1. An-S  | 3.0         | ultra acidic                                | Al 727–506 mg/kg | 9. An-L18   | neutral                                      |
|         |             |                                             | NEL 201 000 mg/kg; PAH C10-C40 121 000 mg/kg; *Cr 182 mg/kg; *Cu 2 102 mg/kg; *Zn 6 946 mg/kg; *Ba 3 652 mg/kg; *Pb 4 066 mg/kg |
| 2. An-N  | 3.32        | ultra acidic                                | As 400 mg/kg; Mn 302,4 mg/kg; *Zn 21,4 mg/kg | 10. An-Pop3 | 7.45 slightly alkaline                        |
|         |             |                                             | As 25 mg/kg; *Sb 5825 mg/kg; *Zn 150 mg/kg; *Cu 60 mg/kg; Pb 70 mg/kg; Hg 0,5 mg/kg |
| 3. An-Pop4 | 3.85       | extreme acidic                              | As 25 mg/kg; *Sb 1022 mg/kg; *Zn 150 mg/kg; Cu 60 mg/kg; Pb 70 mg/kg | 11. An-ZK | 7.51 slightly alkaline                        |
|         |             |                                             | As 25 mg/kg; *Sb 1022 mg/kg; *Zn 150 mg/kg; Cu 60 mg/kg; Pb 70 mg/kg |         |                                              |
| 4. An-Pop1 | 4.52       | very strong acidic                           | As 363 mg/kg; Sb 93 mg/kg; Fe 82.8 mg/kg; Al 5,5 % | 12. An-G | 7.7 slightly alkaline                        |
|         |             |                                             | Mg 344 mg/l; *Fe 463 mg/l; *Mn 36,5 mg/l; *Al 107 mg/l; *Cu 3263 μg/l; *Zn 12600 μg/l; *Cd 15 μg/l |         |                                              |
| 5. An-P  | 5.25        | strong acidic                                | Mg 344 mg/l; Fe 463 mg/l; *Mn 36,5 mg/l; *Al 107 mg/l; *Cu 3263 μg/l; *Zn 12600 μg/l; *Cd 15 μg/l | 13. An-KD | 8.25 medium alkaline                        |
|         |             |                                             | Mg 344 mg/l; Fe 463 mg/l; *Mn 36,5 mg/l; *Al 107 mg/l; *Cu 3263 μg/l; *Zn 12600 μg/l; *Cd 15 μg/l |         |                                              |
| 6. An-Sm | 5.4         | strong acidic                                | *Cu 8186 mg/kg; *Zn 25108 mg/kg; *Pb 2964 mg/kg; *Mn 2647 mg/kg; *Cd 8,76 mg/kg | 14. An-KF | 8.49 medium alkaline                        |
|         |             |                                             | Mg 344 mg/l; Fe 463 mg/l; *Mn 36,5 mg/l; *Al 107 mg/l; *Cu 3263 μg/l; *Zn 12600 μg/l; *Cd 15 μg/l |         |                                              |
| 7. An-Kmi | 5.4         | strong acidic                                | As 200 mg/kg; *Sb 2099 mg/kg; *Zn 200 mg/kg; Cu 70 mg/kg; Pb 115 mg/kg | 15. An-SL | 8.6 strong alkaline                        |
|         |             |                                             | As 200 mg/kg; *Sb 2099 mg/kg; *Zn 200 mg/kg; Cu 70 mg/kg; Pb 115 mg/kg |         |                                              |
| 8. An-Pop5 | 6.05       | medium acidic                                | As, Sb, Cr, Cs | 16. An-Aral | 8.6 strong alkaline                        |
|         |             |                                             | As 200 mg/kg; *Sb 2099 mg/kg; *Zn 200 mg/kg; Cu 70 mg/kg; Pb 115 mg/kg; Hg 0,5 mg/kg |         |                                              |

* Exceeded values of elements; NEL, non-polar extractables; PAH, polyaromatic hydrocarbons.

For checking the growth of the *A. niger* strains we used SDA broth. Upon culturing the strains on the control medium, we did not record any large differences in the size and growth rate of the colonies. The largest colonies, with a diameter of 5 cm, were recorded in a large majority of strains, namely An – S and An – N (which come from ultra acidic soil environment), An – Pop 4 (from extremely acidic soil environment), An – Pop 1 (from the very strong acidic soil environment), the strains An – P, An – Sm and An – Kmi (all from a strong acidic environment), An – L18 (from a neutral environment), An – ZK and An – G (from a slightly alkaline environment), and An – KF and An – SL (from a medium and strong alkaline environment). We recorded colonies with a diameter of 4.6 cm in the An – KD
strain (from a medium alkaline environment). We recorded an average value of 4.4 cm for the An – Pop 3 strain (from a slightly alkaline soil medium), followed by the An – Aral strain, which had colonies of diameter 4.4 cm. The smallest colonies, with a diameter of 3.8 cm, were formed by the An – Pop 5 strain, which comes from a medium acidic environment. Since the differences in the size of the colonies are minimal, it can be assumed that the impact of ecological factors was not manifested in this case, or it was very small to negligible.

Pronounced differences were observed after five days of cultivation, particularly in the size of the colonies and the intensity of sporulation of the strains on diagnostic nutrient media. The largest colonies were formed by strains on the nutrient medium for lipase activity, where we also observed the most intense sporulation. Next were the strains on media for esterase activity and protease activity, and the smallest colonies were formed by strains on nutrient medium for cellulase activity (Figure 1).

Figure 1. Enzymatic activity of the control strain An – G: SDA (a), LA (b), EA (c), CA (d) and PA (d).

Considerable differences were recorded in the size of the colonies also within strains cultivated on the same nutrient medium. The control strain An – G formed the smallest colonies when tracking LA, EA and PA compared with the other strains. Figure 2 shows the differences between the strains An – G and An – L18 isolated from the neutral to slightly alkaline soil media (6.85 – 7.7 pH) and strains An – Pop 1 and An – Sm isolated from very-to-strong acidic media (4.52 – 5.4 pH) when monitoring esterase activity.

Figure 2. Esterase activity of strains An – G (a), An – L18 (b), An – Pop 1 (c) and An – Sm (d) on Tween 80 medium.

The production of the monitored enzymes of the A. niger strains is shown in table 2. When culturing the strains on SBA broth, the colonies had an average size of 2 ± 0.1 to 3.1 ± 0.1 cm. The largest colonies were formed by the An – Pop 1, An – Pop 3 and An – KD strains. The smallest colonies were clearly formed by the control strain An – G, which, however, showed the most intense lipase activity, as the medium stained quickly and the so-called “halo” effect was formed. Strains An – Pop 4, An – P, An – L18, An – ZK and An – SL showed only very weak lipase production (figure 3). In other A. niger strains, the production of lipase was not confirmed, which means that these A. niger strains either do not produce the enzyme at all or that production is so low that discolouration of the SBA diagnostic medium does not occur at all.
Figure 3. Lipase activity of strains An – G (a), An – Pop 4 (b), An – ZK (c), An – SL (d), An – L18 (e) and An – P (f) on Spirit Blue medium.

The enzyme esterase has a function in soil similar to that of lipase. Both enzymes support the breakdown of organic matter by catalysing the hydrolysis of lipid compounds [10]. When monitoring esterase activity on the Tween 80 diagnostic medium, rapid growth and intense sporulation of A. niger strains were observed, with a mean colony size of 2.5 ± 0.1 to 3 ± 0.1 cm. Yellow staining around the colony growth was observed only in the An – L18 strain, and no similar reaction was observed in the other strains. When cultured on Gelatine P3 broth for determining protease activity, A. niger strains formed colonies with an average size of 1.5 ± 0.1 to 2 ± 0.1 cm. However, no halo effect or an opaque zone, which indicates the formation of this enzyme, appeared around the growth of the colonies. On the basis of some studies, the production of the protease enzyme may be inhibited by the occurrence of above-limit Cd values in the environment [11]. This element was present in the majority of the examined localities.

Upon monitoring cellulase activity on the CongoRed medium, very slow growth of A. niger strains was observed, with an average colony size of 1.5 ± 0.1 cm. No production of the cellulase enzyme, which would have been found in a similarly opaque arc around the colony, was observed. Similar results were reported with the monitoring of cellulase activity of Penicillium species taken from environments with above-limit values of the elements As, Zn, Sb and Pb [12].

Table 2. Growth of A. niger (SDA) strains and their lipase (LA), esterase (EA), protease (PA) and cellulase (CA) activity.

| Strain     | SDA | LA | EA | PA | CA |
|------------|-----|----|----|----|----|
| An-S       | ++  | +  | +/-| -  | -  |
| An-N       | ++  | +  | +/-| -  | -  |
| An-Pop 4   | ++  | ++ | +/-| -  | -  |
| An-Pop 1   | ++  | +  | +/-| -  | -  |
| An-P       | ++  | ++ | +/-| -  | -  |
| An-Sm      | ++  | +  | +/-| -  | -  |
| An-Kmi     | ++  | +  | +/-| -  | -  |
| An-Pop 5   | +   | +  | +/-| -  | -  |
| An-L18     | ++  | ++ | +  | -  | -  |
| An-Pop 3   | +   | +  | +/-| -  | -  |
| An-ZK 5    | ++  | ++ | +/-| -  | -  |
| An-G       | ++  | ++ | +/-| -  | -  |
| An-KD      | +   | +  | +/-| -  | -  |
| An-KF      | ++  | +  | +/-| -  | -  |
| An-SL      | ++  | ++ | +/-| -  | -  |
| An-Aral    | +   | ++ | +/-| -  | -  |

Explanations: SDA (control of growth of strains), ++ very good growth/enzyme production; + good growth/weak enzyme production; +/- good growth/enzyme not formed; - weak to very weak growth/enzyme is not formed.
Monitoring the enzymatic activity of microorganisms confirmed the inhibitory effect of potentially toxic elements on the production of various enzymes in several studies. Potentially toxic elements, such as Pb, As, Cd and others, reduce the production of some enzymes, such as urease, catalase, arylsulfatase, phosphatase and protease [13, 14]. Potentially toxic elements had a similar effect on the enzymatic activity of *Aspergillus fumigatus*, *Byssoschlamys spectabilis*, *Cladosporium pseudocladosporioides*, *Epicoccum nigrum*, *Hamigera avellanea*, *H. insecticola*, *Chaetomium globosum*, *Neosartorya fischeri*, *Paeclomyces variotii*, *P. carneus*, *Pencillium chrysogenum var. chrysogenum*, *P. citreonigrum*, *P. daleae*, *P. sacculum*, *P. restrictum*, *Bjerkandera adusta* and *Irpex lacteus*, which were isolated from soil and sludge in a mining area [7].

The production of enzymes depends on several factors, such as pH, temperature and composition of the nutrient medium, for example. However, the environmental factors in which the species of microscopic filamentous fungus originates also play an important role [8]. Depending on these environmental factors, the physiological properties of microscopic filamentous fungi also subsequently change, which can be expressed by a change in enzymatic activity [15, 16]. The production of enzymes is a relatively rapid reaction to changes in conditions, which allows them to be used to evaluate the state of the soil microbiota, e.g. the impact of various potentially toxic elements [17].

4. Conclusion

Differences in the growth and size of colonies, as well as weak enzymatic activity in strains taken from a contaminated environment, are with the highest probability caused by the negative impact of above-limit values of potentially toxic elements that occurred in the majority of the localities. The achieved results confirmed the direct influence of environmental factors on the physiological properties of the studied strains of *Aspergillus niger*. We recorded enzymatic activity when monitoring lipase production, where we observed the most intense activity in the strain that was isolated from an uncontaminated environment (An – G). As a result of the impact of potentially toxic elements that exceed the limit values in all substrates taken from mining sites, the production of enzymes was probably suppressed in strains taken from contaminated environments.

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