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Responses of *Aspergillus niger* to selected environmental factors

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Abstract

Four wild type strains of *A. niger* were collected from soil and stream sediments representing environments with variable level of As, Sb, Al, Fe, Cd, Cu, and Zn contamination. Banská Štiavnica-Šobov (S), Pezinok-Kolársky vrch (P) and Slovinky (Sl) represent contaminated localities. Locality Gabčíkovo (G) was as a control site. The influence of toxic elements in these substrates on fungal growth, colony size, enzymatic activity, production of organic acids and their pelletization in water suspensions with montmorillonite was studied. The aim of our study was to find out how the wild type strains from (contaminated) environment will behave in different model solutions. We also wanted to add some new information in this area of study, because that there is some gap in the available knowledge.

Introduction

Aspergillus niger strains belong to the most widespread microscopic filamentous fungi in the environment (Nováková *et al.* 2012; Šimonovičová 2013). This strain is very often used to study variable processes, such as heavy metal accumulation or bioleaching (Gan *et al.* 2016; Peťková *et al.* 2013; Xu *et al.* 2014), production of organic acids (Hu *et al.* 2016) or different enzymes (Akhter *et al.* 2011; Mrudula and Murugammal 2011). Environmental factors affecting the fungus growth include type of soil and its properties such as pH, contents of organic matter and heavy metals and potentially toxic elements; all these represent primary indicators with very rapid influence on microorganisms (Šimonovičová 2014). Physiological properties of microorganisms changed in dependence on these factors. Environmental pollution strongly affected not only biochemical, but also macro- and micro-morphological characteristics of *A. niger* strains (Šimonovičová *et al.* 2013). Four wild type strains of *A. niger* were collected from soil and stream sediments representing environments with different of contamination. The influence of toxic elements in these substrates on fungal growth, colony size, enzymatic activity, production of organic acids and their pelletization in water suspensions with montmorillonite was studied. The aim of our study was to find out how the wild type strains from (contaminated) environment will behave in

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different model solutions. We also wanted to add some new information in this area of study, because that there is some gap in the available knowledge. Fungal pellets have many advantages of easy harvest, low fermentation broth viscosity and high yield of proteins (Zhang and Zhang 2016). It was discovered, that mycelial pellets are effective as a biomass carrier for the immobilization of bacteria for the degradation of target pollutants (Zhang *et al.* 2011), for degradation of lignin in water and bioremediaton of soil contaminated with pentachlorophenol PCB (Rubilar *et al.* 2009).

Experimental

Microscopic fungi

Four wide types of *A. niger* strains were isolated from terrestrial substrates and stream sediment from Šobov, Pezinok, Gabčíkovo and Slovinky study sites in Slovakia (Fig. 1). The sampling was done from surface horizons at the 10-20 cm depths. The samples were transported and stored in PVC sacks at 4 °C in the dark. Prior to chemical analyses, samples were air-dried at room temperature, homogenized, and then passed through a 2-mm sieve, then stored in a field-moist condition at 4 °C in the dark. All procedures were carried out according to Čurlík and Šurina (1998), Šimanský (2011) and Hrivňáková *et al.* (2011). Total organic carbon content was measured by dichromate oxidation (Nelson and Sommers 1996). The isolation of fungal strains was realized using the dilution plating method (dilution of 10^{-4} CFU) from 10 g of substrate. Dilutions were plated on Sabouraud Maltose Agar (SAB) (Himedia, Mumbai, India) for isolation. Studied strains were isolated from mixed culture of soil microscopic filamentous fungi. Pure cultures of all strains were cultivated in an incubator at 25 °C for 5-7 days on SAB and identified according to micromorphological features and PCR analyses (Šimonovičová *et al.* 2013; Jesenák *et al.* 2015).

Growth and colony size of A. niger *wild type strains*

The growth and the size of colonies of all studied *A. niger* wild type strains were observed after cultivation on SAB agar (Sabouraud Dextrose Agar, it means Mycological peptone, Dextrose and Agar – HiMedia,Mumbai, India) in a temperature-controlled oven at 25 °C for 5-7 days in three replicated runs. Influence of the original substrates from Šobov, Pezinok, Gabčíkovo and Slovinky sites was studied by adjustment of pH values of SAB agar to pH 3-5-7 and 9.

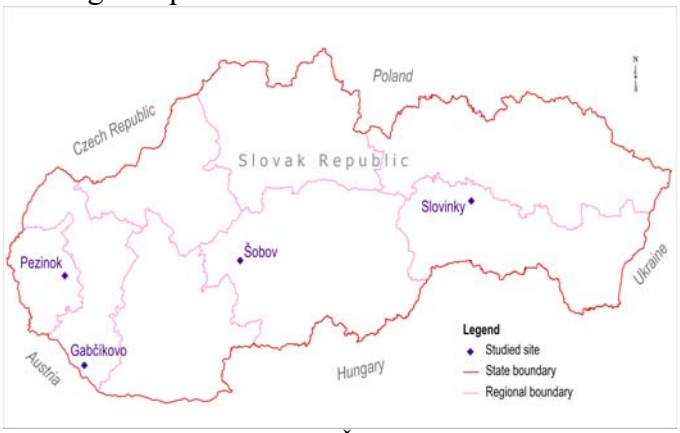

Fig. 1. Study of polluted sites Šobov, Pezinok, Slovinky and Gabčíkovo. The last mentioned locality was the control site.

Enzymatic activity of A. niger *wild type strains*

Enzymatic activity of the strains was studied using diagnostic culture media specific for each individual enzyme as follows: cellulase activity (CE) on CongoRed medium, esterase activity (EA) on Tween 80 medium, lipase activity (LA) on Spirit Blue medium and protease activity (PA) on Gelatine P3 medium. Productivity of enzyme is visible as so-called "halo" effect (Kraková *et al.* 2012; Šimonovičová and Čerňanský 2016) which is a creating zone or a ring of certain diameter around the fungal colony.

Production of organic acids of A. niger *wild type strains*

Pre-treatment of samples. The samples were stabilised at -4 °C. The samples were defrosted in water bath at 20 °C before HPLC analysis. Then, all samples were homogenised and filtered through regenerated cellulose membrane filters with pore size 0.45 μm.

HPLC analysis. Analysis of the samples was realized using a HPLC device with a PDA detector (YoungLin 9100). The mobile phase used to analysis included methanol and water; their

Table 1. Characteristics of the soils from the natural sites where the fungal strains were isolated.

Strain	Locality	pH	Substrate
An - S	Banská Štiavnica-Šobov	3.12 ultra acidic	Dystric Cambisol (contaminated and eroded without vegetation); % C_{ox} 0. 49; Al 727 – 506 mg/kg
An - P	Pezinok	5.3 strongly acidic	Stream sediment; % C_{ox} 7.2; As 363 mg/kg; Sb 93 mg/kg; Fe 82.8 mg/kg
An - G	Gabčíkovo	7.7 slightly alkaline	Eutric Fluvisol; % C_{ox} 6.3, without any contamination, vegetation is *Salici-Popuetum*
An - Sl	Slovinky	8.5 strongly alkaline	Technosol without vegetation; % C_{ox} 0.3 – 0.8; As 305 – 511 mg/kg; Cd 8.6 – 13.4 mg/kg; Cu 7 372 – 9 227 mg/kg; Pb 2 964 – 8 078 mg/kg; Zn 24 786 – 47 291 mg/kg

concentration during analysis was changed from initial ratio 10:90 up to 90:10. Analysis of the samples was carried out at 25 °C. The column (GraceSmart, RP 18, 150 mm length, OD 4.6 mm) for selective separation of analytes was used. The flow rate was 1 mL.min^{-1} and the wave length of PDA detector ranged from 222, 210, 200 to 235 nm (Mackuľak *et al*. 2011).

Pelletization of A. niger *wild type strains*

The fungal pellets were prepared in a 60 mL SAB medium with pH adjusted to 3, 5, 7 and 9 (respectively) enriched with a 10 ml suspension of conidia from each strain and 1 g of montmorillonite. Controls at each adjusted pH value were without montmorillonite. Cultivation was carried out using a shaker Unimax 2010 (Heidolph, Germany) at 135 rpm. Then, the pellets were carefully washed with large amount of distilled water and stored in Petri dishes where number and size of the pellets were measured. Microstructure of the pellets was recorded using a digital stereomicroscope Leica DMD 108.

Results and Discussion

Microscopic fungi

In spite of the fact, that *Aspergillus niger* belongs to cosmopolitan strains of soil filamentous fungi (Domsch *et al*. 2007; Klich 2002), it has not been isolated from all soil types in Slovakia (Šimonovičová 2013). On the other hand this strain was very frequent-to dominant in contaminated soils and substrates (Šimonovičová *et al*. 2016). This strain belongs to metal tolerant fungi (Anahid *et al*. 2011; Iram *et al*., 2009, 2013). Four wild type strains of *A. niger* were used in this study that originate from soils and stream sediments from various localities in Slovakia (Fig. 1, Table 1).

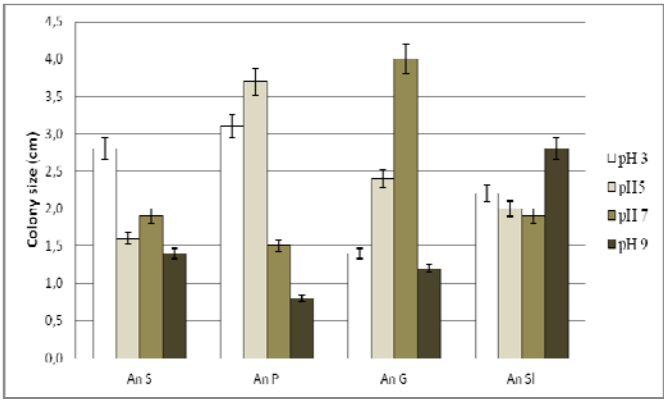

Fig. 2. Colony size of four *A. niger* wild type strains depending on pH of the growth substrate.

The first strain An-S was isolated from Dystric Cambisol (contaminated and eroded) on the locality Šobov, near Banská Štiavnica. This ultra-acidic soil lacks vegetation and also is very poor on organic material. From stream sediment on the locality Pezinok-Kolársky vrch (Pezinok) was isolated the second strain An-P. The substrate on this site is strongly acidic and rich on organic material, but contaminated with very high amount of As, Sb and Fe (Table 1).

From Eutric Fluvisol, a slightly alkaline substrate rich on organic material from the locality Gabčíkovo, was isolated the third strain – An-G. This strain was taken as a control strain, because the locality lacks any contamination. The strongly alkaline Technosol without vegetation on the locality Slovinky was the source of the fourth strain An-Sl. The corresponding content of organic

material is minimal, and the soil is contaminated with several toxic elements including As, Cd, Cu, Pb and Zn (Table 1).

Table 2. Production of organic acids (mg/L) by the studied *A. niger* wild type strains.

Sensitivity mg/L	**0.095**	**0.1**	**0.1**
Strain	**formic acid**	**acetic acid**	**oxalic acid**
An-S	0.108	0.143	0.173
An-P	0.144	0.1	0.175
An-G	0.238	0.1	0.228
An-Sl	0.164	0.11	0.138

Growth and colony size of A. niger *wild type strains*

Average sizes of colonies growing on the cultivation media with different pH were as follows: 2.37 cm at pH 3, 2.42 cm at pH 5 and 2.32 cm at pH 7 (Fig. 2). The largest colonies were always recorded under conditions most similar to their original environment, i.e. An-S formed colonies of 2.8 cm at pH 3, An-P of 3.7 cm at pH 5, An-G of 4 cm at pH 7 and An-Sl of 2.8 cm at pH 9. An exception was the strain An-S1 isolated from strongly alkaline substrate that formed colonies of quite similar size at different pH (2.2 cm at pH 3, 2 cm at pH 5 and 1.9 cm at pH 7) (Fig. 2). Even though microscopic filamentous fungi occur in soils and other terrestrial substrates of various pH, it is obvious that they prefer acidic or neutral environmental conditions (Chen *et al.* 2013; this study).

Enzymatic activity of A. niger *wild type strains*

Relatively slow growth of all *A. niger* strains were recorded on the substrate assigned for esterase activity assessment and very low growth, almost negligible, was recorded on the substrates for proteases and cellulases. The growth of all *A. niger* strains was very good on the substrate for lipases. The so-called "halo" effect was recorded for all strains. The obtained results confirm a higher lipase activity for the An-G strain compared with the other strains studied. Inhibition of metabolism as well as of enzymatic activity can be an expression of the quality of the original environment from which the fungal strains were isolated. The lowest metabolism and enzymatic inhibition was recorded for the strain An-G isolated from unpolluted environment. However, the real influence of the metal(loid) contaminants and other soil properties such as (low) carbon content, humidity etc. on metabolism of the other studied strains (An-P, An-S, An-Sl) must be further studied.

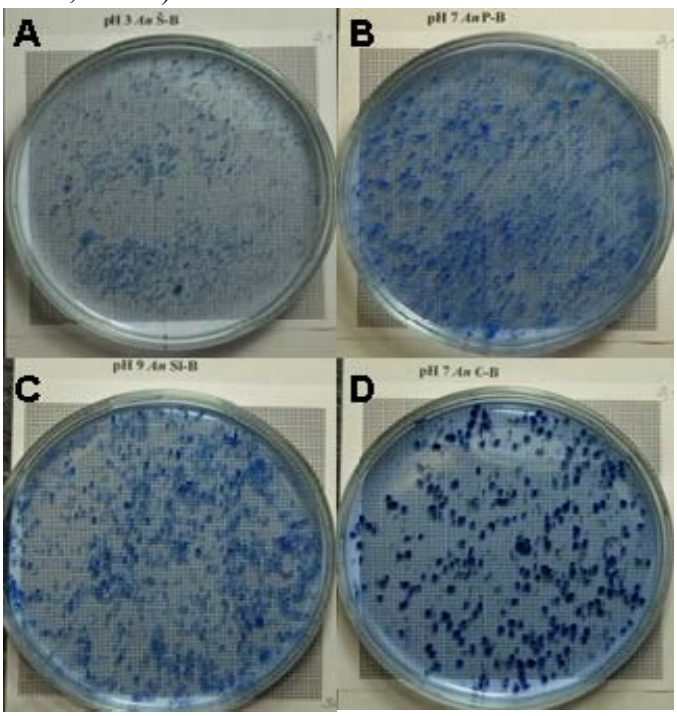

Fig. 3. Significantly higher number of pellets with the lower diameter (compared to pellets without clay minerals) as a consequence of the montmorillonite presence using *A. niger* strains isolated from Šobov at pH 3 (A), Pezinok at pH 7 (B), Gabčíkovo at pH 7 (C) and from Slovinky at pH 9 (D).

Production of organic acids of A. niger *wild type strains*

According to production of organic acids by *A. niger* strains (Table 2) the results of HPLC analysis suggests the ability of all the strains to decompose organic pollutants in contaminated soil samples. Such decomposition products can include lower acids, notably oxalic-, formic- and acetic acids.

The abundance of individual acids (their ratio) in the samples varied, likely due to enzymatic activity of the particular *A. niger* strain. The highest concentrations of organic acids, especially of oxalic acid, were recorded in the pre-treated sample An-G of the control strain from Gabčíkovo as a probable consequence of the highest lipase activity of the microorganism.

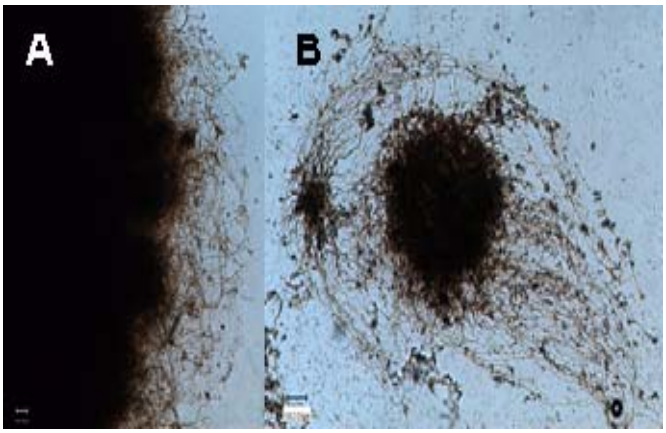

Fig. 4. Filaments of pellets in the control sample An-G (A) and changing the shape of the pellets in the presence of montmorillonite (B).

Partial intermediates of decomposition of organic acids during bioleaching gradually separated organic acids due to disruption of the equilibrium in term of lysis of the cells of *A. niger* strains. Due to disruption of the equilibrium, the complete degradation of organic acids e.g. to acetone, methanol, aldehyde *etc*. was reached (Amiri *et al.* 2012). The *A. niger* strains effectively degrade pollutants to simple acids with no significant negative ecological impacts to the environment. Subsequently, such produced substances can be utilized by other microorganisms as a source of carbon or energy. These processes can stimulate decomposition procedures of resistant types of pollutants occurred in landfills confirmed by seepages (Xu *et al.* 2014). Metabolites of microscopic filamentous fungi such as organic acids and amino acids affect pH of the medium. Acidification of the medium supports the mobility of metals, especially the transport mechanism of metal from the environment into the fungal cell and backwards (Gadd 2004).

Pelletization of A. niger *wild type strains*

One of the factors influencing the growth of pellets is the presence of inorganic powders. These are, for example, clay minerals, which are a regular part of soils and sediments. In our case, we studied the influence of clay mineral montmorillonite on the growth of pellets. We find out that in the presence of montmorillonite there was significantly higher number of pellets; however, the diameter of the pellets was lower (Fig. 3A-D) throughout the growing period. This claiming is valid for each strain tested.

This phenomenon can be explained by several reasons. Most probably higher amount of nucleation sites which are constituted by microscopic particles of clay minerals is responsible for increasing of the number of pellets coupled with spherical inhibition of fungal growth expressed in the diameter changes. Another possible reason is a relative increase in the length of fungal filaments in pellets (Fig. 4A-B).

According to control experiments, the largest pellets were produced by the An-S strain at pH 3 (2.67 mm) and pH 9 (3.04 mm), followed by the An-P at pH 5 (5.28 mm) and pH 7 (4.7 mm), the An-G at pH 3 (4.39 mm) and pH 5 (3.65 mm) and An-Sl at pH 9 (5.35 mm), (Fig. 5A-D).

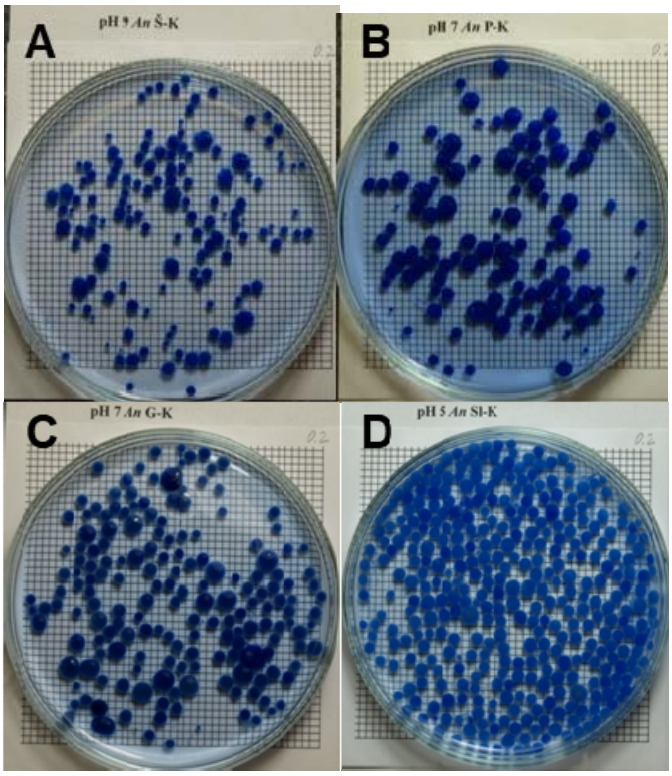

Fig. 5. Pellets of different sizes in the control samples. *A. niger* strains isolated from the localities Šobov at pH 9 (A), Pezinok at pH 7 (B), Gabčíkovo at pH 7 (C) and from Slovinky at pH 5 (D) with a diameter 5.35 mm.

According to Fomina and Gadd (2002), the changes in size of the pellets, shape and length of their mycelia of *Cladosporium cladosporioides* and *Humicola grisea* was also confirmed. All these changes can be explained by a significant increase in the number of inorganic particles in the culture medium.

Conclusions

The obtained results confirm the effects of environmental factors such as pH values of the substrate, % C_{ox} and contents of potentially toxic elements on physiological properties of *Aspergillus niger* strains. Influenced were especially the growth of colonies and the metabolism. Lipase activity in the strain An-G was higher compared with the other studied strains. Pollutants such as As, Sb, Al, Cd, Cu, Pb and Zn in all substrates except for those from the Gabčíkovo site inhibited the fungal metabolism as well as enzymatic activity. This effect was probably even enhanced by extremely low content of organic carbon in the samples from the Šobov and Slovinky sites. On the other hand, higher content of organic carbon in the substrate does not increase enzymatic activity in case of the samples An-P and An-G. The production of pellets was significantly reduced in the presence of montmorillonite. In this case, all strains produced a lot of pellets with small sizes. The mechanisms behind smaller pellet formation are unknown but might represent a fungal adaptation to presence of toxic compounds. Also, it can be assumed application of fungal pellets in remediation of landfills, where the contents of metal(loid)s are higher, especially during bioleaching of mining wastes with low contents of organic carbon.

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