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ORIGINAL ARTICLE

Differences in metabolites production using the Biolog FF Microplate™ system with an emphasis on some organic acids of *Aspergillus niger* wild type strains

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Abstract

This paper investigates the differences in some metabolites using Biolog FF Microplate™ system and the production of organic acids such as β-hydroxybutyric, p-hydroxyphenylacetic, and others. Another group of organic acids such as gluconic, oxalic and citric acid were studied during cultivation in a liquid medium. Four different *Aspergillus niger* (An) wild type strains were used as a model organism. Three strains, from the Banská Štiavnica – Šobov (An – Š), Pezinok (An – P) and Slovinky (An – Sl) localities were isolated from contaminated old mining areas with soil with ultra acidic to strong alkaline reactions. The fourth strain isolated from the Gabčíkovovo (An – G) locality was used for comparative purposes. According to the RAMP analysis, the strains are clustered into two groups, An – Š and An – P (similarity 82%), An – G and An – Sl (similarity 64%) which correlates with the pH values of the original environment. However, significant differences were found in metabolic processes in the reaction with a wide range of organic acids. In general, the reactions with D-lactic acid and D-malic acid correlate with the results of the RAMP analysis of fungal genotype similarities, the An – Š and An – P strains had an identical negative reaction, and an identical positive reaction was found in the An – Sl and An – G strains. During incubation the wild-type strains produced substantial amounts of gluconic acid, oxalic acid and small amounts of citric acid. The appearance and accumulation of organic acids was found to be highly pH dependent with the most active strain isolated from an ultra-acidic environment. The comparative strain differs entirely in the production of oxalic acid.

Keywords *Aspergillus niger* · Biolog FF Microplate™ system · Contamination · Metabolites · Organic acids · RAMP analysis

Introduction

Fungi are valuable producers of many important metabolites including organic acids. Aspergilli are successfully employed in the biotechnology sector for the production of organic substances which are used in a wide variety of applications (Rodrigues 2016; Upton et al. 2017). The *Aspergillus niger* strain of aspergilli is highly versatile metabolically, as it has an innate ability to secrete various organic acids including malic, oxalic, gluconic, itaconic, kojic and citric acids. According to Liaud et al. (2014); Li et al. (2011, 2016) the screening of 66 strains of filamentous fungi clearly showed the extreme potential of *Aspergillus niger* in organic acid production. Organic acids have a wide range of applications in the food and pharmaceutical industries; for example citric acid, is extremely useful thanks to its versatility and non-toxicity (Show et al. 2015).

Nowadays, it would be useful to isolate environmental fungal producers of valuable metabolites (Goldberg et al. 2006). Many filamentous fungi are able to live in environmental burdens conditions in extreme stress with the presence of toxic elements (Šimonovičová et al. 2013; Qayyum et al. 2016; Mohammadian et al. 2017). These fungal strains may have often enormous biotechnological potential and can be

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considered as a source of useful organic acids to be exploited in the mycoremediation of contaminated sites through the bioleaching of Cd, Cu, Zn, Pb, Mn, Al from red mud (Ren et al. 2009; Zeng et al. 2015; Urík et al. 2015, 2017; Mohanty et al. 2017), and the bioaccumulation of Cr, Ni (Shivakumar et al. 2014; Remenárová et al. 2020) or As (Singh et al. 2015). Organic acids play a key role in the bioavailability of selenium (Dinh et al. 2017) and can also utilize Fe(III) citrate as an iron source (Odoni et al. 2017).

On the other hand, organic acids production can be used not only in biotechnology. As shown Lee et al. (2013) the secondary metabolite neosartorin was identified as an *Aspergillus lentulus* specific compound. And that metabolite-based chemotaxonomic classification is an effective tool for the classification of *Aspergillus* spp. with species-specific activity.

The aim of this study was to determine potential differences in the production of metabolites and the ability to metabolize various substrates such as carbon sources by four *Aspergillus niger* wild type strains isolated from soils of different localities and under various stress conditions: from ultra-acidic to strong alkaline, with a minimal amount of organic matter and with excessive values of toxic elements as As, Cd, Cu, Mn, Sb and Zn.

Differences in the production of metabolites in the utilization of selected organic acids were determined using the modern Biolog FF Microplate™ system.

Materials and methods

Fungal strains

Four *Aspergillus niger* (An) wild type strains were used as experimental material. Three of the strains were isolated from an environment contaminated by former mining activities. All of the localities differ in the basic chemical analysis of the content of potentially toxic elements, organic matter and soil reaction. The first strain of *A. niger* labeled as An – Š was isolated from Dystric Cambisol (contaminated and eroded) from the locality Šobov near the town of Banská Štiavnica. The second strain of *A. niger* labeled as An – P was isolated from a stream sediment of the Blatina River near the town Pezinok. The third strain of *A. niger* labeled as An – Sl was isolated from Alkaline Technosol at the Slovinky site. All of these localities are known as mining areas with hundred year-old activities. The fourth strain of *A. niger* labeled as An – G was isolated from Haplic Fluvisol in a floodplain forest near the uncontaminated control locality of Gabčíkovo (Šimonovičová et al. 2013, 2017a).

Cultivation of fungal strains

The cultivation of all *A. niger* strains were carried out for 10 days at 25 °C under laboratory conditions in SDB (Sabouraud Dextrose Broth, Himedia, Mumbai, India). 5 ml of inoculum with *A. niger* conidia were added to 95 ml of SDB. 100 ml of SDB were used as a control. During the cultivation carried out also regular sampling of the liquid medium for pH measurement and organic acid analyses in the 2nd, 4th, 6th, 8th and 10th days of cultivation. The SDB medium was also measured as a control.

DNA extraction and RAMP analysis

Fungal DNA was isolated using the DNeasy Plant mini purification kit (Qiagen, Hilden, Germany). Briefly, the fungal isolates were inoculated in SDB (Sabouraud Dextrose Broth, Himedia, Mumbai, India) and incubated at 28 °C until growth. Later the fungal pellets were separated from the broth by filtration with sterile filter paper and the DNA was extracted according to the manufacturer's instructions. The resulting fungal DNA was stored at -20 °C and subsequently used as a template in the appropriate RAMP (Random Amplified Microsatellite Polymorphism) PCR approach.

RAMP-PCR was applied in order to characterize the isolates by applying a protocol similar to a previous study (Pangallo et al. 2012). 50–100 ng DNA were added to the reaction mixture (final volume 25 µl) containing 2 U SuperHotTaq DNA Polymerase (Bioron), 2.4 $mmol^{-1}$ $MgCl_2$, 200 μmol^{-1} dNTP, 60 pmol of the microsatellite primer T14 (AAT GCC GCA G) and 60 pmol of the random primer K7 (CAA CTC TCT CTC TCT). The PCR program consisted of initial denaturation at 95 °C for 5 min, 30 cycles (95 °C for 45 s; 30 °C for 60 s with ramping 0.1 °C s^{-1} to 50 °C; 50 °C for 60 s with ramping 0.1 °C s^{-1} to 68 °C; 68 °C for 90 s) and a final polymerization at 68 °C for 10 min. Five microliters of RAMP-PCR products were separated on 1.8% agarose gel for 4.5 h at 2.3 V cm-1 in TAE buffer. Gels were stained with ethidium bromide, visualized under UV light, photographed and digitalized. RAMP profiles were analyzed by BioNumerics ver. 6 software (Applied Maths, Kootrijk, Belgium) using Pearson's correlation coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) algorithms.

All experiments were performed in triplicates. Under static conditions, *A. niger* formed mycelia which were twice filtered using blue filter paper KA 4 (BRAND, Czech Republic) and 20 ml of SDB solution were removed to determine the fungal organic acids.

Biochemical characteristic of *A. niger* strains

The phenotypic properties of the tested *A. niger* wild type strains were evaluated using Biolog FF Microplate™ system (Biolog Inc., USA). The system serves for the identification and phenotypic characterization of microorganisms based on highly accurate and patented biochemical tests (Bochner

2009). Using FF-microplates, a unique biochemical profile can be obtained for individual strains; the phenotypic fingerprinting of tested fungi accurately reflects their catabolic potential and can also be used to identify microscopic fungi using more detailed phenotypic profiling.

In 96-well Biolog FF-microplates, the studied fungal isolates react with the pre-treated substances for 48–96 h of incubation at 26 °C. The isolated strains for identification were first cultivated on 2% Malt Extract Agar medium (HiMedia Laboratories, India) at 26 °C for 5–7 days to induce conidia formation. The conidia were then inoculated into a solution (IF) supplied by the manufacturer and diluted to a recommended cell density of 65%. This suspension was then inoculated according to the Biolog protocol to FF-MicroPlate plates in the amount of 100 µl per well and the MicroPlate was incubated at 26 °C to allow the phenotypic fingerprint to form. During incubation, the fungal cells react with the test substance in the microplate wells which the fungi can use as a carbon source for growth. Positive metabolic reactions cause the reduction of the tetrazolium dye, resulting in varying purple colour intensity; the negative wells remain colourless, as does the negative control well (A1) with no carbon source. After incubation, the results of each test are evaluated and compared with the Biolog system database. If a match is found, the instrument reports successful species identification including probability and similarity levels with the pre-set database.

For metabolic profiling we selected reaction tests between the studied strains of fungi and the following organic acids, which make part of the Biolog FF MicroPlate test system: D-galacturonic acid, D-gluconic acid, D-glucuronic acid, 2-keto-D-gluconic acid, γ-amino-butyric acid, bromosuccinic acid, fumaric acid, β-hydroxy-butyric acid, γ-hydroxy-butyric acid, p-hydroxyphenylacetic acid, α-keto-glutaric acid, methyl ester D-lactic acid, L-lactic acid, D-malic acid, L-malic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, succinic acid and mono-methyl ester succinic acid.

Determination of organic acids produced by *A. niger*

Organic acids and common inorganic anions in the culture broth were determined by ion chromatograph Dionex ICS-5000 (Sunnyvale, ca., USA). The separation of analytes was carried out in the IonPac AS11–HC column using an electrolytically generated 1–30 mM KOH gradient elution. Individual anionic species were determined by suppressed conductivity detection. The chromatographic method enables the good resolution of individual anionic species (Fig. 1). Even the early eluting peaks, gluconate, lactate and acetate are well resolved on the AS11-HC column.

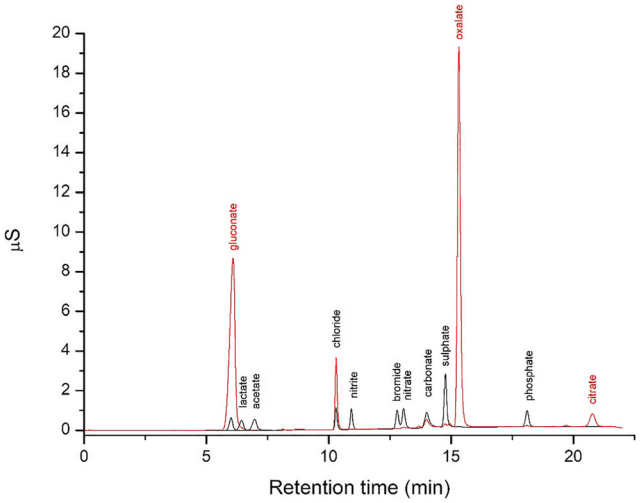

Fig. 1 Overlaid chromatograms of a standard anion solution (black) and the SDB liquid medium metabolized by *Aspergillus niger* (red). Significant peaks of gluconate, oxalate and citrate as well as the peaks of common inorganic anions chloride, sulphate and phosphate appeared in the chromatograms of culture broth

Results and discussion

The *A. niger* Tiegh. species is a cosmopolitan heterotrophic microscopic fungus occurring in several environments (Ilondu and Nweke 2016; Gniadek et al. 2017) with a preference for soils including contaminated soils (Carranza et al. 2017; Šimonovičová et al. 2013, 2016, 2017a, 2019).

The chemical characteristics of the substrates of isolated *A. niger* wild type strains are very different (Table 1). The soil reaction of Dystric Cambisol (contaminated and eroded) in the Banská Štiavnica – Šobov locality is ultra-acidic (< 3.5), with an extremely high amount of aluminium but lacking in organic material. The stream sediment of the Blatina River near Pezinok is contaminated with As, Sb, Fe and Al. The soil reaction of the substrate is strong acidic (5.25) and lacking in organic material. The tailing pond at the Slovinky locality, which is as high as 113 m, is lacking in organic material and contaminated with arsenic, cadmium, copper, manganese, lead and zinc. There is a strong alkali soil reaction of the substrate (8.6). The toxic elements achieved exceeded values (*) at all three of these localities. Halpic Fluvisol at the control Gabčíkovo locality has a slightly alkali (7.7) soil reaction and a sufficient amount of organic matter (Šimonovičová et al. 2017a, b).

The RAMP analysis of the analysed strains confirmed a certain similarity of all profiles (up to 50%). Strains clustered into two groups, the An – P and An – Š isolates had an 82% similarity and were more related than An – G and An – Sl isolates which had a 64% similarity (Fig. 2).

Different responses were identically confirmed in all the tested *A. niger* isolates while utilizing acids D-galacturonic acid, γ-amino-butyric acid, bromosuccinic acid, fumaric acid,

Table 1 Chemical characteristic of substrates as a source of *Aspergillus niger* wild type strains at the Šobov, Pezinok, Slovinky and Gabčíkovo localities

Strain	Locality/substrate	Chemical characteristic of substrate			References
		pH H_2O	$\%C_{ox}$	elements	
An – Š	Šobov Dystric Cambisol (contaminated and eroded)	3.12	0.49	*Al 506–727 mg/kg	Šimonovičová et al. 2013, 2017b, 2019
An – P	Pezinok stream sediment	5.25	0.72	*As 363 mg/kg; Sb 93 mg/kg, Fe 82.8 mg/kg	Šimonovičová et al. 2013, 2017b, 2019
An – Sl	Slovinky Alkaline Technosol	8.6	0.8	*As 511 mg/kg; *Cd 8.76 mg/kg; *Cu 8186 mg/kg; *Mn 2647 mg/kg; *Pb 2964 mg/kg; *Zn 25107 mg/kg	Šimonovičová et al. 2017a, 2019
An – G	Gabčíkovo Haplic Fluvisol in floodplain forest	7.7	2.7		Šimonovičová et al. 2013, 2017b

*exceeded values of toxic elements in substrates; in Slovakia there are no limits for Sb and Fe

β-hydroxy-butyric acid, γ-hydroxy-butyric acid, L-lactic acid, L-malic acid, sebacic acid, succinic acid.

However, in the reaction with a wide range of acids, significant differences were found in metabolic processes which allow the identification of typical enzymatic profiles of the evaluated *A. niger* strains (Table 2). In particular, the reactions with D-lactic acid and D-malic acid correlate with the results of the RAMP analysis of fungal genotype similarities; the An – Š and An – P strains had an identical negative reaction, while an identical positive reaction was found in the An – Sl and An – G strains. The reactions of the An – P strain, which reacted negatively in all the tests of this group, are also very interesting. Experimental data, obtained on the basis of both quantitative and kinetic analysis of the reactions, reveal significant biochemical properties correlating genotypes with phenotypes, thus completing a unique identification profile for individual strains.

In the Biolog test system, organic acids that metabolize the tested *A. niger* strains with a clearly positive result were also recorded. These tests can be considered to be characteristic of the biochemical profiling of the studied *A. niger* wild type strains. In addition, based on the observed values quantifying these metabolic reactions, their course and thus the number of enzymes produced can be deduced, resulting in varying purple intensity due to the kinetics of the reduction of the tetrazolium dye. These are tests with D-gluconic acid, D-glaucuronic acid, 2-keto-D-gluconic acid, quinic acid and D-saccharic acid (Table 3).

D-gluconic acid is an important organic acid that results from D-glucose oxidation. The ability to produce it has been described in a large group of microorganisms, particularly microscopic filamentous fungi (Cochrane 1958; Lockwood 1975). The biological metabolism of gluconic acid oxidation in microorganisms is associated with the production of gluconic acid reductases (Eschenfeldt et al. 2001; Kuorelahti et al. 2005). Other enzymes have been isolated from bacteria that can convert 2.5-diketo-d-gluconic acid to 2-keto-L-gluconic acid (Matthews and Halimi 2015), but the general gluconate pathways of fungal D-gluconic acid appear to differ significantly from the bacterial pathway (Ramachandran et al. 2006) and products may even be species-specific (Kuivanen and Richard 2017). D-gluconic acid and 2-keto-D-gluconic acid, key enzymes in the metabolism of both acids, have been included among the characteristic biochemical parameters of *A. niger* wild type strains, of which the An-G strain showed the highest production (up to twice as much intensity compared to the A – Š strain).

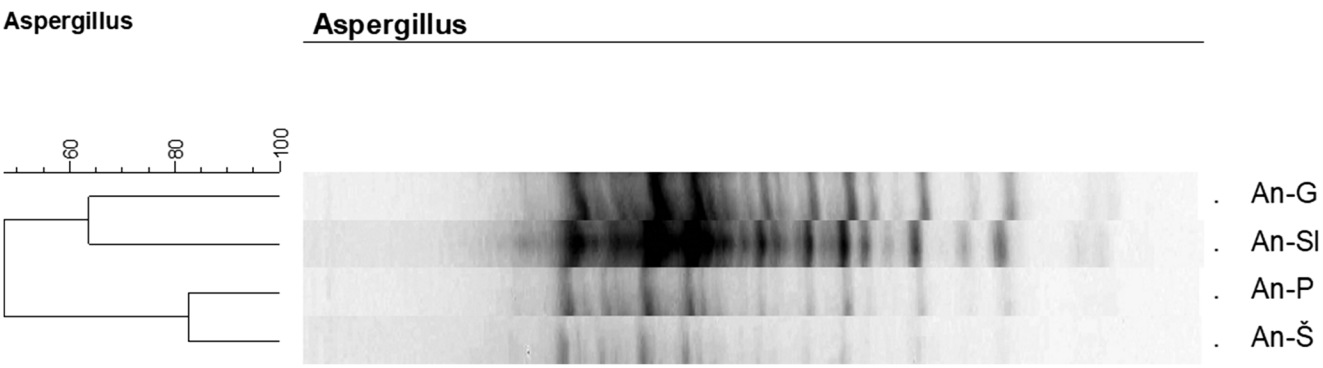

Fig. 2 RAMP analysis of four *Aspergillus niger* strains. They were grouped by using Pearson correlation and UPGMA clustering

Table 2 Biochemical fingerprints of *Aspergillus niger* wild type strains – differences in the utilization of selected organic acids

Organic acids	Fungal strains			
	An-Š	An-P	An-Sl	An-G
β-Hydroxy-butyric	+/–	–	+/–	–
p-Hydroxyphenylacetic	+	–	+	–
α-Keto-glutaric	+/–	–	+/–	–
D-Lactic	–	–	+	+
D-Malic	–	–	+	+
Succinamic	+/–	–	–	–
Succinic	+/–	–	–	–

+/– variable response, there is uncertainty about the interpretation of results

D-Glucuronic acid is a hexuronic acid derived from D-glucose. The basis of catabolic D-glucuronate pathways in *A. niger* appears to be an enzyme from the group of reductases (2-keto-glucuronate reductase), which differs significantly from other known D-glucuronate pathways confirmed in other microorganisms (Kuivanen et al. 2016). The intensity of D-glucuronic acid metabolism was observed in all studied *A. niger* strains, with the An-Sl strain showing the highest activity.

The metabolic pathways of β-hydroxyphenylacetic acid in *A. niger* were published as early as 1976 as part of the conversion of D, L-phenylalanine (Kishore et al. 1976). This pathway includes hydroxymandelic acid, which is a further oxidation and decarboxylation metabolized to ring-cleaved products of hydroxybenzoate acids. The highest metabolic activity in the degradation of p-hydroxyphenylacetic acid was observed in the An – Š strain, which was up to 1.5 times higher compared to the An – Sl strain; however, this metabolic activity was not observed at all in the remaining 2 strains.

Table 3 Biochemical fingerprints of *Aspergillus niger* wild type strains – differences in the production of metabolites in the utilization of organic acids as carbon source

Organic acids	Fungal strains			
	An-Š	An-P	An-Sl	An-G
D-Gluconic	+ <357>	+ <608>	+ <722>	+ <774>
D-Glucuronic	+ <192>	+ <493>	+ <847>	+ <571>
2-Keto-D-Gluconic	+ <411>	+ <343>	+ <771>	+ <840>
Quinic	+ <750>	+ <775>	+ <672>	+ <951>
D-Saccharic	+ <747>	+ <556>	+ <786>	+ <412>

Analysis of colour development in metabolic reaction, <x> value of the positive metabolic reaction expressed by the of purple color due to the reduction of the tetrazolium dye. Gray color – very close values indicate genotypic similarity in this region of the genom (correlation of genotype with phenotype)

Quinic acid and D-saccharic acid (D-glucaric acid) are metabolized by all of the studied *A. niger* strains with a positive reaction of varying intensity. While in quinic acid utilization the results of tested *A. niger* strains are almost equivalent, enzymatic differences were observed in D-saccharic acid metabolism.

D-glucaric acid is a dicarboxylic acid analogue of D-glucose, formerly known as D-saccharic acid. The data published in the study (Zakes 1969) has demonstrated that glucaric acid is catabolized in the fungi *A. niger* by means of a pathway involving a dehydrogenase enzyme and other enzymes catalysing the breakdown of keto-deoxy-glucarate. The major metabolic intermediate products of the carbohydrate acids metabolism are pyruvate and alpha-ketoglutaric acid; they can be further degraded by enzymes up to CO_2. There were noticeable differences in the metabolism of the D-glucurate fungal *A. niger* strains, where the metabolic activity decreases away from the strain An – Sl > An – Š > An – P > An – G.

The mutual similarity between the An – Š and An – P strains in one group and the similarity of the An – Sl and An – G strains in the other group were also confirmed in this group of tests. This conclusion is confirmed by the very close values of the course of the reactions, in particular in the case of D-gluconic acid, 2-keto-D-gluconic acid and quinic acid (Table 2).

The production of fungal organic acids was measured in the course of growth of *A. niger* wild type strains in the 2th, 4th, 6th, 8th and 10th days of cultivation. The liquid medium changed in pH values from weak acidic (pH 6.21 control medium) to extreme acidic (2nd day of cultivation) and ultra-acidic (from the 4th to 10th days of cultivation) as shown in Fig. 3a–d.

A. niger produces weak organic acids such as gluconic acid, oxalic acid and citric acid, and their accumulation depends on the pH of the culture medium (Yang et al. 2017). Figure 4a-d shows the cumulative production of organic acids and pH evolution during the incubation of *A. niger* strains in an SDB liquid medium with an initial glucose concentration of 20 g L^{-1}. The pH gradually decreased as a result of the accumulation of protons in the fermentation broth.

The reaction involving the conversion of glucose to gluconic acid by filamentous fungi is catalyzed by the enzyme glucose oxidase (Mischak et al. 1985; Witteveen et al. 1992). The enzyme is induced in the presence of high levels of glucose in the medium, pH of around 5.5 and elevated oxygen levels (Ramachandran et al. 2006). Glucose oxidase is formed early in fermentation and converts a significant amount of

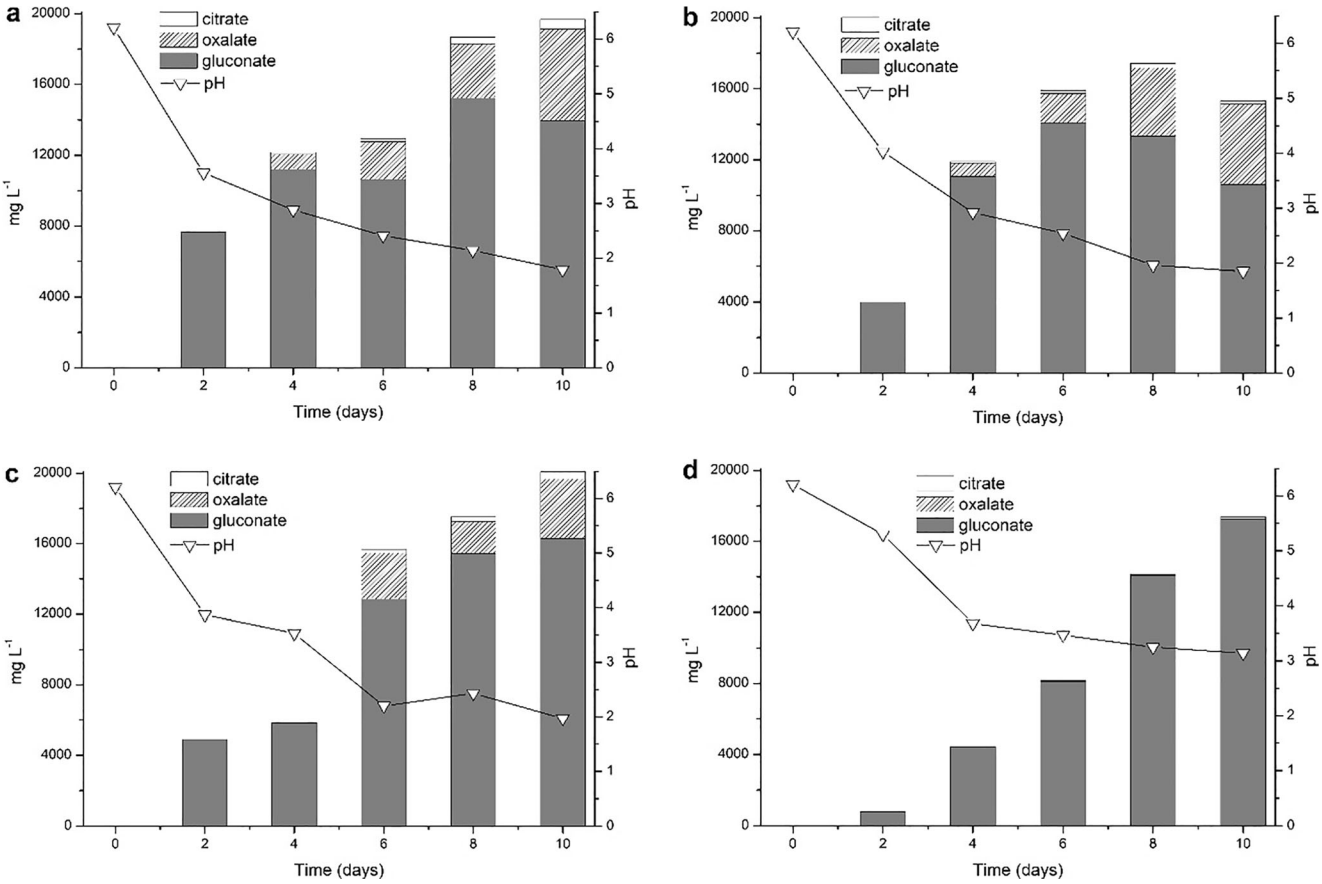

Fig. 3 The cumulative production of organic acids and pH changes during the incubation of *Aspergillus niger* isolated from Šobov (**a**), Pezinok (**b**), Slovinky (**c**), Gabčíkovo (**d**) localities

glucose into gluconic acid. The concentration of gluconic acid achieved 17.25 g/l (strain An – G, Fig. 3d), which corresponds to a carbon yield of 80%. However, due to the extracellular location of the enzyme, it is directly influenced by the external pH and is inactivated at pH lower than 3.5 (Wolschek and Kubicek 1999; Show et al. 2015). Following its production, gluconate was apparently taken up and catabolized further in the wild-type strain An – P (Fig. 3b). The ability to metabolize gluconic acid was confirmed in all four tested wild strains which resulted in positive metabolic reactions in Biolog FF MicroPlate test system (Table 3).

In contrast to gluconic acid formation, the biosynthesis of oxalate and citrate by *A. niger* requires the catabolism of glucose via several enzymatic steps (Kubicek et al. 1994). *A. niger* possesses a pyruvate carboxylase located in the cytosol which forms oxaloacetate from pyruvate and carbon dioxide (Cleland and Johnson 1954; Kubicek et al. 1979; Bercovitz et al. 1990). Glycolytic pyruvate can therefore be converted to oxaloacetate without being transported to the mitochondria (Kubicek et al. 1994). Oxalate biosynthesis occurs by the hydrolysis of oxaloacetate catalyzed by oxaloacetate acetylhydrolase which is located in the cytosol (Kubicek 1987; Kubicek et al. 1988). Oxalic acid is produced in large quantities by a variety of fungi, including saprotrophic and phytopathogenic species (Dutton and Evans 1996).

The main factors in the production of fungal organic acids in a natural environment are determined by physicochemical soil properties, including pH, and metal oxide content (Dinh et al. 2017). Under laboratory conditions, the effect of the pH of the medium appeared to be the main factor governing oxalic acid production by *A. niger* (Walaszczyk et al. 2018). Our observations indicate that the production of oxalate began when the medium pH dropped below 3.5 (Figs. 3 and 5a). At that pH level the fungi concurrently secreted multiple organic acids, particularly gluconic, oxalic and citric acid. The comparative strain (An – G) was deficient in producing oxalate (Fig. 4a). Oxalate is the most powerful proton-producing organic acid that efficiently acidifies the surrounding environment at a given pH. The production of gluconate is not such an effective method of acidifying the medium. This is apparent from the evolution of the pH of the nutrient broth at the An – G strain that which oxalate production (Fig. 3d). Despite the ongoing production of gluconate, with its highest concentration achieved at that strain, the medium pH did not drop below 3.

According to (Gadd et al. 2014) oxalate is a key metabolite that plays a significant role in many mineral transformations

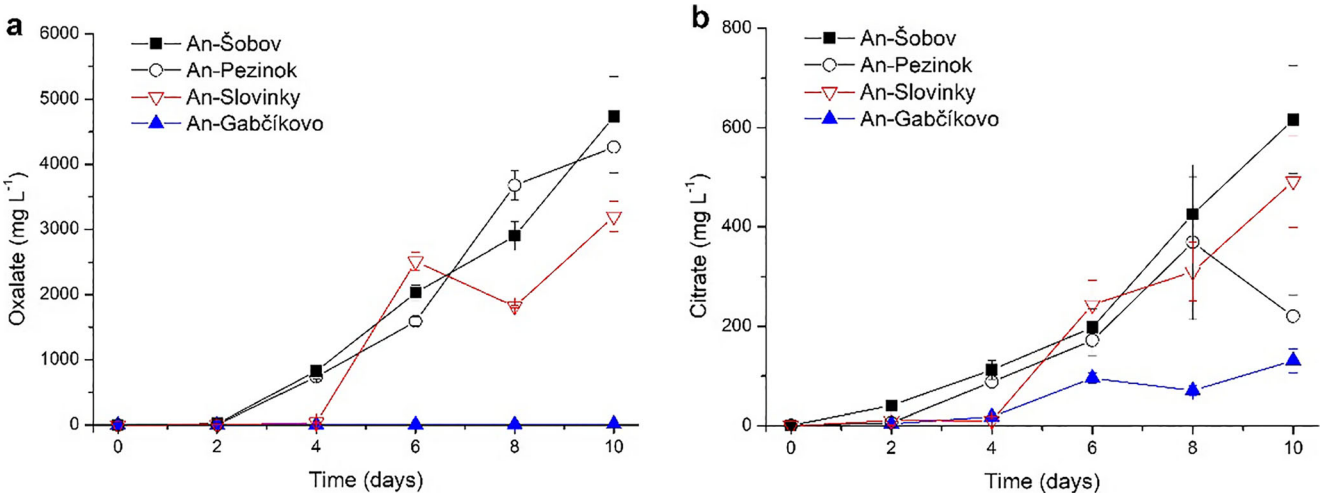

Fig. 4 Oxalic (**a**) and citric acid (**b**) production by individual strains of *Aspergillus niger*

mediated by fungi. Metal and mineral transformations are central to geomycological processes, including nutrient and element cycling, metal transformations, bioweathering and biomineral formations.

Ruijter et al. (1999) studied oxalic acid production using an *A. niger* mutant lacking glucose oxidase to prevent interference by gluconic acid production. They reported that oxalic acid production was optimal at a pH ranging from 4 to 6. The production of oxalic acid decreased below pH 4. A significant amount of oxalic acid was still being produced at pH 2, but was very low at pH 1.5. However, the lack of oxalate at lower pH levels can be explained by the finding that when the culture medium is below pH 2.5, oxalate decarboxylase, the enzyme that degrades oxalate to CO_2 and formate, is synthesized (Emiliani and Bekes 1964). Oxaloacetate acetylhydrolase (OAH), the enzyme responsible for oxalate formation in *A. niger*, cleaves oxaloacetate to oxalate and acetate. Interestingly, acetate was never detected in the culture broth during oxalate production. If OAH is the only enzyme involved in oxalate production, the acetate formed must be very efficiently utilized (Ruijter et al. 1999). In our samples, acetate was never detected in the culture broth during the 10-day incubation period of these strains.

During the incubation of *A. niger*, the non-buffered medium quickly acidified, which facilitated the synthesis of citric acid. The most important environmental factors that trigger the accumulation of citric acid include: excessive concentrations of carbon source, high levels of dissolved oxygen, low pH, and suboptimal concentrations of ammonia and phosphate and certain trace metals (Kubicek et al. 1985; Gadd 1999; Papagianni 2007). In our experiments, the production of citric acid commences at pH values below 3.5 (Figs. 3a-d and 5b). The biochemical mechanism by which *A. niger* accumulates citric acid has attracted much interest. Citrate formation requires at least one mitochondrial step, i.e. citrate synthase which is located exclusively in the mitochondria (Kubicek et al. 1994; Karaffa and Kubicek 2003; Papagianni 2007). Citrate synthase catalyzes the reversible condensation

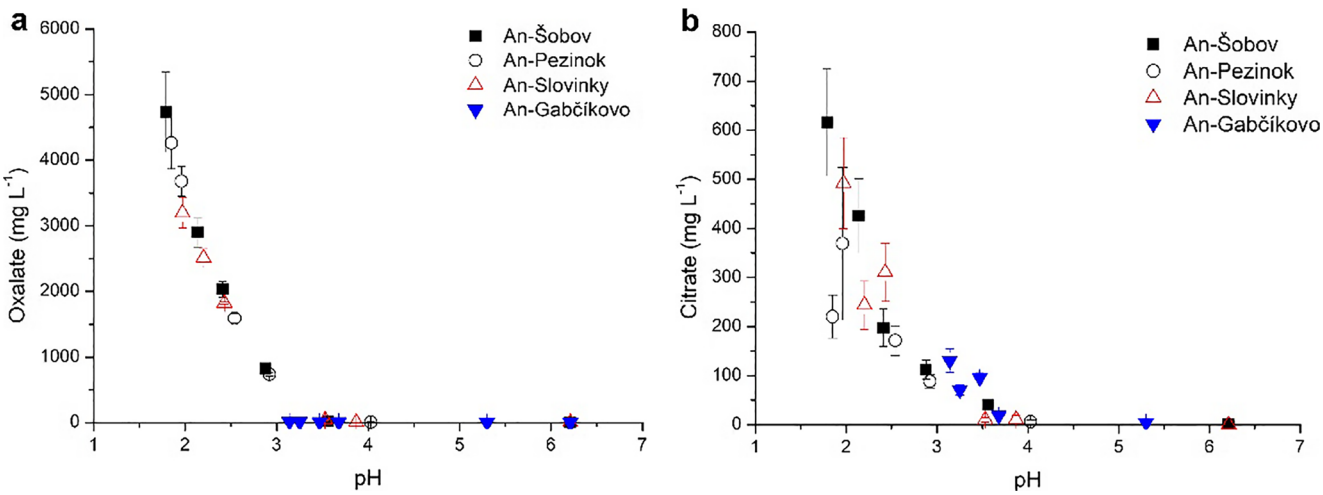

Fig. 5 Oxalate (**a**) and citrate production (**b**) as a function of culture pH during the incubation of various strains of *Aspergillus niger* in the SDB medium

reaction between oxaloacetate and acetyl CoA (Krebs 1970). The pH of non-regulated *A. niger* cultures can fall to a value 1.4 and 2.0 owing to high citric acid production (Kristiansen and Sinclair 1978; Schrickx et al. 1995). An *A. niger* strain lacking both glucose oxidase and oxaloacetate acetylhydrolase activity produced citric acid from sugar substrates in a regular synthetic medium at near neutral pH and, under these conditions, production was completely insensitive to Mn^{2+} ions (Ruijter et al. 1999). These findings show that a major reason for the low pH requirement in traditional commercial citric acid fermentation is the inhibition of gluconic acid and oxalic acid by-production.

Conclusions

Our results demonstrate the sequential production of organic acids from glucose by four *A. niger* wild type strains isolated from different environments. It seemed that depending by their origin, the analyzed strains produced substantial amounts of gluconic acid, and moderate amounts of oxalic and citric acids. The production of organic acids seems to fit the general strategy of *A. niger* and other fungi to generate acidity.

All four *A. niger* strains rapidly converted glucose into gluconic acid. Gluconic acid produced early in the fermentation was seen to be consumed in the later stage of incubation, at least at the An – P strain. Positive metabolic activity in gluconate utilization was confirmed in all four strains by the Biolog FF microplate system test. The oxalate deficient An – G strain produced the largest quantity of gluconate which corresponds to a carbon yield of 80% of the initial amount of glucose in the medium. Gluconate is not such a powerful proton-producing acid compared to oxalate. The pH value of the nutrient broth did not drop below 3 due to a lack of oxalate production by the An – G strain. The production of organic acids by *A. niger* has been shown to be dependent on environmental pH. According to Andersen et al. (2009), the production of organic acids by *A. niger*, and specifically oxalate and citrate, leads to the most efficient acidification of the medium based on the external pH, while the production of gluconate is not an effective method of acidifying the medium. It therefore seems likely that the efficient conversion of glucose to gluconate by *A. niger* has evolved not as a way of acidifying the medium, but rather as a mechanism to rapidly make glucose unavailable to competing organisms.

The results revealed the enormous biotechnological potential of the studied wild type strains of *A. niger* as powerful producers of organic acids which can be exploited in mycoremediation and mineral processing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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