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EXPERIMENTAL ARTICLES

Formation of Organic Acids by Fungi Isolated from the Surface of Stone Monuments

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Abstract— Capacity of the fungi isolated from the surface of stone monuments for acid formation was studied in cultures under various carbon sources and cultivation conditions. The composition of organic nutrients was adjusted according to the results of investigation of the surface layers from the monuments in urban environment. The primary soil formed at the surface of the stone monuments under urban conditions was shown to contain a variety of carbon and nitrogen sources and is a rich substrate for fungal growth. Oxalic acid was produced by fungi grown on media with various concentrations of sugars, sugar alcohols, and organic acids. Malic, citric, fumaric, and succinic acids were identified only at elevated carbohydrate concentrations, mostly in liquid cultures. Oxalic acid was the dominant among the acids produced by *Aspergillus niger* at all experimental setups. Unlike *A. niger*, the relative content of oxalic acid produced by *Penicillium citrinum* decreased at high carbohydrate concentrations.

Keywords: micromycetes, organic acids, carbon sources, cultivation conditions, stony substrate

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Natural rocks are usually inhabited by microorganisms belonging to different taxonomic and ecological groups; micromycetes are often predominant in lithobiontic communities [1–3]. The growth of fungi on the surface of rocky substrates occurred at the expense of compounds originating from the environment or produced by the other members of lithobiontic community. Micromycetes are the most active degraders of the stony monuments and constructions that work by changing physicochemical properties of rocky surfaces, resulting in damage and destruction [2].

The surface of stony constructions in urban environments contains all the elements necessary for microbial growth: C, N, P, S, Na, K, Ca, Mg, Mn, and Cl [4, 5]. For instance, surface layers of granite contained polysaccharides, proteins, nucleic acids, melanin, products of the polysaccharide degradation (furfuran, methyl furfuran, furan, methyl furan, and levoglucan), fatty acids (C_7–C_{20}) with predominance of palmitic and linoleic acids, carotenoids, steroids, as well as pyrrol-, pyridine-, and indole derivatives [4, 6]. Oxalates, amino acids, and other organic compounds which can be used as substrates for the growth of stone-degrading fungi were found on the surface of marble monuments [7]. It is known that fungi can utilize mono-, di-, and polysaccharides, sugar alcohols, and organic acids as carbon sources [8, 9]. However, until now it remains unclear which compounds occurring on the surface of stony monument are used by micromycetes for their growth and development.

The excretion of organic acids is the most effective factor of fungal impact on the rocky surfaces [10]. The acidifying activity of fungi is often considered an adaptive reaction to environmental changes. Thus, it was shown that the excretion of oxalic acid is important for detoxification of heavy metals and some other xenobiotics [11, 12]. The acidification of medium promotes extraction of phosphorus, potassium, aluminum, and iron from minerals and transformation of some of them into available forms [13, 14]. In spite of increased interest in this problem, many ecological aspects of the acid-forming activity of fungi remain unclear.

The aim of the present work was to study the effect of the low-molecular substances occurring on the surface of rocky monuments on growth of the stone-degrading micromycetes and production of organic acids.

MATERIALS AND METHODS

Objects of the study. To determine composition of organic substances from the monuments with the signs of biodegradation, the samples were withdrawn from the surfaces of marble- and granite monuments in the Alexander Nevsky Lavra (central region of St. Petersburg) in 2011–2012 over the spring–autumn period. The monuments were made from Italian marble, limestone (Pudostkii and Putilovskii), and granite (grey and rose). The samples were taken from a dirty surface (initial soil formed by abscised leaves and moss), as well as from the gypsum-enriched patina.

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The study was carried out with fungi *Penicillium citrinum* (Thom) L 4/09 (isolated from the surface of damaged monument in the Alexander Nevsky Lavra) and *Aspergillus niger* (Tiegh) Ch 4/07 (isolated from a damaged monument in the Historical and Archaeological Reserve, Chersonese, Crimea) which were maintained in the Collection of Microorganisms, Laboratory of Mycology, Faculty of Biology and Soil Science, St. Petersburg University.

Cultivation of fungi. Fungi were grown in media containing (g/L): $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_20$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.002. Concentrations of carbon sources varied in different variants: glucose (10, 20, 30, 50, and 70 g/L); fructose (10, 30, 50, and 70 g/L); saccharose (10, 30, 50, and 70 g/L); mannitol (30 g/L); sorbitol (30 g/L); calcium gluconate (10 and 30 g/L); potassium oxalate (10 and 30 g/L). The surface cultivation was carried out in conventional liquid media usually applied in biochemical studies of fungi and on agar media supplemented with calcium carbonate (0.2%) at 25°C for 15 days. Biomass was determined gravimetrically and expressed in mg/50 mL of liquid medium or in 5 mL of agar medium.

Sample preparation for chromatography. The samples withdrawn from the monument surfaces were dried, weighed, and extracted thrice with methanol in a ratio of 0.02 g/mL for 24 h. The extracts were evaporated under vacuum at 40°C; the residue was dissolved in pyridine to obtain trimethylsilyl (TMS) derivatives [15]. For isolation of organic acids, the culture broth (CB) was acidified with 0.1 N HCl to pH 1.0 to obtain free oxalic acid. In the case when fungi were grown on solid medium, agar was preliminarily dissolved in water (1 mL of agar medium per 10 mL of water) under heating and filtrated. Then an aliquot of filtrate was passed through a KU-2-8 cation-exchange resin to obtain free organic acids.

For removing excess sugars, the samples were passed through an AN-2FN anion-exchange resin; acids were displaced with 0.2 N NaOH and passed again through the cation-exchange resin. The obtained aqueous solution of organic acids was evaporated; dry residue was dissolved in pyridine, and TMS-derivatives were obtained in the presence of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [15, 16].

Gas chromatography–mass spectrometry. The carboxylic acids produced by fungi and extracts of samples withdrawn from the stone surface were analyzed by gas chromatography–mass spectrometry (GC–MS) on an Agilent device equipped with a MSD5975 mass-selective detector and an HP-5MS column (30 m x 0.25 mm). The programmed temperature range from 70 to 320°C was scanned at 4°C/min. The process was monitored using an Agilent ChemStation software package; the data were processed with the use of the AMDIS program (http://www.admis.net/index.html) and conventional databases (NIST2005 and Wiley6). The quantitative processing of chromatograms was performed by using C_{18} hydrocarbon as an internal standard with the aid of the UniChrom program (http://www.unichrom.com unichrome.shtml).

RESULTS AND DISCUSSION

The samples of initial soil containing residues of abscised leaves, which were withdrawn from the surface of marble- and granite monuments, contained sugars (glucose, fructose, arabinose, galactose, and unidentified disaccharides), sugar alcohols (glycerol, erythritol, ribitol, arabitol, mannitol, sorbitol, and myoinositol), amino acids (valine, serine, threonine, phenylalanine, and proline), and gluconic acid. Uridine and phosphate were found in trace amounts. Spring samples contained additionally succinic and malic acids, as well as such amino acids as leucine, isoleucine, and glutamine. Reliable difference in the composition of the spring- and autumn samples was not determined because of high variability in their quantitative and qualitative composition. Typical chromatogram of extract of initial soil taken from a monument surface is shown in Fig. 1. The total amount of the low-molecular carbon substrates suitable for fungi (sugars and sugar alcohols) varied from 1.5 to 7.6 mg/g dry weight (3 mg/g on average). The total amount of amino acids was 0.1–0.5 mg/g of substrate.

Sugar alcohols and mono- and disaccharides were the predominant low-molecular components in the samples taken from the surface of marble- and granite monuments. The initial soil formed on the monument surface is the richest medium for the fungi development. In the gypsum crusts at white Italian marble and in the samples of initial soil, high content of organic acids, mainly oxalic acid (up to 10 μg/g of gypsum crusts and to 300 μg/g of initial soil with moss) was found.

Based on the results obtained, in experiments in vitro on the study of the micromycete growth and organic acid production, we used media containing mono- and disaccharides, sugar alcohols, and salts of gluconic and oxalic acids. All the studied carbon sources except for oxalic acid were easily utilized by micromycetes (Fig. 2). No mycelium growth was observed in the medium with potassium oxalate as the sole carbon source. Biomass of fungi increased with increasing concentration of the studied sugars.

Oxalic acid prevailed among organic acids excreted by micromycetes grown on all the studied media. The total amount of produced organic acids increased with carbohydrate concentration. High glucose content (over 30 g/L) in liquid medium promoted intense synthesis of citric acid (up to 64.0 mg/g of mycelium) and gluconic acid (up to 47.7 mg/g of mycelium) by *A. niger* (Fig. 3). An increase in glucose concentration

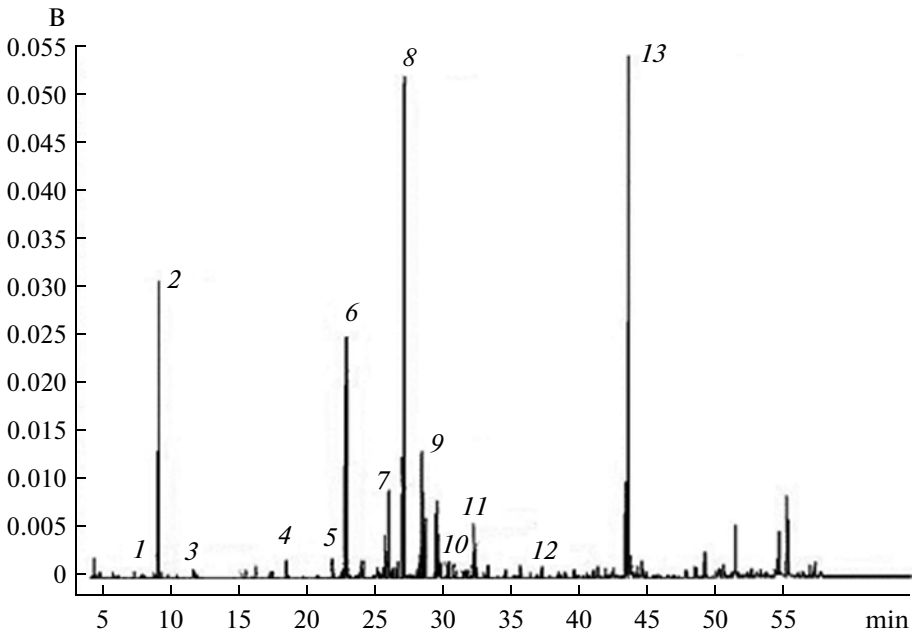

Fig. 1. Chromatogram of extract of the initial soil withdrawn from the surface of the E.Kh. Minikh marble monument in the Alexander Nevsky Lavra (St. Petersburg): valine (*1*); glycerol (*2*); proline (*3*); glutamine (*4*); ribitol (*5*); C_{18}, internal standard (*6*); α-glucose (*7*); sorbitol (*8*); β-glucose (*9*); myoinositol (*10*); ribitol (*11*); uridine (*12*); unidentified disaccharides (*13*).

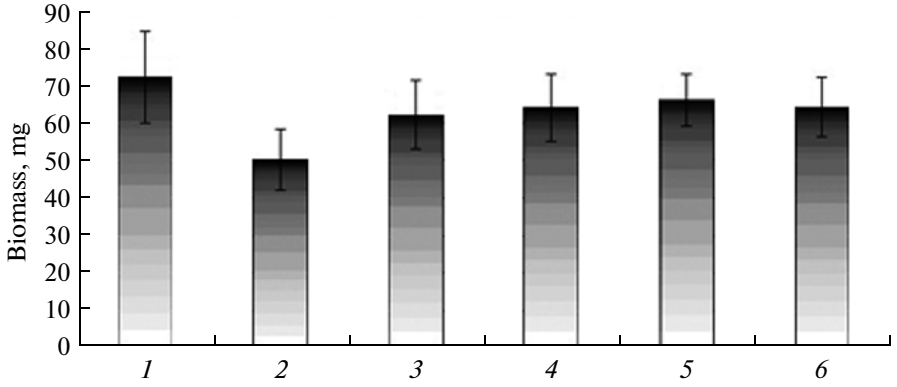

Fig. 2. Biomass of *Penicillium citrinum* grown on agar media with sugar alcohols, carbohydrates, and gluconic acid: glucose (*1*); fructose (*2*); saccharose (*3*); mannitol (*4*); sorbitol (*5*); gluconic acid (*6*).

up to 50 g/L induced excretion of succinic, fumaric, and malic acids by *P. citrinum* grown on agar medium supplemented with $CaCO_3$ (table). In this case, the amount of oxalic acid per biomass unit (productivity of mycelium) decreased, although its absolute value in medium increased at high glucose concentrations. *A. niger* grown on this medium produced only oxalic and gluconic acids; amounts increased with glucose concentration.

The production of succinic, fumaric, and malic acids by *P. citrinum* grown on media with high fructose concentrations (50–70 g/L) was more intense as compared with that in glucose- and sugar-containing media (table).

The production of oxalic and gluconic acids was the highest on media with glucose. A reliable increase in production of succinic and gluconic acids was revealed in liquid media with fructose and glucose, respectively (Fig. 4).

Sugar alcohols were also easily metabolized by fungi with formation of organic acids. The amounts of organic acids produced on media with mannitol and sorbitol were similar to those formed on carbohydrate-containing media. The content of oxalic acid in CB of *P. citrinum* grown on medium with sorbitol (30 g/L) was 15% as high as that observed in the presence of glucose at the same concentration and 25% higher than that on the medium with fructose. On the con-

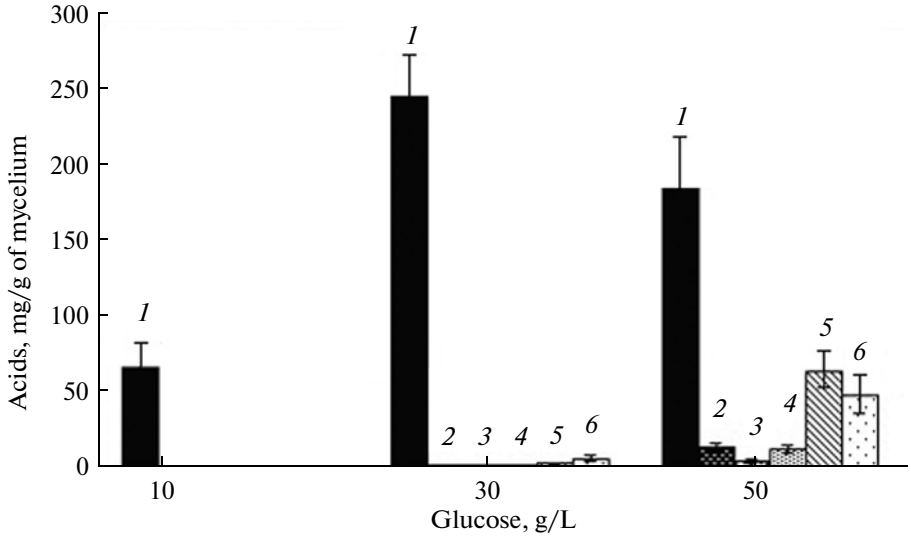

Fig. 3. Organic acids produced by *A. niger* grown in liquid Czapek medium with different concentrations of glucose: oxalic acid (*1*); succinic acid (*2*); fumaric acid (*3*); malic acid (*4*); citric acid (*5*); gluconic acid (*6*).

trary, the amounts of succinic, malic, fumaric, citric, and gluconic acids produced on this medium were lower than those on carbohydrate-containing media.

When *A. niger* was grown in liquid media with sorbitol and mannitol (30 g/L), the oxalate content of CB was not reliably different from that on the medium containing glucose at the same concentration; whereas the amount of citric acid was three- to fivefold higher than that on glucose-containing medium. Only traces of succinic, fumaric, and malic acids were found.

In agar medium with calcium gluconate (30 g/L), oxalic acid was revealed; its amount was similar to that on glucose-containing medium but higher than on media with saccharose and fructose.

Based on the obtained data on the effects of carbon sources occurring at the stone surface on fungal growth and organic acid production, it can be concluded that the main groups of these compounds (mono- and disaccharides, sugar alcohols, and organic acids) are suitable substrates for the mycelium growth and excretion of organic acids. It is considered that mono- and disaccharides, mainly glucose and saccharose, are the best carbon substrates for production of organic acids by fungi [17–20]. According to our results, fungi grown on media containing glucose, gluconic acid, and sugar alcohols excreted the largest amount of oxalic acid.

It is suggested that physiological role of oxalic acid is associated with its synthesis as a by-product in the course of the acetyl-CoA metabolism through the glyoxylate and the Krebs cycles. The oxidation of malate into oxaloacetate, a direct precursor of oxalate, is accompanied by the NADH formation. Then oxaloacetate is hydrolyzed by oxaloacetate hydrolase to oxalic and acetic acids; the former is excreted into the medium, whereas acetic acid is used for the synthesis of acetyl-CoA [21]. Oxaloacetic acid can be also formed through pyruvate carboxylation in cytoplasm [28]. Thus, the oxalate synthesis is an energy-efficient process, and the excreted oxalic acid is a by-product of sugar oxidation.

Increased carbohydrate concentration in the medium (more than 30 g/L) promoted excretion of citric, malic, fumaric, and succinic acids by fungi. These acids are probably synthesized via the Krebs cycle; unlike oxalic acid, they are intermediates capable of further incorporation into metabolic reactions. We think that one of possible mechanisms involved in the excretion of these acids from the cells is "catabolic inactivation," i.e. the inhibition of some respiratory enzymes by high concentrations of sugars [9, 22]. Besides the carbon source, a ratio between nitrogen and carbon sources in the medium is also an important factor affecting the organic acid production by fungi. High C/N ratio is favorable for the excretion of acids, intermediates of the Krebs cycle [23]. It can be suggested that in our experiments, increased production of citric, malic, succinic, and fumaric acids in the media with high carbohydrate concentrations is also due to the fact that fungi are unable to metabolize completely high concentrations of sugars under relatively low nitrogen concentration.

Among tested sugars, fructose was the most suitable carbohydrate for the synthesis of fumaric, malic, and, especially, succinic acid, possibly because of its ready involvement in glycolysis with subsequent formation of organic acids in the Krebs cycle. Glucose and saccharose are probably involved mainly in biosynthetic processes [9].

Mannitol and sorbitol were efficiently metabolized in the course of mycelium growth and acid formation.

The effects of glucose, fructose, and saccharose at different concentrations on production of organic acids by *P. citrinum* grown on agar medium supplemented with $CaCO_3$

Acids	Carbohydrate concentration, g/L	Sugars					
		glucose		fructose		saccharose	
		acid, mg/g of mycelium	acid concentration, μg/mL	acid, mg/g of mycelium	acid concentration, μg/mL	acid, mg/g of mycelium	acid concentration, μg/mL
Oxalic	10	49.6 ± 6.6	182.6 ± 45.6	23.5 ± 5.9	103.8 ± 43.8	13.4 ± 1.5	48.2 ± 5.4
	30	33.4 ± 1.8	486.5 ± 55.1	20.2 ± 2.5	323.2 ± 64.2	19.8 ± 3.5	237.6 ± 40.0
	50	28.3 ± 0.8	510.8 ± 28.5	17.7 ± 2.0	73.4 ± 19.9	24.4 ± 8.1	405.0 ± 40.0
	70	4.7*	99.8*	3.3 ± 0.2	24.9 ± 1.1	19.7 ± 5.3	394.0 ± 106.0
Succinic	50	0.7 ± 0.1	13.3 ± 2.6	3.0 ± 0.4	31.5 ± 8.9	0.3 ± 0.1	5.0 ± 1.7
	70	0.7*	1.5*	16.8 ± 4.2	50.4 ± 14.1	1.2 ± 0.3	24.0 ± 6.0
Fumaric	50	0.6 ± 0.1	11.4 ± 3.2	1.4 ± 0.4	5.9 ± 1.3	0.5 ± 0.1	8.3 ± 1.7
	70	0.3*	6.1*	20.1 ± 3.1	60.3 ± 11.6	1.6 ± 0.2	32.0 ± 3.4
Malic	50	6.7 ± 0.9	13.1 ± 1.9	0.5 ± 0.1	2.2 ± 0.7	0.3 ± 0.1	4.9 ± 1.6
	70	0.4*	9.39*	5.6 ± 0.5	16.6 ± 2.2	0.9 ± 0.4	18.0 ± 6.6
Gluconic	50	8.9 ± 0.9	190.1 ± 20.3				
	70	4.1*	88.3*				

* The growth of mycelium was not observed in all of the replicated experiments.

Metabolism of sugar alcohols is closely associated with the carbohydrate metabolism [24, 25]. The oxidation of mannitol to fructose by mannitol dehydrogenase was established in 1978 [26] and confirmed later [27]. In *Aspergillus niger*, D-sorbitol can be also transformed into D-fructose by sorbitol dehydrogenase, which is encoded by gene *sdhA* [26]. Thus, the acid formation by fungi grown on sugar alcohols most likely did not involve special biosynthetic mechanisms and occurred through the sugar formation.

Gluconic acid was excreted into CB by *P. citrinum* in the course of sorbitol- and fructose metabolism. It is considered that, unlike other acids produced by fungi, gluconic acid is synthesized mainly extracellularly through the functioning of glucose oxidase and gluconolactonase localized outside the cytoplasmic membrane [23, 27]. Our results suggest that formation of extracellular gluconic acid from glucose is mainly associated with functioning of extracellular glucose oxidase; however, the biosynthesis of gluconic acid from the other sugars and sorbitol can proceed through an alternative mechanism. The formation of gluconic acid can be extremely important for micromycetes inhabiting rocky substrates; it can be utilized by fungi in lithobiontic community. Moreover, this acid is a favorable substrate for production of oxalic acid, which plays a key role in chemical degradation of natural rocks.

Our experiments showed that synthesis of organic acids by fungi depended not only on the carbon source concentration, but also on the cultivation conditions (Fig. 5). Liquid medium was the most favorable for the excretion of organic acids: the amounts of oxalic, citric, malic, succinic, and gluconic acids produced in liquid medium were five- to eightfold higher than those formed on solid medium, possibly, because of higher availability of carbohydrates.

The stimulatory effect of calcium carbonate on production of organic acids was revealed in many studies. Supposed mechanisms responsible for this phenomenon have included neutralization of produced acids by Ca^{2+} ions, increased carbonic acid content of the medium, and alkalization of CB [28, 29]. In previous experiments, we revealed stimulatory effect of calcium on hyperproduction of oxalic acid by fungi of the genera *Penicillium* and *Aspergillus* grown in the presence of $CaCO_3$ [30]. Results of the present study confirm this finding.

On the whole, the results of this study demonstrate that organic substances (sugars, sugar alcohols, and

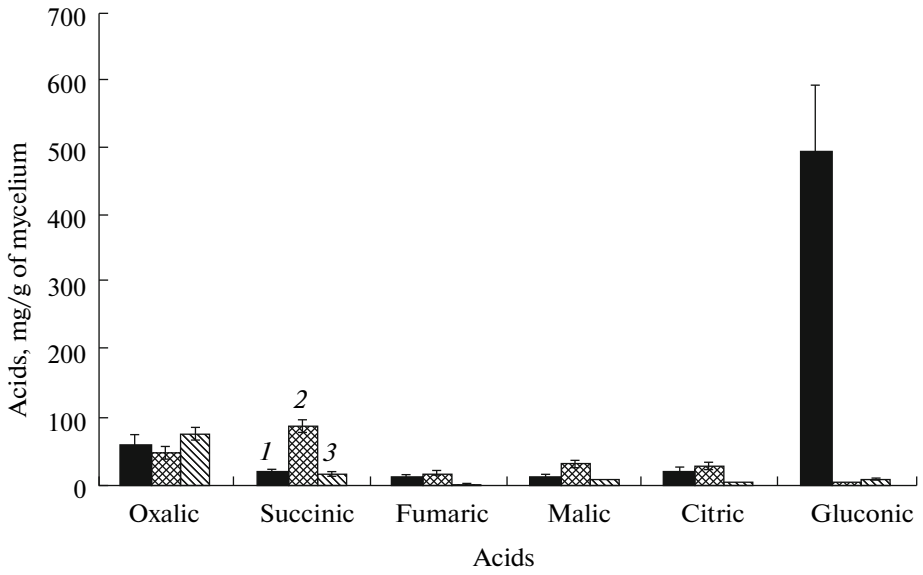

Fig. 4. Organic acids produced by *P. citrinum* grown in liquid media containing 30 g/L of: glucose (*1*), fructose (*2*), and sorbitol (*3*).

organic acids), which are accumulated on the surfaces of stony monuments in urban environments, are suitable carbon sources for the development of micromycetes and the organic acid excretion. Oxalic acid is a by-product of fungal metabolism in the presence of various carbon sources; it is the main compound excreted into the medium. Under experimental conditions, the major amount of oxalic acid was excreted on media containing glucose, gluconic acid, and sugar alcohols. The excretion of oxalic acid occurred even at relatively low concentration of organic substances in the medium. On the contrary, malic, citric, fumaric, and succinic acids were produced only at increased content of carbohydrates, mainly in liquid media. In lithobiontic communities, the excretion of these acids by fungi apparently does not have an important ecological role. At the same time, oxalic acid produced by micromycetes in large amounts is an important factor of the monument destruction under urban environments; especially if monuments are made from carbonate rock.

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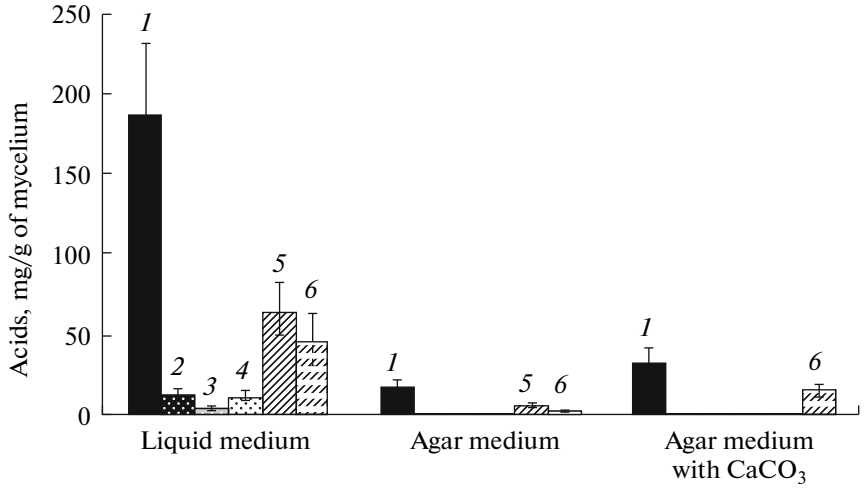

Fig. 5. Production of organic acids by *A. niger* grown on different media with glucose concentration of 50 g/L: oxalic acid (*1*); succinic acid (*2*); fumaric acid (*3*); malic acid (*4*); citric acid (*5*); gluconic acid (*6*).

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