ISOLATION AND PRIMARY SELECTION OF MICROMYCETES PRODUCING CITRIC ACID

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Abstract

78 strains of mycelial fungi were isolated from various soil samples of the Republic of Uzbekistan. A cup express method has been developed for the primary assessment of the acid-forming ability of mycelial fungi belonging to the genus *Aspergillus* and *Penicillium*. As a result of screening, two strains with the highest acid-forming ability were selected. It has been established that in natural strains in the dynamics of growth, the maximum concentration of citric acid (CA) biosynthesis is observed at the end of the lag phase or in the phase of exponential growth. In the growth dynamics of natural strains 5 and 8, the maximum biosynthesis of CA was observed within one or two hours, respectively.

Keywords: citric acid, producer, *A.niger*, express selection method, biosynthesis, spore count, mycelial fungi.

Introduction

Citric acid (CA) (2-hydroxy-1,2,3-propane tricarboxylic acid, formula $C_6H_8O_7$) is an important commercial product found in almost all tissues of plants and animals [1]. It is known that the first and last reactions in the tricarboxylic acid cycle (TAC) include the formation of CA from oxaloacetate, acetyl-CoA and water with the help of citrate synthase, which ultimately generates chemical energy in the form of adenosine triphosphate [2].

In 2017, the world production of CA was 2 million tons [3] and there is a constant increase in the level of CA production all over the world. Only the food industry consumes about 70% of the total amount of CA produced in the world, while the remaining 30% is used by other industries [4, 5]. CA is currently predominantly produced in China, which accounts for approximately 60% of world production [6]. All over the world, CA is recognized as a generally recognized safe substance "GRAS" (generally recognized as safe) and approved by the experts of joint FAO/WHO Committee on Food Additives. Representatives of the genus *Aspergillus*, in particular *A.niger, A.oryzae, A.flavus, and A.terreus* are the most important for commercial use, as they are able to produce a wide range of low molecular weight organic acids, including citric acid, in large quantities [7 - 9]. Starting from the 1950s, the discovery of the TAC contributed to the development of the production of CA by the deep method, which was first developed in the USA [10]. Currently, CA and its salts are widely used in food, cosmetics, pharmaceuticals, biomedicine (nanodrugs, tissue engineering), agriculture, and other fields [11, 12, 13, 14, 15].

It is known that the most difficult step in testing both newly isolated microorganisms and their mutant forms is the development of an express method that allows you to quickly select forms with the necessary properties.

For primary selection and subsequent selection of yeast mutants producing citric acid from n-alkanes, a solid nutrient medium containing $CaCO_3$ was modified [16]. Avchieva P.B. and Vinarov A.Y. proposed a method for selecting producers of organic acids based on the use of liquid indicator media for the yeast *Candida lipolytica* [17]. For quick selection of mutants, selective media with citrate and acetate have been developed, as well as an express method for detecting active CA producers in yeast strains of *Yarrowia lipolytica* on solid media with $CaCO_3$ and indicator bromcresol green, including the limiting concentration of amine nitrogen (60 mg/l) and excess glucose. On the medium with $CaCO_3$, in the process of acid isolation around the colonies of yeast cultures, $CaCO_3$ dissolution

zones of various sizes appeared, corresponding to the amount of acids formed [18, 19].

However, the analysis of literature data shows that at present, an express method for a preliminary rapid assessment of the detection of organic acids for mycelial fungi is not described in the available literature. Also, a small number of studies affect the study of the effect of the initial amount of conidia suspension on the biosynthesis of CA by micromycetes. At present, CA is widely represented in the Republic of Uzbekistan by foreign companies. Obtaining a local active strain - a producer of citric acid in the future can help reduce the demand for the use of imported products.

Purpose of the research

The aim of the work is to isolate CA producers, develop an express primary screening method for assessing acid-forming ability, and study the effect of A. *niger* spore abundance on CA biosynthesis.

Materials and Methods

The object of the study was 78 strains of mycelial fungi isolated from various substrates of the Republic of Uzbekistan. For the isolation of mycelial fungi, Czapek-Dox and Suslo-agar media were used [20]. The soil with a profile of 5 and 10 cm, rhizospheric soil, flowers of the persimmon plant, and jam from cherry and strawberry fruits contaminated with microscopic fungi served as a substrate for the isolation of mycelial fungi.

Cultivation of mycelial fungi. Primary screening for the acid-forming ability of the strains was carried out on agar media:

1. Czapek-Dox medium (CD) with a $CaCO_3$ content of 6 g/l;
2. Modified Czapek-Dox medium (MC) with NH_4NO_3 - 2 g/l, instead of $NaNO_3$, which is part of the composition of the medium, $CaCO_3$ - 6 g/l and trace elements according to Burholder (mg/l): KJ - 0.1, B - 0.01, Mn^{2+} - 0.01, Zn^{2+} - 0.03, Cu^{2+} - 0.01, Mo^{2+} – 0.013.

3. SSN (selective solid nutrient) medium as follows (g/l): NH_4NO_3 –2.0, K_2HPO_4 – 1.0, $MgSO_4*7H_2O$ – 0.5, KCl – 0.5, $FeSO_4*7H_2O$ – 0.1, sucrose – 30.0, agar – 15.0, distilled water 1000 ml. The SSN medium additionally also contains trace elements according to Burholder and a solution of vitamins.

The medium is sterilized at 0.5 atm for 20 minutes. Sterile $CaCO_3$ (6 g/l) and vitamin solution (2 ml/l) were added to a sterile nutrient medium (50-55°C) immediately before pouring into Petri dishes. The composition of the vitamin solution included (mg/10 ml): thiamine nitrate -10.0; riboflavin-2.0; pyridoxine hydrochloride- 8.0; nicotinamide – 100. After complete solidification of the medium in Petri dishes, conidia of 6 daily cultures of mycelial fungi were sown by injection. According to the zones of dissolution of chalk around the colony, the acid formation of the studied strains was assessed. Incubation was carried out for 48-55 hours in a thermostat at 28°C. Further, after incubation, to assess the efficiency of acid formation, the diameter of the mycelial fungi colony (A) and the diameter of the chalk dissolution zone around the colony (B) were measured on the ruler - a template for measuring the size of the zones (Hi-Media), the width or size of the zone (C) of chalk dissolution around colonies in mm, C=B-A [21].

Deep cultivation. The selected strains were cultivated in a liquid nutrient medium (LNM) of the following composition (g/L): NH_4NO_3 – 1.5, K_2HPO_4 – 1.0, $MgSO_4*7H_2O$ – 0.5, KCl – 0.5, $FeSO_4*7H_2O$ – 0.1, sucrose- 35.0, trace elements according to Burholder, distilled water 1000 ml. The cultivation of mycelial fungi was carried out in Erlenmeyer flasks with a volume of 250 ml containing 50 ml of the LNM nutrient medium. The medium was inoculated with a suspension of mycelial fungal conidia containing $5*10^3$ and $1*10^6$ spores/ml. The number of conidia was determined in a Goryaev chamber [20]. The duration of cultivation is 124 hours, at a temperature of 28 °C. At the end of the cultivation, the flasks with the deep culture of the fungus were preliminarily heated in a water bath at 80°C for 45 min to disinfect the fungi. Then the flasks were cooled to room temperature, the fungal mycelium was separated from the culture liquid by filtration, and then the

biomass content was determined [22]. Determination of the pH of the culture liquid was determined by the potentiometric method.

The quantitative content of CA was determined chemically according to the method of Zhabolovskaya N.A. et al. [23]. The method is based on the oxidation of citric acid with permanganate to acetone dicarboxylic acid, which, upon bromination, turns into pentabromoacetone. The amount of pentabromoacetone is estimated spectrophotometrically. In the process of carrying out the determinations, reagents were used in the following quantities: 1.0 ml of the test solution was added to a measuring tube with a ground stopper. Then 0.5 ml of 40% H_2SO_4 solution and 0.25 ml of 30% KBr solution were added. After vigorous stirring, 1.0 ml of a 5% $KMnO_4$ solution was added to the mixture and left for 10 min with occasional stirring. After completion of the reaction, 5.0 ml of a saturated solution of Mohr's salt ($[NH_4]2[SO_4]FeSO_4*6H_2O$) was added to remove excess manganese dioxide and bromine, and then 5.0 ml of distilled chloroform. The contents of the tube were shaken vigorously for one minute, while pentabromoacetone was extracted with chloroform. The upper aqueous layer was discarded after separation, the lower layer was used to measure the extinction of pentabromoacetone at a wavelength of 300 nm on a UV-5100 spectrophotometer (METASH, China). The amount of CA in the analyzed sample was determined from the corresponding calibration curve. The selected strains were identified according to the generally accepted method based on their cultural and morphological features [24, 25].

Results and Discussion

78 strains of mycelial fungi were isolated from various substrates in our region; on the basis of cultural and morphological characteristics, 53 strains were assigned to the species A. *niger*, 25 strains to the genus *Penicillium*. Further, according to the zones of dissolution of chalk, which is formed due to the release of organic acids, acid formation was assessed.

The study of the acid-forming ability of the isolated natural 78 strains on the CD medium did not allow the selection of producers with the necessary properties. Although, there was an intensive growth and development of these strains.

Many researchers note that CA biosynthesis by A.*niger* fungi on hydrocarbon media increases NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl are used as nitrogen sources [26, 27]. Kim K.S. et al. found that an increase in the amount of intracellular ammonium ions can lead to the prevention of citrate inhibition of phosphofructokinase activity, which will lead to an overproduction of CA [28]. In this regard, NH_4NO_3 was introduced into the composition of the MC medium. It was found that one strain belonging to the species A. *niger* 8 had a high acid-forming ability on the MC medium, where the size of the chalk dissolution zone was 5.0 mm, 16 strains did not show acid-forming ability (Table 1). It should be noted that the zones of chalk dissolution on the MC medium around the colony did not have visually clear transparency. In this regard, it was necessary to develop a medium that made it possible to more clearly visually assess the size of the dissolution zone around the colony of mycelial fungi. It was revealed that many strains had a high acid-forming ability on the SSN medium compared to the above medium (9; 23; 6p; 18p; 21p) (Table 2).

The highest acid-forming ability had two strains 5 and 8 belonging to the species A. *niger*, where the size of the chalk dissolution zone reached 5.6 - 6.5 mm. Only 6 strains did not show acid-forming ability. Thus, it was found that selection on the SSN medium compared to the "MC" medium makes it possible to more clearly visually assess the size of the chalk dissolution zone around the colony and increases the frequency of detection or selection of cultures.

In order to identify acids, as well as to check the results obtained by the cup express method, the selected strains were cultivated in a liquid nutrient medium (LNM). To assess the biosynthesis of AC on LNM, strains 5 and 8 belonging to the species *A. niger* were selected, which had the largest zones of chalk dissolution.

The effect of different amounts of spore suspension on CA biosynthesis in the dynamics of growth by strains 5 and 8 on the LNM medium showed that with an initial amount of spore suspension of $1 * 10^6$, CA biosynthesis by strains 5 and 8 was characterized by a low value of CA accumulation and in the growth dynamics at 96 hours of cultivation, CA biosynthesis did not exceed 1.2 and 1.3 g/l (Figure 1 A and B). Whereas, during the inoculation of the medium with suspensions of conidia of strains 5 and 8 in the amount of $5 * 10^3$ in the dynamics of growth, the highest concentration of CA was found at 96 and 120 hours of cultivation and amounted to 1.1 g/l (strain 5) and 4.1 g/l (strain 8). It was found that in the dynamics of growth of strains 5 and 8, the pH value of the culture liquid when using low doses of a suspension of $5*10^3$ spores/ml compared to using 1 million spores/ml was characterized by an intense decrease in the pH of the medium. It should be especially noted that at the initial stage of growth of strains 5 and 8 within 24 hours, when using small doses of spores, the most intensive decrease in the pH value of the culture liquid from the initial level is observed (from pH 5.5 to pH 2.8 and 3.1, respectively).

Table 1.

Acid-forming ability of strains of the species *A.niger* and the genus *Penicillium* on the solid medium "MC"

Number of strains	Amount of strains	Dissolution zone diameter, mm
7; 16; 18; 20; 25; 26; 30; 33; 38; 46; 50; 22p*; 23p*; 27p*; 28p*; 33*	16	0
27; 32; 40; 43; 59; 60; 4p*; 5p*; 20p*; 24p*; 29p*; 34p*; 35p*;	13	Up to 1,5
10; 12; 14; 15; 17; 19; 24; 28; 34; 36; 37; 41; 44; 45; 47; 52; 8p*; 14p*; 26p*; 31p*	20	Up to 2,5
1; 4; 11; 13; 21; 29; 31; 35; 39; 42; 57; 58; 7p*; 9p*; 12p*; 19p*; 25p*; 30p*	18	Up to 3,5
2; 3; 5; 6; 9; 22; 23; 6p*; 18p*; 21p*	10	Up to 4,5
8	1	Up to 5,5

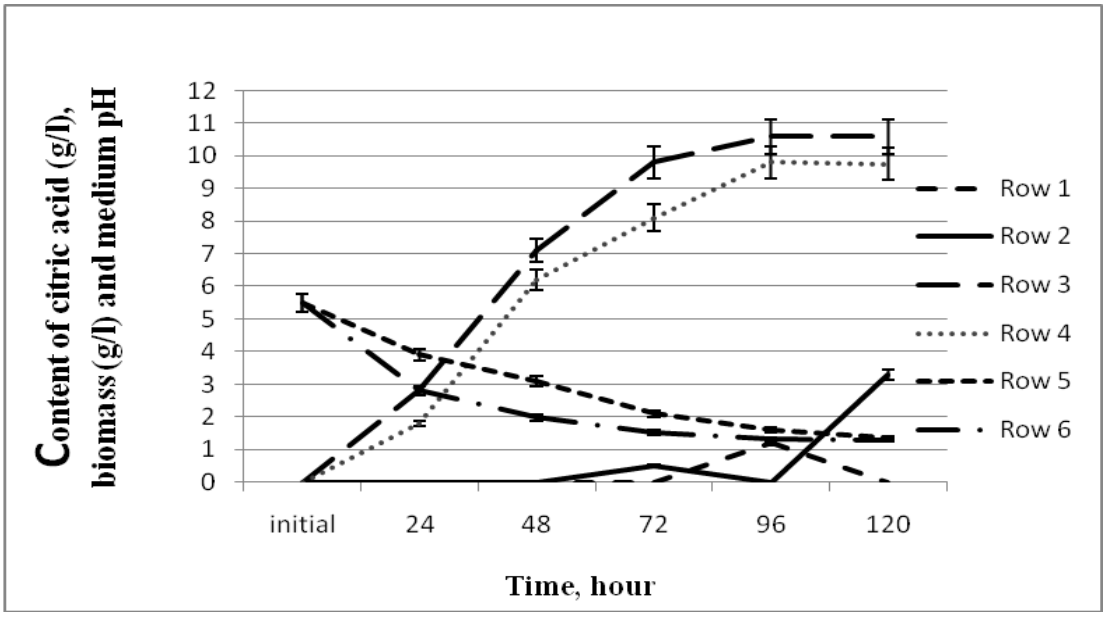
Table 2

Acid-forming ability of strains of the species *A.niger* and the genus *Penicillium* on the solid medium "SSN"

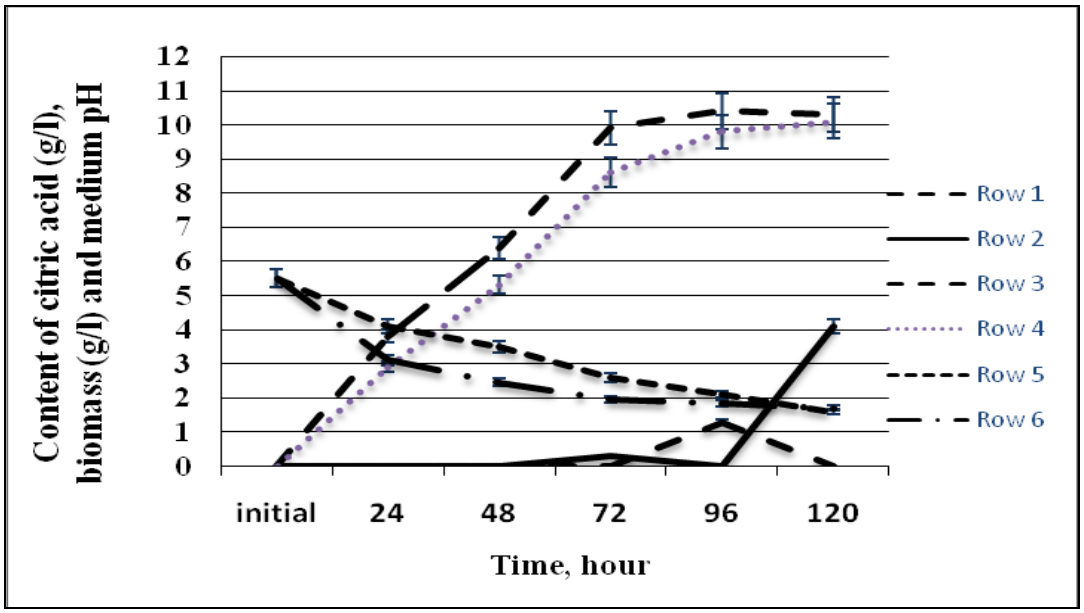
Number of strains	Amount of strains	Dissolution zone diameter, mm
26; 33; 38; 22p*; 24p*; 33p*	6	0
7; 16; 20; 25; 46; 50; 23p*; 27p; 34p*; 35p*	10	Up to 1,5
17; 18; 24; 27; 32; 34; 43; 59; 4p*; 5p*; 14p*; 28p*; 29p*	13	Up to 2,5
1; 4; 6; 10; 11; 13; 15; 19; 28; 30; 35; 36; 40; 41; 42; 47; 52; 60; 8p*; 9p*; 20p*; 30p*; 31p*	24	Up to 3,5
2; 3; 12; 14; 21; 22; 29; 31; 37; 39; 44; 45; 57; 58; 7p*; 12p*; 25p*; 26p*	18	Up to 4,5
9; 23; 6p*; 18p*; 21p*	5	Up to 5,5

5; 8	2	Up to 6,5

Note: * - strains belon to the genus *Penicillium*



A



B

Figure 1. Influence of different number of spores on CA biosynthesis, biomass content and pH of the medium in the dynamics of growth by strains 5 (A) and 8 (B).

1 - CA content during inoculation of the medium with spore suspensions $1*10^6$.
2 - CA content during inoculation of the medium with spore suspensions $5*10^3$.
3 - Biomass content during inoculation of the medium with spore suspensions $1*10^6$.
4- Biomass content during inoculation of the medium with spore suspensions $5*10^3$.

5. The pH value of the medium during the inoculation of the medium with spore suspensions $1*10^6$.
6. The pH value of the medium during the inoculation of the medium with spore suspensions $5*10^3$.

It was found that the biomass content of strains 5 and 8 in the dynamics of growth using a $1*10^6$ spore suspension, compared with the use of small doses of inoculum, increased intensively, and by 120 hours of cultivation, the biomass of strains 5 and 8 was 10.9 and 10.3 g/l (respectively) . The relationship between the accumulation of CA in the medium and the growth rate of mycelium has a relationship characteristic of products of primary metabolism. According to some authors, the release of acid occurs most intensively in the first days of growth, and then slows down [29]. According to other data, the largest amount of CA accumulates at the end of the lag phase and at the very beginning of the stationary phase of growth [30].

Due to the fact that when using a small number of spores of strains 5 and 8, compared with using a larger number of spores, it was characterized by an intensive decrease in the pH of the medium in the initial stage of growth, up to 96 hours of cultivation, it was necessary to conduct studies to determine the CA, the biomass content and pH of the medium and at other hours of cultivation. The results of the study showed that with the inoculation of $5*10^3$ spores/ml, the maximum concentration of CA biosynthesis in the growth dynamics of strain 8 was observed in the initial hours of cultivation (at the end of the growth lag phase) for 14 and 16 hours and the CA content was 30 g/l (Figure 2 B). It should be noted that during the growth dynamics of strain 8, the maximum biosynthesis of CA continued intensively for two hours. Subsequently, a sharp decrease in CA biosynthesis was observed down to zero values with an increase in it in the stationary phase of growth for 120 hours of cultivation. However, in the stationary phase of growth, the accumulation of CA was 7 times lower compared to the biosynthesis of CA at the end of the lag - phase of growth and amounted to 4.2 g/l. It was found that in strain 5 under similar conditions (with inoculation of $5*10^3$

spores) as compared to strains 8, the maximum concentration of CA biosynthesis in the growth dynamics is observed in the exponential growth phase at 50 hours of cultivation and the CA content was 6.28 g/l. It should be noted that the highest content of CA in strain 5 was 5 times lower than in strain 8 (Figure 2 A).

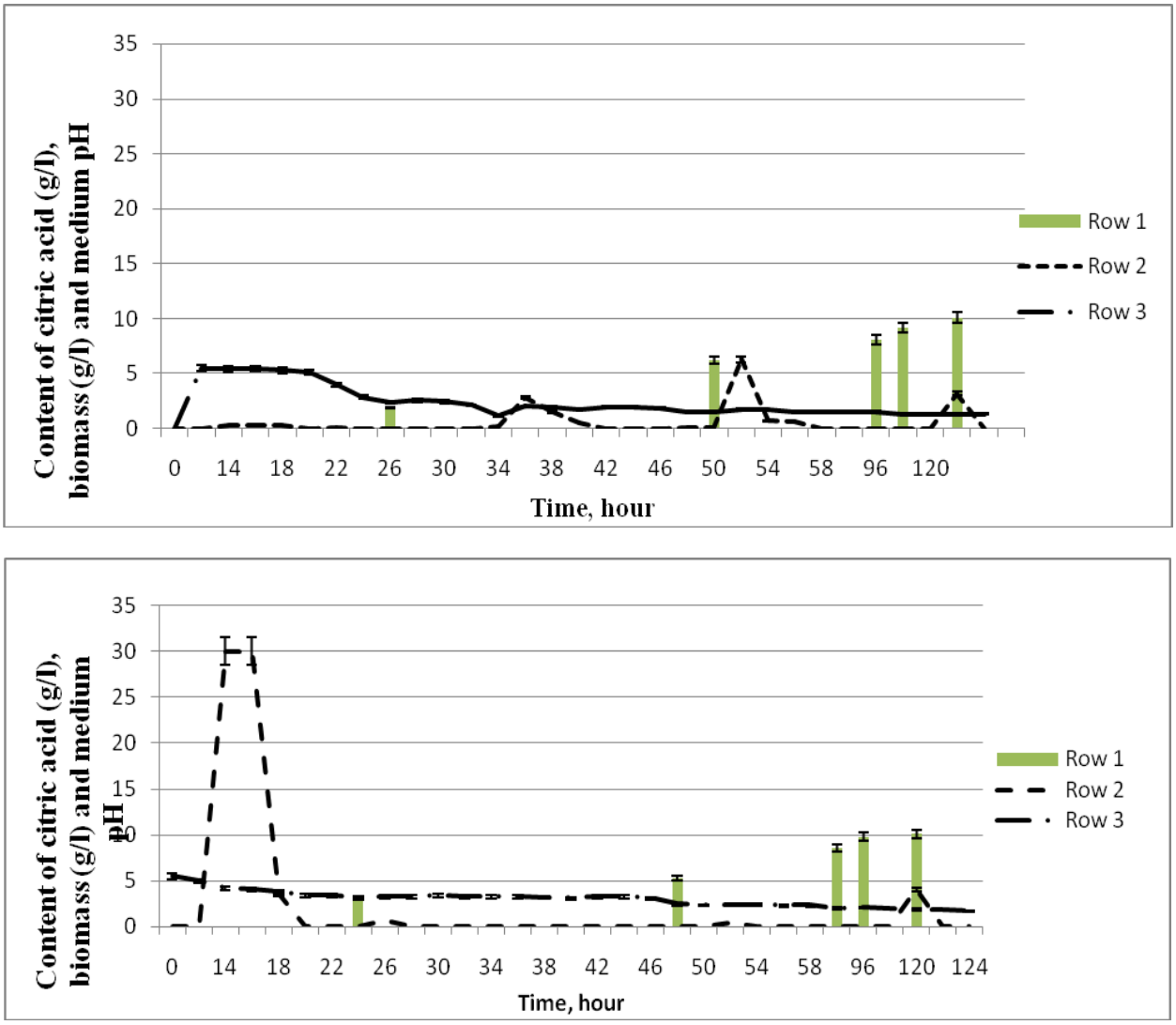


Figure 2. Biosynthesis of CA in the growth dynamics of the species *A. niger* strains No. 5 (A) and No. 8 (B). Row 1 - biomass of the strain; Row 2 – biosynthesis of CA; Row 3 – medium pH

It was found that at the end of the lag phase at the maximum concentration of CA in strain 8, the pH value of the culture fluid did not exceed 4.5, while in the stationary phase of growth at the second peak of CA formation, the pH of the culture fluid was 1.72. In strain 5, both in the exponential growth phase and in the

stationary growth phase (120 hours), at the highest concentration of CA, the pH of the medium was more acidic, and the pH of the medium was 1.71 and 1.31.

It is known that, with a sufficient amount of oxygen, the pyruvate molecule is decarboxylated to form acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex. Acetyl-CoA interacts with oxaloacetate and forms CA; this reaction is catalyzed by the enzyme citrate synthase [31, 32]. In mitochondria, CA is included in subsequent reactions of the Krebs cycle. It is assumed that the excretion of di- and tricarboxylic acids of the Krebs cycle occurs with the participation of transport proteins [33, 34]. It is also known that a significant part of CA is transported via the citrate/malate transporter from mitochondria to the cytosol [35]. Also, it is assumed that the detection of the maximum amount of CA at the end of the lag phase or the exponential phase of mycelium growth is associated with the participation of transport proteins.

Conclusion

Thus, CA producers have been identified and selected, and an express method has been developed for the initial assessment of the acid-forming ability of filamentous fungi belonging to the genus *Aspergillus* and *Penicillium* on a solid SSN medium. It has been established that during the growth dynamics of natural strains of *A. niger*, the maximum biosynthesis of CA intensively continues for only one or two hours. It has been shown that for the intensification of CA biosynthesis, the optimal initial amount of inoculum is of great importance and depends on the physiological characteristics of the producing strains.

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