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Production of citric acid from fermentation solution by cultivation *Aspergillus niger-1* strain on an extract obtained from topinambur (*Helianthus tuberosus*) artichoke tubers

Inna E. Melkumyan[1]*, Baghish A. Harutyunyan[1], Luiza S. Manukyan[1], Samvel Kh. Stepanyan[2], Gohar G. Oganezova[1], Nelly S. Avetisyan[1], Marina A. Melkumyan[1]

[1]SPC «Armbiotechnology» of NAS of RA, 14 Gyurjyan Str., 0056 Yerevan, Armenia; [2]Institute of Chemical Physics of NAS of RA, 5/2 Paruyr Sevak Str., 0014 Yerevan, Armenia.

[*]**Corresponding author:** Inna E. Melkumyan, Scientific Researcher of the SPC "Armbiotechnology" of NAS of Armenia, 14 Gyurjyan, Yerevan 0056, Armenia.

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ABSTRACT

Background: Citric acid is an organic hydroxycarboxylic tribasic acid. It is widely distributed in plants: in berries, citrus fruits, for example, in lemons, up to 9% of dry weight. Citric acid is used in many industrial processes, but it is most frequently used as a flavoring ingredient and preservative in food products. Because of its beneficial antioxidant properties, citric acid helps control acidity and aids in digestion. Citric acid is traditionally produced by microbiological synthesis using beet molasses as the primary raw material during the metabolism of the producer strain *Aspergillus niger-1.*

Objective: Nowadays, the microbial synthesis of citric acid also depends on the utilization of readily available and reasonably priced products as raw materials. This study reports on the biosynthesis of citric acid by the producer strain *Aspergillus niger-1* on an extract derived from the tubers of the Jerusalem artichoke plant that contains inulin.

Currently, the synthesis of citric acid with microbes is also associated with the use of readily available products as raw materials. This study describes the biosynthesis of citric acid during the growth of the producer strain *Aspergillus niger-1* on an extract derived from Jerusalem artichoke tubers and containing inulin.

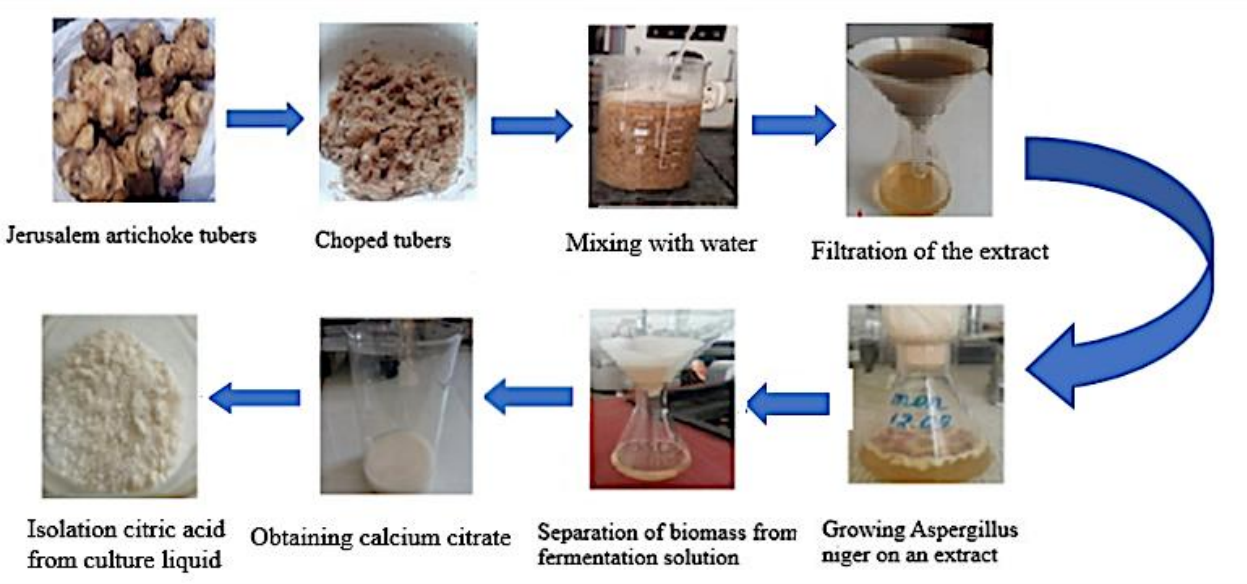
Methods: To prepare the extract, water was added to the crushed Jerusalem artichoke tubers in a ratio of 1:2.
The inulin content in the extract was determined spectrophotometrically at a wavelength of 490 nm. Fungi were grown on a shaker for submerged cultivation for seven days. HPLC and neutralization methods were used to determine the concentration of citric acid in the samples.

Results: After a series of treatments, 2.0 liters of aqueous extract were obtained from crushed Jerusalem artichoke *(Helianthus tuberosus)* tubers, which contained about 6.5–7.0% inulin. On the fifth day of culture development, the fungus fermentation solution had the highest concentration of citric acid (5.8%), of which 4.3% citric acid was found in the supernatant. Ultimately, 3.5 g of milky-white crystalline powder was isolated from the supernatant, the yield of which was 60.3% of the amount of citric acid in the fermentation solution.

Conclusion: To date, the aqueous extract from Jerusalem artichoke tubers has not been utilized as a carbon source for the microbiological synthesis of citric acid. Due to its high inulin content, resistance to environmental stressors, and minimal agricultural requirements, we proposed studying the possibility of obtaining citric acid from inulin-containing raw materials—Topinambur tubers *(Helianthus tuberosus)*—using microorganisms.

Novelty of the study: The scientific novelty lies in obtaining citric acid from an aqueous extract from tubers of topinambur, which is an alternative to the classic method of obtaining citric acid from beet molasses. Since this is a multi-stage process, molasses is an expensive raw material.

Keywords: Topinambur *(Helianthus tuberosus)*, inulin-containing extract, *Aspergillus niger*, fermentation solution, supernatant, citric acid powder, food additives



Graphical Abstract: Preparation of extract, cultivation of *Aspergillus niger*, and obtaining citric acid

INTRODUCTION

Citric acid is a hydroxy carboxylic organic acid that contains three hydroxyl groups (Figure 1). The chemical formula is $C_6H_8O_7$ [1]. This acid is mainly found in various plants, particularly citrus fruits, where it reaches up to 9%. Citric acid is of great practical importance. Two to three million tons of citric acid are produced worldwide annually [2]. About 75% of the total production is used in the food industry, and 25% in the medical, pharmaceutical, and chemical, metallurgical industries, etc. [3]. Citric acid has the status of an additive food as a flavoring and preservative that is used in the preparation and storage of bakery and confectionery products as a leavening agent for dough (which gives the baked dough splendor and airiness), drinks, including dry drinks, vegetable and fruit canned products, etc. [4,5]. Functional foods are also very important in the food industry. They play an important role in reducing the risk of diseases, providing additional health benefits in addition to their main nutritional value. Citric acid can be considered a functional food. It regulates acidity and promotes digestion due to its beneficial antioxidant properties [6]. In addition, citric acid is used in medicine as a preservative for blood transfusions, in pharmaceuticals as part of medicinal preparations that stimulate metabolic processes, and in various household cleaning products [7].

Figure 1. The structural formula of citric acid with three COOH groups.

In the past, citric acid was obtained mainly from citrus fruits. Lemon juice remained the main source of citric acid for a long time. The growing demand for citric acid and its limited production from plant sources prompted researchers to look for other sources of its production, one of which was microbial production based using producer strains to find this product through metabolic processes [8]. Currently, citric acid is also produced by microbial synthesis, resulting in increased productivity, reduced time, space costs, and economic efficiency [9]. Fungi *Aspergillus, Penicillium, Pichia, Saccharomyces, Yarrowia*, and some bacterial strains showed a high percentage of citric acid production [10-12]. The production of citric acid from organic substances involves a complicated biochemical process that demands carefully chosen conditions, such as the carbon source, pH level, aeration, etc. [13]. In industrial production, citric acid is predominantly produced with the help of the fungus *Aspergillus niger,* which has a higher productivity. Furthermore, these fungi are simple to cultivate and can grow on a range of affordable raw materials [14].

For many decades, the attention of researchers has been focused on the study of microbial synthesis of citric acid from various raw materials. For example, beet molasses is usually used for industrial production of citric acid, with the participation of mycelial fungi of the *Aspergillus niger* species. Molasses is a raw material obtained by processing sugar beets and contains a significant amount of sucrose (40-55%). However, molasses is not a standard raw material; it contains impurities, and each batch of it must be subjected to special biochemical research and processing before being put into production. Traditional technology for producing citric acid from molasses has a limited raw material base and is a multi-stage process. Currently, cane molasses,

starch, corn or sweet potato hydrolysates, palm oil, or soybean oil are also widely used for the production of citric acid. [15-19].

However, plants containing inulin have not previously been explored as a source for citric acid production. The objective of this research is to create a method for producing citric acid by cultivating the fungus *Aspergillus niger* using an extract derived from Topinambur tubers (*Helianthus tuberosus*) that is rich in inulin. Jerusalem artichoke is a perennial herbaceous plant. The Jerusalem artichoke is a hardy, prolific, and low-maintenance herbaceous plant that can withstand drought and frost without needing special attention. Jerusalem artichoke belongs to the Asteraceae family, like sunflower, and differs from it precisely by the presence of underground tubers, which are its edible part and contain a large amount of inulin. The inulin content in tubers is 15-20%. [20]. Inulin ($C_6H_{10}O_5$) is an organic substance from the group of polysaccharides, a fructose polymer, also called fructan, consisting of over 90% fructose molecules, which can be obtained in the form of an amorphous powder and crystal. It readily dissolves in hot water and has a limited solubility in cold water. It has a sweet taste. Like starch, inulin serves as a reserve substance. It is found mainly in plants of the Asteraceae family. In Jerusalem artichoke tubers, the inulin content reaches up to 20%, in dahlia tubers, up to 15%, and in onion bulbs, up to 5% [21]. Given the simplicity of the Jerusalem artichoke and the rich presence of inulin in its tubers, we suggested a technique to produce citric acid by growing a strain of the mold *Aspergillus niger* on a water extract derived from Jerusalem artichoke tubers.

MATERIALS AND METHODS

Strain and nutrient medium: The subject of the research was the mold fungus *Aspergillus niger-1*, sourced from the MDC SPC

« Armbiotechnology» NAS RA. The fungus was grown on a synthetic Czapek-Dox agar medium with the following composition: agar - 20.0 g/l, KCl - 0.5 g/l, sucrose - 20.0 g/l, $NaNO_3$ - 2.0 g/l, KH_2PO_4 - 1.0 g/l, $MgSO_4 \cdot 7H_2O$ - 0.5 g/l, $Fe_2SO_4 \cdot 7H_2O$ - 0.01 g/l, agar - 20.0 g/l, pH 6.8 [22]

Preparation of Jerusalem artichoke tuber extract: After harvesting, tubers of topinambur were taken to the laboratory, where they were cleaned of soil residues. To obtain the extract, the tubers were crushed using a Toplab TL-500 device (Toplabindia, Mumbai, India). Two liters of distilled water were added to 1 kg of crushed tubers and mixed thoroughly for approximately one hour at a temperature of 80°C. A sieve with a 1 mm hole was used to remove the suspension containing foreign impurities, and then the mixture was filtered [23].

Measurement of inulin content in Jerusalem artichoke tuber extracts: To measure the inulin content, 1 mL of Jerusalem artichoke extract was diluted 100 times. Then, 2 mL of Selivanov's reagent (0.1 g of resorcinol dissolved in 50 mL of 20% hydrochloric acid solution) was added to 1 mL of the diluted extract. The mixture was heated in a water bath at 100°C for 15 minutes, after which it was cooled. A UV-6300PC VWR International spectrophotometer (located in Pennsylvania, USA) was employed to measure the colored complex generated during the hydrolysis of inulin at 490 nm. To quantify inulin, a standard curve was constructed using different concentrations of inulin (0.25, 0.5, 0.75, and 1.0 mg/mL) [23].

Sample preparation: The *Aspergillus niger-1* strain was cultivated for seven days at 30°C on Czapek-Dox agar to produce a new culture. The fresh culture was then grown in a 100 ml extract made from Jerusalem artichoke tubers that contained inulin. The flasks were kept on a shaker for submerged cultivation at 30^0C and pH 6.78. Fungi-free extract served as a control. The following steps were involved in sample preparation: samples for analysis were taken on the third, fifth, and seventh days of culture growth in the extract. A paper filter was then used to

filter the samples. For additional examination, the filtrate that was produced was gathered and kept in a freezer.

Determination of quantitative content of citric acid: For quantitative determination of citric acid, the following were used: control: 5 ml of distilled water, standard solution: 10 ml of water was added to 500 mg of standard sample of citric acid (Sigma), and the studied solutions, which were a sterile extract and culture liquids. 5 ml of each sample was taken and placed in 10 ml test tubes. Then, 0.1 ml of 0.1% alcohol solution of methylene red was added to each of them. The quantitative content of citric acid was determined by titration, which was carried out with 0.5 normal sodium NaOH solution according to the following formula:

$$x\ (\%) = \frac{Ns \times V \times Ex}{q \times 10} \times 100,$$

where x is the content of citric acid (%), Ns is the normality of the titrated solution, V is the volume of the titrated substance, Ex is the equivalent amount of the test solution, E citric acid = 70.047, q is the sample of the analyzed substance [24].

Determination of organic acids using HPLC: Citric acid was determined by the reversed-phase HPLC method using UV detection. A liquid chromatograph "Waters 2695 Separations Module" (USA) with an ultraviolet detector "Waters 2487" was used. Chromatographic column for separation of organic acids "Altima C 18", 5 microns, 250×4,6 mm, was used as the stationary phase; separation of organic acids was carried out in the isocratic mode of elution, 0.5% MeOH, 0.5% CH3CN in 1000 ml of water was used as the mobile phase, the flow rate was 1 ml/min. Detection was carried out at a wavelength of 210 nm, column temperature 30 °C [25].

Isolation of citric acid from culture liquid: To obtain citric acid, the culture liquid was separated from the biomass, then chalk was added to the culture liquid, heated to 80°C in a water bath, and thoroughly mixed for 25 minutes until a precipitate of calcium citrate was formed. The end of the reaction was determined using a pH meter. The reaction was considered complete at a pH of 6.5-6.8. Then, calcium citrate was dissolved in sulfuric acid, heated for 20-25 minutes in a water bath, and mixed. After this, the mixture was centrifuged to separate the gypsum, obtaining a supernatant. The volume of the resulting supernatant was measured and placed in an incubator for evaporation at a temperature of 40°C.

The yield of citric acid: The final yield of citric acid from the supernatant was determined using the following formula:

$$Yield\ of\ citric\ acid\ (\%) = \frac{b}{a} \times 100$$

where (b) is the mass of the final crystalline citric acid powder and (a) is the quantity of citric acid generated by fungi.

Analysis of statistics: In this study, each experiment was conducted a minimum of three times. The data were analyzed using Minitab 17.1, a statistical software developed by Minitab Inc. in Pennsylvania, USA, employing a one-way ANOVA with Dunnett's test ($p<0.05$).

RESULTS AND DISCUSSION

Jerusalem artichoke tuber extract containing inulin: After adding water to the crushed tubers and a series of specific sequential mechanical and thermal treatments - heating and filtration, about two liters of aqueous extract with a pH of 6.78 were obtained. The amount of inulin was determined using Selivanov's reagent, a standard inulin curve, and a spectrophotometer. When hydrolyzing inulin with the reagent, a colored complex of red-cognac color was formed. About 6.5-7% of inulin was calculated in the extract from Jerusalem artichoke tubers.

Determination of citric acid by a new modified method: After sterilization of the obtained extract at a pressure of 0.5 atmospheres for 20 minutes, the *Aspergillus niger-1* strain was cultivated for seven days on this extract to obtain citric acid. Samples were taken on the third, fifth and seventh days of the experiment. A sterile extract from Jerusalem artichoke tubers and distilled water served as a control. To determine citric acid, 5 ml of water, extract, 5.0% citric acid solution, and culture fluid were added to test tubes. Then 0.1 ml of 0.1% alcohol solution of methylene red was added to each test tube, because of which the test tubes with the citric acid solution and culture fluid acquired a red color, the test tube with water did not change color, and the test tube with the extract, to a very small extent. This indicated the absence of citric acid in the sterile extract or its presence in trace amounts. As a result of adding sodium hydroxide solution, the solution's color shifted from red to yellow (Figure 2).

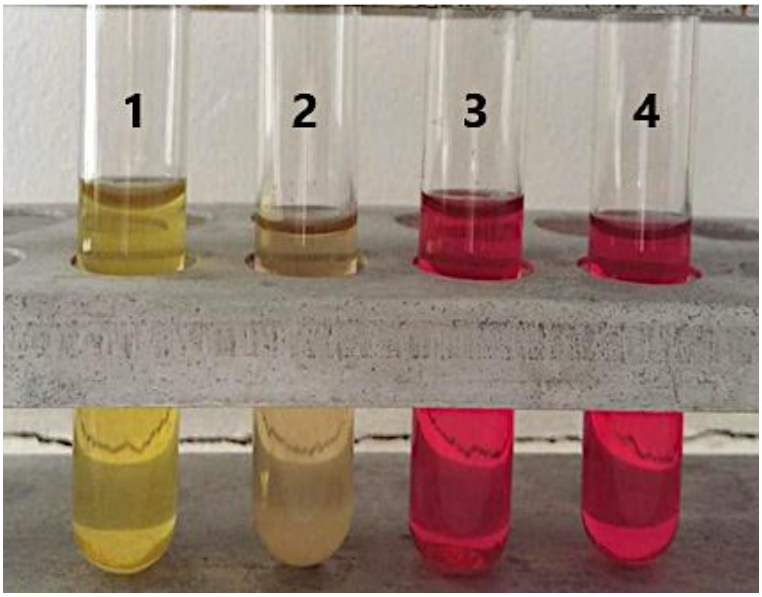


Figure 2. Determination of acidity using methylene red: 1. Distilled water 2. Sterile extract medium 3. 5.0 % citric acid solution 4. Aspergillus niger culture medium.

The research on the bioconversion of inulin-containing extract into citric acid revealed that a significant quantity of citric acid was produced by the third day of incubation, reaching 1.9%. On the fifth day of the experiment, its concentration rose to 5.8%, which is 2.9 times higher compared to the third day (Table 1). A decrease in pH from 3.5 to 2.77 occurred due to the acceleration of citric acid synthesis, due to good aeration during deep cultivation (Figure 3).

Table 1. Citric acid content in samples on different days of growth.

Sample collection day	Volume of NaOH (ml)	Amount of citric acid (%)
3	21.6	1.9
5	66.1	5.8
7	26.2	2.3
Control CA	57.0	5.0

By the seventh day of fermentation in the experiment, the citric acid content decreased by 2.5 times compared to the fifth day. The culture began utilizing citric acid as a carbon source after the exhaustion of the nutrient medium, resulting in a decrease in its concentration. Because of adequate aeration, respiration in submerged cultivation takes place more quickly than in surface cultivation, resulting in a notably earlier exhaustion of nutrients. In addition, the *Aspergillus* strain started to produce spores on the sixth day. Therefore, it can be concluded that the *A. niger* strain examined began the sporulation process as a means of survival due to the minimal sugar present in the medium (Figure 4).

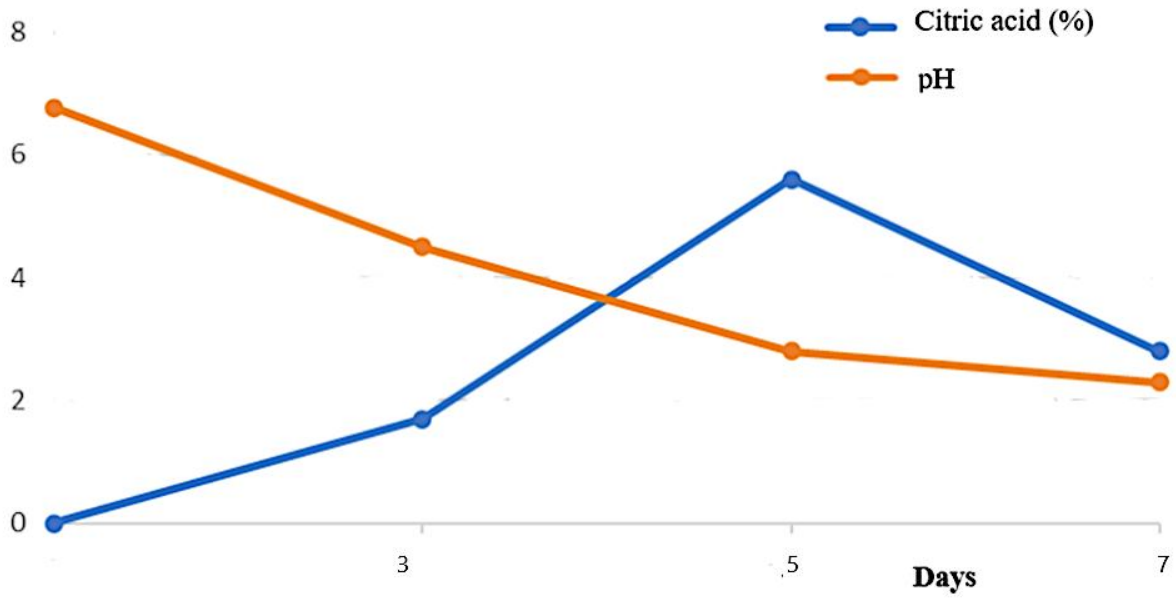


Figure 3. Amount of citric acid and pH values in samples taken on different days of growth of the strain Aspergillus niger-1.

Yield, isolation, and identification of citric acid: According to the HPLC data analysis, the highest amount of citric acid formation was observed on the fifth day of growth when the *Aspergillus niger-1* strain was grown on Jerusalem artichoke tuber extract containing approximately 6.5–7% inulin. As a result, it was calculated that the starting material, inulin, produced citric acid with a yield of about 82%. Considering this data, on the fifth day of growth of the culture, 100 ml of a sample was taken, which was filtered through paper and activated carbon, to separate the biomass from the culture liquid. As a result of filtration, 80 ml of the enzymatic solution was obtained. The pH of the solution was measured and was about 2.7-2.8. Next, the resulting filtrate volume was neutralized with chalk. The boiling point of the reaction was raised to 85°C and kept for 20 minutes, stirring thoroughly. The results showed that after adding chalk to the enzymatic solution, calcium citrate precipitate formed, accompanied by the release of carbon dioxide bubbles. As a result of the reaction, the pH value rose to 6.6.

Next, concentrated sulfuric acid was added to the resulting precipitate. The resulting mixture was heated to a boil for 30 minutes, stirring carefully. When Ca citrate was converted to citric acid, gypsum precipitated, which was removed from the reaction mixture by centrifugation, separating the supernatant liquid with citric acid. To clarify the citric acid, activated carbon was added, then filtered. The volume of filtered liquid was 35 ml.

Due to the data obtained by HPLC analysis, 4.3% citric acid was obtained from 35 ml of supernatant, the yield of which was about 74% of the amount of citric acid initially contained in the fermentation solution on the

fifth day of fungal growth. (Figure 5, Table 2). Then the supernatant was placed in a thermostat at 40°C for 3 days. After evaporation of the water mass, citric acid remained, which has the form of yellowish crystalline grains. The mass of the crystals was 3.5 g, and the degree of extraction of citric acid from the supernatant was 81%.

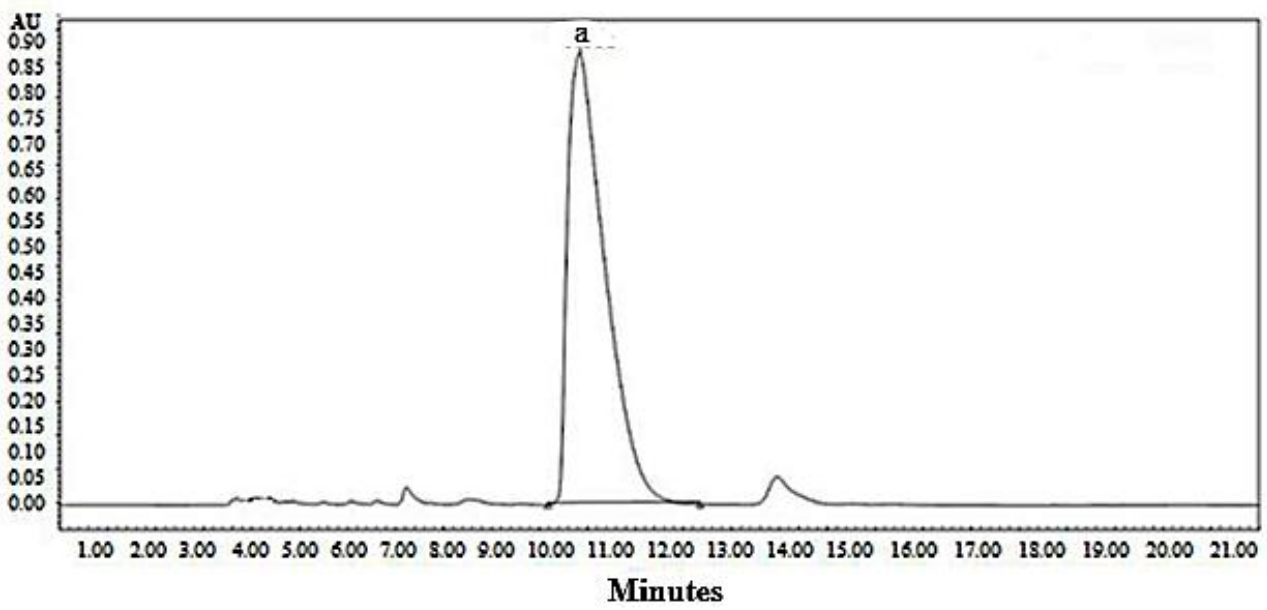


Figure 4. HPLC of organic acids in supernatant: a-citric acid.

Table 2. The content of organic acids of the supernatant.

	Name	Retention Time	Area	Height	Conc. mg/ml
1.	Citric acid	10.835	37050519	696602	43.0

DISCUSSION

This study details the quantitative analysis of citric acid in samples collected on various days during the growth of cultures of *Aspergillus niger.* Additionally, it outlines a procedure for extracting citric acid from the cultural liquid of the fungus *A. niger*, which was cultivated using an extract derived from Jerusalem artichoke tubers. Mixing 1 kg of Jerusalem artichoke tubers with water in a 1:2 ratio yields 2.0 liters of extract containing approximately 6.5% inulin. With deep cultivation of mold fungus, the maximum amount of citric acid is observed on the fourth-fifth day of growth, because of which about 5.8% is synthesized in the culture liquid, which is about 80-81% of the initial inulin-containing raw material. Ultimately, 3.5 g of milky-white crystalline powder was isolated from the supernatant, the yield of which was 60.3% of the amount of citric acid in the fermentation solution.

The scientific novelty lies in obtaining citric acid from the aqueous extract of Jerusalem artichoke tubers, which represents an alternative to the classical method of producing citric acid and differs from the method of obtaining it from beet molasses. The practical application is that in the future, its salts will be obtained citrates, intended for use in various industries. For example, the food industry uses sodium citrate as a leavening agent. Additionally, magnesium citrate is used in medicine as a drug that effectively helps with constipation.

There is much data in the literature on the synthesis, yield, and production of citric acid by various microorganisms during their growth on various raw material sources. For example, some researchers using

the *A. niger GCB-75* strain and various concentrations of cane molasses (150 g/l and 10 g/l) obtained 31.1 g/l and 25.8 g/l of citric acid, respectively [26]. Other authors presented the synthesis of citric acid using *A. tubingensis* and *A. niger* strains for the production of citric acid. Sorted kitchen waste, raw molasses, and sucrose were used as substrates. The results showed that the highest yield, namely 16.14±0.03 g/L, was obtained on the *A. tubingensis* fermentation medium containing 15% sucrose and 2.0% methanol, while 15.97±0.01 g/L was obtained on the *A. niger* fermentation medium containing the same amount of sucrose and 1.0% methanol [27]. Other authors, culturing *Aspergillus niger* on cane molasses with a sugar concentration of 15%, which was optimized with the addition of 0.4 g/l magnesium sulfate, 2 g/l potassium ferrocyanide, and 10 g/l ammonium oxalate, obtained citric acid with a yield of 78% [28]

Ling-Fei Wang et al. [28] also used an extract from artichoke tubers as a carbon source and as a microorganism, genetically modified yeast, strain *Yarrowia lipolytica 30*, and subsequently obtained 6.83% citric acid from an extract containing 8.4% of total sugars, and the citric acid yield was 81% over 336 hours. At the same time, it was found that 67.2% of the citric acid in the culture supernatant was recovered [29].

CONCLUSION

The significance of this study is that citric acid was successfully produced from a novel substrate—an extract derived from Jerusalem artichoke tubers. Given the plant's high adaptability, its capacity to thrive in diverse climates, and its minimal agricultural requirements, along with the substantial inulin content found in the tubers, we formulated a method for synthesizing citric acid through the submerged cultivation of the Aspergillus niger-1 fungus using the extract obtained from Jerusalem artichoke tubers. The maximum concentration of citric acid (5.8%) was achieved on the fifth day of culture growth, which indicates a favorable result. As a result, 3.5 g of milky-white crystalline powder was extracted from the supernatant, accounting for 60.3% of the citric acid content in the fermentation solution. Thus, a comparison of the obtained results with the available literature data suggests that the *Aspergillus niger-1* strain shows considerable promise for future applications in citric acid production. Moreover, future work is planned to increase the yield of citric acid by adjusting certain factors (such as pH, inoculum size, incubation temperature, fermentation time, etc.) [33]. The production process of sugar beet molasses, which serves as the main raw material for the microbial synthesis of citric acid, is particularly expensive and labor-intensive, whereas the extraction of citric acid from Jerusalem artichoke tubers is relatively time-efficient and requires less effort. Thus, from the above, it becomes clear that the use of Jerusalem artichoke as a new source to produce citric acid is highly relevant. Further work is also planned to obtain citric acid salts—citrates for use as food additives and antioxidants.

List of Abbreviations: *A. niger,* Aspergillus niger; MDC, Depository Center for Microorganisms; CA, Citric acid; HPLC, High Performance Liquid Chromatography; NaOH, Sodium hydroxide; pH, potential of hydrogen.

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Author contributions: Author Contributions: I.E.M: Methodology; Writing - original draft; Formal analysis; I.E.M: Investigation, Methodology; B.A.H Visualization; Writing - review and editing; M.A.M: Software; Formal analysis; L.S.M: Project administration; Conceptualization; S.Kh.S: Data curation; Validation; Investigation; G.G.O: Visualization; LSM: Investigation; M.A.M: Formal analysis; Investigation; N.S.A: Formal analysis; Supervision.

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