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Improving the citric acid production by mutant strains *Aspergillus niger* using carbohydrate-containing raw materials as a carbon source

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ABSTRACT

The demand for citric acid (CA) as a component of food products, pharmaceuticals, and cosmetics is increasing yearly. The use of adapted micro-organisms that convert naturally occurring carbohydrates into organic acids makes it possible to increase annual CA production significantly. The research aim was to study CA production by the *Aspergillus niger* strain in the medium based on carbohydrate-containing raw materials as a carbon source. We used a fermentation by *A. niger*. Starch hydrolysates were chosen as a nutrient medium. To improve the CA production of *A. niger*, multi-step mutagenesis was performed. This resulted in mutant strain *A. niger* R5/4, which had the highest acidogenic activity among the samples. The study evaluated the effect of temperature on the productivity of the mutant strains. The quantitative content of citric acid was analyzed at different incubation times (144, 168, and 192 h). The effect of the initial medium pH (4.5, 5.0, and 5.5) on acid formation was also investigated. The strain's optimum temperature, pH, and cultivation time parameters were determined. A three-factor, three-level Box-Benken design (BBD) was used to optimize CA production by *A. niger* strain R5/4 on a starch-containing medium. When assessing the impact of temperature on CA production, the ideal range was between 29 and 31 °C.

Keywords: citric acid, chemical mutagenesis, X-ray irradiation, *Aspergillus niger*, deep fermentation

INTRODUCTION

One of the limiting factors for food production development in the Republic of Kazakhstan is the low industrial processing of agricultural commodities, mainly due to insufficient technical and technological equipment in processing industries **[1]**.

Technologies for processing raw materials are sought after and advanced in global scientific practice. Deep processing is an established industry generating stable high revenues for many foreign countries **[2]**. Citric acid (CA) is the most valuable weak organic acid on the international market **[3]**. For example, the demand of the Customs Union countries is about 84 thousand tons of CA per year. Only one operating CA plant in the CIS region is located at the Skidel Sugar Refinery in the Republic of Belarus. The plant's output is 1,500 tonnes of acid per annum, with 800 tonnes for the domestic market and the remainder for export **[4]**. Thus, the production of CA by biotechnological approaches is an essential task all over the world **[3]**. Therefore, by developing CA production in the country, the enterprise producer can supply both the local market and export to neighboring countries.

The estimated market value of CA is projected to exceed $2 billion per kilo, indicating a global demand for CAs based on market trends **[4]**, **[5]**. As a result, it is essential to establish CA production in the Republic of Kazakhstan **[6]**. There are more than 100 patents in producing the CA globally **[3],** but it is essential to get the schema manufacturing given its abundance of raw materials that can be used in its production **[6]**.

The Republic of Kazakhstan has produced about 17 million tonnes of wheat grain annually for the past ten years. Deep processing of grain enables higher added value to be achieved. This is an innovative way to promote the biotechnological production development and the agro-industrial complex of the Republic of Kazakhstan. In this way, the range of valuable products obtained from grain expands, simultaneously increasing production in related industries: food, pharmaceuticals, engineering, metallurgy, construction, petrochemicals, and other sectors of the economy **[4]**, **[7]**.

There is a global trend towards using environmentally sustainable raw materials in CA production. Using various starches (potato, corn, wheat, rice) and their hydrolysates offers industrial potential **[8]**. The unique aspect of their use as a carbohydrate source for microbial CA producers is the simultaneous generation of enzymes catalyzing the hydrolysis of polysaccharides in acidic conditions during fermentation, accompanying the principal product **[9]**.

The CA accumulation process by *A. niger* in the sugar medium is directly related to substrate concentrations, requiring a high initial sugar concentration in the medium **[5]**, **[10]**, and also depends on the nature of the carbon source. Carbohydrates that are quickly metabolized are most efficient for production, with sucrose being the most optimal, followed by glucose and fructose. In contrast, galactose weakly promotes fungal growth and does not contribute to CA accumulation **[10]**.

The consumption of raw materials for CA production can show considerable variation **[11]** showed that the consumption coefficient of raw materials for producing one tonne of CA reduced from 2.7 (for beet molasses) to 1.2 (for corn and wheat starch hydrolysates).

The *A. niger* mold fungus is identified as the foremost microorganism for commercial CA production due to its higher CA production per time unit **[10]**, **[12]**.

Natural microbial strains need to produce by-products to be able to synthesize the desired product in optimal amounts easily. The most commonly used method to improve CA-producing strains is to induce mutations in parental strains using mutagens **[5]**, **[13]**. Gamma rays and ultraviolet radiation are the physical mutagens used **[14]**. UV treatment in combination with chemical mutagens like aziridine, N-nitroso-N-methylurea, ethyl methane sulfonate, and uracil is used to obtain hyperproductive strains. Fluorescent auxotrophic selection is also applied in the investigation of various promoters to obtain *A. niger* strains that are capable of synthesizing CA in higher yields **[15]** due to their ability to either inhibit or stimulate the enzymatic process **[16]**.

The study aims at improving the CA production by the wild strains of *A. niger* on medium-containing carbohydrate feedstocks as a carbon source. The emphasis has been placed on perfecting the fermentation conditions.

Scientific Hypothesis

Isolated wild local Aspergillus strains can be used in citric acid manufacturing. Mutant *A. niger* wild strain could be an efficiency producer in CA manufacturing. Carbohydrate-containing raw materials, because of the cereals processing, could be used as a base nutrition medium in CA production. This allows for introducing modern, resource-saving, and cost-effective technologies in the industrial production of organic acids.

MATERIAL AND METHODOLOGY

Samples

Wild strains of *A. niger* separated from the starch-rich mediums were checked for the possibility of CA production in native and after mutation.

Chemicals

Czapek Dox agar and Czapek Dox broth (HiMedia Laboratories LLC); indicators alizarin red and phenolphthalein (LLP Reaktivsnab, Shymkent, Kazakhstan); sucrose, methanol, ethanol, and isopropanol (LLP Reaktivsnab), chemical components as $(NH_4)_2SO_4$; K_2HPO_4; $MgSO_4 \cdot 7H_2O$, $CaCl_2$, NaOH, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $NaNO_3$; *N*-nitroso-*N*-methylurea (NMU) (LLP Reaktivsnab).

Our experiment used a *composite medium* that concluded starch hydrolysate and sucrose as the carbon sources. The fermentation medium for CA manufacturing consisted of the following ingredients: corn starch maltodextrin with a DE of 18-20%, ammonium nitrate, magnesium sulfate heptahydrate, potassium dihydrophosphate, zinc sulfate heptahydrate, ferrous sulfate heptahydrate, and copper sulfate heptahydrate. The fermentable sugar content in the medium is 140 g/l.

Animals, Plants and Biological Materials

Wild strains (Almaty Region, Kazakhstan) of *Aspergillus niger* were able to consume carbohydrate-containing raw materials as a carbon source and produce CA.

Instruments

pH-meter Mettler Toledo Seven Compact S220-Basic (Mettler-Toledo Instruments Co., Ltd., Shanghai, China); Siemens medical X-ray machine at "Scientific-Research Institute for Radiation Medicine and Ecology" (Semey, the Republic of Kazakhstan); laboratory flasks shaker KJ-201BD (Mettler-Toledo Instruments Co., Ltd.); Erlenmeyer and other flasks types, tubes, paper filters (a filter ash weight was 0.54×10^{-3} g), microbiological needles, loops, and pipettes, Petri plates.

Laboratory Methods

Qualitative analysis of the CA secretion: in hydrolysis has been checked by the yellow zone formation due to adding an acid-base indicator, alizarin red, into the medium. Strains with the most significant CA secretion were selected for the subsequent trials - quantitative screening that was conducted in sterile Czapek Dox broth consisting of 50 ml in 250 ml Erlenmeyer flasks with additional ingredients: 30 g/l sucrose; 2.0 g/l $(NH_4)_2SO_4$; 1 g/l K_2HPO_4; 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.05 g/l $CaCl_2$. After incubation for 7 days at 30 °C, culture liquid from each flask was filtered through filter paper to separate the mycelium. Then, the filter was dried, and the yield of dry fungal biomass was recorded. The supernatant was used to determine CA, and the final pH values were recorded in a sample of 2 ml of culture liquid and 200 ml of distilled water: at the first stage – It was titrated with 0.1N NaOH solution using 1% phenolphthalein as an indicator. In the second stage, pH level was measured using a pH meter.

Mutations of fungal strains: NMU was used at concentrations of 0.005% and 0.015% to induce mutants in *A. niger* **[17]**. The material was washed twice with distilled water after incubation in the mutagenic solution at 30 °C for 45 min. The spores washed from the mutagen were resuspended in water and sown from serial tenfold dilutions on Petri plates containing agarified Czapek Dox medium with the addition of the acid-base indicator alizarin red.

At the physical stage, X-ray irradiation was used as a mutagenic agent to increase citric acid synthesis by *A. niger* **[18]**. Two irradiation regimes were used: the first test series included two irradiation periods of 1.8 s duration, irradiation dose – 3.6 μSv; the second test series had three irradiation periods with time – 1.8 s, irradiation dose – 5.4 μSv. After irradiation, the spores were resuspended in water and sown from serial tenfold dilutions on Petri plates with Czapek Dox agar. Survival rate was calculated as the ratio of colonies grown after cell treatment to those grown in the control (non-exposed strain).

Description of the Experiment

Sample preparation: *Aspergillus niger* pure cultures were isolated from the soil samples in Petri plates with sterilized Czapek Dox agar according to aseptic and antiseptic conditions: incubation at 30 ºC for 3-5 days and to 7 days for obtaining the highest sporulation level.

For quantitatively checking CA production, each inoculum (conidial suspension) was inoculated with 0.5 ml Czapek Dox broth and cultivated in a 150 rpm shaker at 30 °C for 7 days. Then, 2 ml of the cultivate liquid (with 200 ml distilled water) was titred by NaOH and tested at pH level by the pH meter.

For the mutation experimental trials, seven-day *A. niger* cultures grown on Petri plates with Czapek Dox medium were used.

Number of samples analyzed: At the first stage, 17 fungi isolates were studied; after mutagenesis, 383 mutant strains and 18 strains with high acidogenic activity were tested after the mutation factors acting.

Number of repeated analyses: to get significant results, each analysis has been repeated in three times.

Number of experiment replication: 3 parallel replication measurements have been conducted for each tested parameter.

Design of the experiment: We studied the CA production process and the modification of this process by using mutations in CA-producing strains. First of all, all isolated fungi strains were checked in CA production. The best product strains were conducted under chemical and physical factors.

In the next stage, the CA-producing ability of the mutant A. niger strain has been tested as well as checking the best conditions for it.

Seed mycelium cultivation was conducted in a 750 ml flask filled with 50 ml of nutrient medium, followed by 1% conidia suspension on an orbital shaker (160 min^{-1}) and incubated at 30 °C for 36 h.

A liter of the nutrient medium was placed in a 3-liter fermenter and sown with 10 ml of growing mycelium. The flask was placed on a rocker (160 min^{-1}) and incubated for 6-7 days at 30°C. After fermentation, the fungal biomass was separated on a Buechner funnel, and the CA amount and the conversion of sugars to CA in the culture solution were determined.

Determination of fermentation factors was tested by adding different concentrations of nutrients such as $MgSO_4{\cdot}7H_2O$, KH_2PO_4, $ZnSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $NaNO_3$, and ethanol to the growth medium during fermentation. Therefore, the nutrient factors affecting CA production by *A. niger* R5/4 were investigated to optimize the composition of the nutrient medium.

The effect of different inoculum amounts on CA production has been checked. The fermentation medium was inoculated with varying amounts of inoculum – 1, 3, 5, and 7% and incubated for 7 days at 30 °C.

A Box-Benken three-factor three-level design (BBD) was used to optimize CA production by *A. niger* R5/4 strain on a starch-containing medium (14%). Temperatures of 29, 30, and 31 °C were chosen to evaluate the effect of temperature on CA yield. The CA quantitative content was analyzed at different incubation times of 144, 168, and 192 h, and the impact of initial pH on acid formation was also investigated at 4.5, 5.0, and 5.5.

Fermentation efficiency was measured by mycelial dry weight, substrate concentration, and CA concentration. Citric acid mass yield coefficient (Y_{CA}), expressed in grams of CA per gram of substrate consumed, was calculated from formula (1):

$$Y_{CA} = \frac{P}{S} \quad (1)$$

The specific culture productivity, expressed in grams of CA per gram of biomass per hour, was calculated according to formula (2):

$$q_{CA} = \frac{P}{X \cdot t} \quad (2)$$

Where:

P – the total CA amount in the culture liquid at the end of cultivation, g; S – the total amount of substrate consumed, g; t – fermentation time, h; X – biomass in the fermenter, g.

The determination of acid concentration in the fermentation medium was evaluated by titrimetric method **[19]**.

Statistical Analysis

Statistical analysis was performed using SPSS version 22 statistical software for Microsoft Excel spreadsheets. Results were presented as standard deviation (SD) of the mean values from 3 parallel measurements. Data with p <0.05 with a 95% confidence interval were considered statistically significant. The mathematical processing of the measurements was carried out using SigmaPlot 12.5 with a non-linear regression method. Mathematica 12 software was used to visualize the data obtained and calculate the optimum. Statistical testing of the model using Box-Benken design was performed using Student's t-test to analyze variance (ANOVA).

RESULTS AND DISCUSSION

Study corn and wheat starch's physicochemical properties and quality parameters

The physicochemical properties of the raw materials, especially native starches, significantly influence the final properties of maltodextrins used in CA production **[9]**, **[11]**.

The phosphates and lipids influence the functional properties of starch in its composition. The phospholipid content in starch granules is proportionally related to the amylose content, as phospholipids tend to be complex with amylose and long branches of amylopectin and affect starch solubility **[20]**. The study revealed (Table 1) that the amylose amount was higher in wheat starch than in corn starch. The amount of phosphate (0.047%), lipid (0.261%), and amylose (28.3%) prevailed in wheat starch; on the contrary, the amount of phosphate was 0.037%, lipid 0.207%, and amylose 24.7% in corn starch.

Table 1 Physicochemical properties of native starches.

Indicator	Wheat starch	Corn starch
Moisture content, %	13.2 ±0.18	13.4 ±0.17
Phosphates, %	0.047 ±0.003	0.037 ±0.002
Lipids, %	0.261 ±0.001	0.207 ±0.005
Proteins, %	0.231 ±0.04	0.253 ±0.02
Minerals, %	0.237 ±0.01	0.220 ±0.02
Amylose, %	28.3 ±0.8	24.7 ±0.7
Amylopectin, %	71.75 ±0.8	75.3 ±0.7

Note: p <0,05; Mean value ± SD from three repetitions.

Starch hydrolysates – maltodextrins with DE 18-20% obtained by enzymatic corn and wheat starch were used for the cultivation of selected *A. niger* strains (Table 2).

Table 2 Physicochemical properties of starch hydrolysates used for CA production.

Indicators	Maltodextrins	
	wheat starch	corn starch
Mass fraction of dry substances, not less %	97.3	96.2
Mass fraction of reducing agents per dry substance, %	17.8	17.7
pH	6.5	6.5
Mass fraction of total ash per dry substance, %, not less	0.16	0.11
Proteins, %	0.063	0.070
Phosphorus, per P2O5, %	0.031	0.050

Maltodextrins have been sourced, considering that the presence of high protein in starches is particularly undesirable. It causes foaming of syrups during cooking and impedes filtration, resulting in increased syrup losses **[9]**, **[11]**, **[21]**. Protein content between 0.20 and 0.25% and ash content between 0.220 and 0.262% indicate the high quality of the starch samples obtained **[11]**. We have designed a two-stage starch dextrinisation process, which is considered optimal for complete starch liquefaction and the production of high-quality maltodextrin: first stage to DE 8-11%, then attaining DE 12-19% in the second phase **[11]**.

Note that acid formation tests cannot definitively determine which acid accumulates in the medium **[9]**, **[16]**. Strains were grown on mineral broth to identify acids and to verify the results obtained by express methods. The *A. niger* A1 and *A. niger* A6 strains with the highest CA production were then tested for quantitative CA formation (Table 3). The determination was performed in culture liquid at 30±1 °C for 168 h with pH changes according to **[22]** recommendations. CA concentration in the fermentation medium was estimated titrimetrically **[23]**.

Table 3 Quantitative determination of CA in culture liquid.

No.	Strain	CA quantity, g/100ml					
		Wheat maltodextrin			Corn maltodextrin		
		72 h	120 h	168 h	72 h	120 h	168 h
1	*A. niger* A1	1.412 ±0.12	7.64 ±0.04	10.4 ±0.05	1.345 ±0.13	8.75 ±0.07	11.21 ±0.2
2	*A. niger* A6	0.815 ±0.11	5.51 ±0.26	8.05 ±0.03	0.748 ±0.10	5.6 ±0.25	8.9 ±0.05

Note: $p < 0.05$; Mean value ± SD from three repetitions.

Table 3 shows that the CA levels after 168 hours of cultivation were between 8.05 and 10.4 g/100 ml on wheat maltodextrin medium and between 8.9 and 11.21 g/100 ml on corn maltodextrin medium. This indicates that products resulting from cereal processing can be used in CA manufacturing. Therefore, a closed cycle for cereal processing can be implemented to improve the economic efficiency of CA production. As noted **[3]**, this approach can efficiently produce organic acids and meet the demands of a market that is increasingly focused on a circular economy. A schema for the production of CA using sweet potato peels was published by **[24]** and found it to be a viable and sustainable option for manufacturing. proposed using cocoa pod husks to produce CA and demonstrated its potential for the future **[5]**.

As fermentation progressed, a gradual decrease in pH (Figure 1) was observed in all experiments, indicating that CA accumulated in the medium. These data agree with those of **[25]**, who found that the initial pH of 6.5 was gradually reduced to 1.5 during fermentation in a control production medium **[22]** suggests the maintenance of a low pH (less than 2.0), as a higher pH (around 4.0) may result in gluconic acid accumulation. In addition, an intermediate acidity between 1.8 and 2.5 is recommended by **[25]**.

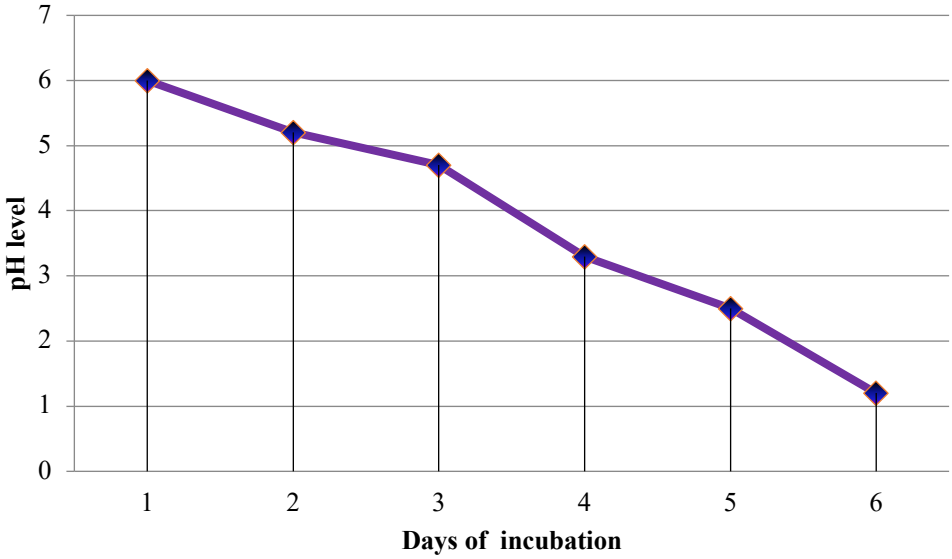

Figure 1 pH change during fermentation of *A. niger* A1.

Therefore, the *A. niger* A1 strain was selected (from now on, *A. niger* CA20).

Mutant strain production

Following one step of NMU 0.005% mutagenesis, 302 mutant lines were isolated, and following 0.015%, 81 lines were isolated. As shown, the survival rates for the strains decrease as the NMU concentration increases. A qualitative assay method based on pH change was used to select mutants with improved acid production capacity **[14]**, **[26]**

From 383 mutant strains, 18 strains with high acidogenic activity (diameter of yellow zone around colony more than 15 mm) were selected. In 211 cultures, the indices were between 8 mm and 15 mm, and 172 were less than 8 mm.

Mutant strains with the highest acidogenic rates were selected. The acidogenic activity was quantified by measuring the volume of titratable acid using 1% phenolphthalein as an indicator. Acid synthesis rates were calculated after 168 h. Analysis of the results showed that acid production increased in the mutant strains. After 168 h of cultivation, acid production ranged from 12.05 to 20.30 g/l (Table 4).

Table 4 CA yield in mutant strains after the first chemical mutagenesis step.

No.	Mutant strains	CA quantity, g/l
1	*Aspergillus niger* CA20-1(0.005)	15.80 ±0.9
2	*Aspergillus niger* CA20-2(0.005)	18.10 ±1.5
3	*Aspergillus niger* CA20-3(0.015)	15.30 ±0.2
4	*Aspergillus niger* CA20-4(0.005)	16.09 ±0.3
5	*Aspergillus niger* CA20-5(0.015)	12.80 ±2.1
6	*Aspergillus niger* CA20-6(0.015)	15.05 ±1.9
7	*Aspergillus niger* CA20-7(0.015)	12.05 ±1.5
8	*Aspergillus niger* CA20-8(0.005)	19.56 ±0.2
9	*Aspergillus niger* CA20-9(0.015)	13.40 ±0.8
10	*Aspergillus niger* CA20-10(0.015)	12.63 ±1.7
11	*Aspergillus niger* CA20-11 (0.005)	20.30 ±0.5
12	*Aspergillus niger* CA20-12(0.005)	18.90 ±2.1
13	*Aspergillus niger* CA20-13(0.005)	18.24 ±0.5
14	*Aspergillus niger* CA20-14(0.005)	15.03 ±0.9
15	*Aspergillus niger* CA20-15(0.005)	16.80 ±0.8
16	*Aspergillus niger* CA20-16(0.005)	18.70 ±0.3
17	*Aspergillus niger* CA20-17(0.005)	13.11 ±1.5
18	*Aspergillus niger* CA20-18(0.005)	15.71 ±0.4
19	Control *Aspergillus niger* CA20	11.21 ±0.2

The most excellent acid-forming ability of *A. niger* CA20-11(0.005) was reached after 7 days, resulting in 20.3 g/l CA, almost twice as high as the control wild strain. Since the spore survival rate was maximal and the mutant strains obtained had a high acid-forming ability, the recommended dosage of NMU application for further studies was 0.005%.

For the second mutagenesis step, the strains with the highest acid production rate, *A. niger* CA20-8 (0.005) and *A. niger* CA20-11 (0.005), were used. After 0.005% NMU action, 57 strains of *A. niger* CA20-8 (0.005) and 118 strains of *A. niger* CA20-11 (0.005) were isolated. Similarly, mutants were selected using a qualitative analysis method based on pH change and a quantitative analysis method. This resulted in 11 strains with 20.30 g/l activity (Table 5).

Table 5 CA yield in mutant strains after the second chemical mutagenesis step.

No.	Mutant strains	CA quantity, g/l
1	*Aspergillus niger* CA20-8(0.005)-1	22.80 ±1.2
2	*Aspergillus niger* CA20-8(0.005)-2	21.10 ±1.3
3	*Aspergillus niger* CA20-8(0.005)-3	22.30 ±0.7
4	*Aspergillus niger* CA20-11 (0.005)-4	25.09 ±0.5
5	*Aspergillus niger* CA20-11 (0.005)-5	22.80 ±1.1
6	*Aspergillus niger* CA20-11 (0.005)-6	25.05 ±0.9
7	*Aspergillus niger* CA20-11 (0.005)-7	23.05 ±0.2
8	*Aspergillus niger* CA20-11 (0.005)-8	20.56 ±0.2
9	*Aspergillus niger* CA20-11 (0.005)-9	23.40 ±0.7
10	*Aspergillus niger* CA20-11 (0.005)-10	26.63 ±0.4
11	*Aspergillus niger* CA20-11 (0.005)-11	21.30 ±0.5
12	Control *Aspergillus niger* CA20	11.21 ±0.2

As a result of these studies, a mutant strain of *A. niger* CA20-11 (0.005)-10 was obtained, which has 2.4 times the acidification capacity of the original strain.

The mutants produced by γ-irradiation are not considered genetically modified organisms, so their release into the environment after use is permitted, making biotechnological products cost-effective **[5]**, **[27]**. Using radiation achieves a high mutation probability. By exposing spores rather than mycelium to X-rays, micromycetes develop higher stress tolerance levels, minimizing unwanted side effects **[28]**.

Following radio irradiation of the *A. niger* strain CA20-11 (0.005)-10 at a dose of 3.6 μSv, 37 strains were isolated. Some of these colonies showed changes in spore color, colony growth rate, and pigment formation (Figure 2).

Figure 2 Cultural, morphological, and physiological characteristics of the strain CA20-11 after irradiation (3.6 μSv): A. Single colony at the Czapek Dox agar; B. Morphological study CA20-11 strain; magnification x40; C, D. – quality testing of the CA production – at the third (C) and seventh (D) day.

The mutation process affects the enzyme system of the mutant fungi, potentially increasing the activity of the enyzmes and improving the fungi's conforming activity or stability **[29]**. For instance, mutagen action can enhance the production of CA, which is useful in manufacturing. So, all mutant strains were tested for acidogenic activity in this way. Seven strains showed higher rates of CA formation than the parental strain (refer to Table 6). Some authors **[5]**, **[30]** found increased levels of protein engineering and CA production, respectively, in mutant strains of fungi and A. niger B6-CCT 7717.

Table 6 CA yield in mutant strains after radio irradiation (3.6 µSv) of *A. niger* CA 20-11 (0.005)-10.

No.	Mutant strains	CA quantity, g/l
1	*Aspergillus niger R3/6-1*	32.2 ±1.9
2	*Aspergillus niger R3/6-2*	26.8 ±1.1
3	*Aspergillus niger rR3/6-3*	28.1 ±0.5
4	*Aspergillus niger R3/6-4*	26.7 ±1.3
5	*Aspergillus niger R3/6-5*	33.5 ±0.2
6	*Aspergillus niger R3/6-6*	27.9 ±0.7
7	*Aspergillus niger R3/6-7*	27.3 ±0.9
8	Control *Aspergillus niger CA20-11 (0,005)-10*	26.6 ±0.4

In a second set of experiments, *A. niger* CA20-11(0.005)-10 received 5.4 µSv, which significantly reduced survival. A total of 21 mutant strains were isolated, and their CA productivity was analysed quantitatively. All strains tested showed a wide variation in CA synthesis parameters (Table 7). The highest CA yield was found in *A. niger* R5/4-8 (37.5 g/l) and the lowest in *A. niger* R5/4-11 (15.7 g/l).

Table 7 CA yield in mutant strains after radio irradiation (5.4 µSv) of *A. niger* CA 20-11 (0.005)-10.

No.	Mutant strains	CA quantity, g/l
1	*Aspergillus niger* R5/4-1	34.4 ±0.5
2	*Aspergillus niger* R5/4-2	28.7 ±1.8
3	*Aspergillus niger* R5/4-3	31.1 ±0.4
4	*Aspergillus niger* R5/4-4	33.5 ±0.2
5	*Aspergillus niger* R5/4-5	17.1 ±1.7
6	*Aspergillus niger* R5/4-6	25.5 ±1.1
7	*Aspergillus niger* R5/4-7	29.9 ±0.4
8	*Aspergillus niger* R5/4-8	37.5 ±0.7
9	*Aspergillus niger* R5/4-9	29.4 ±0.9
10	*Aspergillus niger* R5/4-10	27.5 ±1.1
11	*Aspergillus niger* R5/4-11	15.7 ±1.8
12	*Aspergillus niger* R5/4-12	22.1 ±1.3
13	*Aspergillus niger* R5/4-13	21.4 ±0.5
14	*Aspergillus niger* R5/4-14	36.1 ±0.6
15	*Aspergillus niger* R5/4-15	23.9 ±0.5
16	*Aspergillus niger* R5/4-16	19.8 ±1.1
17	*Aspergillus niger* R5/4-17	27.0 ±0.9
18	*Aspergillus niger* R5/4-18	35.1 ±0.4
19	*Aspergillus niger* R5/4-19	29.1 ±1.8
20	*Aspergillus niger* R5/4-20	34.2 ±0.5
21	*Aspergillus niger* R5/4-21	19.6 ±1.8

Thus, radio irradiation of the parental strain *A. niger* CA20-11 (0.005)-10 at a dose of 5.4 µSv resulted in *A. niger* R5/4-8 (hereafter referred to as *A. niger* R5/4) with the highest acidogenic activity (37.5 g/l).

Optimization of culture medium composition for *Aspergillus niger* R5/4

Aspergillus spp. is used in industry to produce citric acid, grow aerobically, and are heterotrophic **[16]**. The most important factors for citric acid production are fermentation time, medium pH, carbon sources, and nitrogen concentration **[24]**. These factors have a stimulating effect on the process. Therefore, we experimented to select the optimal conditions. The optimal maltodextrin-based nutrient medium composition for A. niger R5/4 with DE 18-20% was performed on a semi-pilot scale in a Labfors 5 Infors-HT fermenter (Switzerland), 3.6 l capacity with 1-2 l working volume but were took care not only of these four points and enlarged tested parameters.

Various concentrations of essential nutrients such as NH_4NO_3, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ were included in the fermentation medium to boost product yield (Table 8).

Table 8 Effect of some micronutrients on CA biosynthesis by *A. niger* R5/4.

Ingredients	Concentration	CA yield (g/l)			Biomass (g/l)		
		96 h	120 h	144 h	96 h	120 h	144 h
NH_4NO_3	1 g/l	6.46	7.52	8.75	0.75	0.95	1.30
	2 g/l	7.50	8.00	9.00	0.72	1.05	1.25
	3 g/l	7.51	8.28	9.80	0.82	1.25	1.62
	4 g/l	7.50	8.08	9.05	0.92	1.30	1.72
KH_2PO_4	2 g/l	6.32	7.01	8.62	0.68	0.92	1.20
	3 g/l	5.66	6.84	7.95	0.98	1.35	1.96
	4 g/l	6.03	7.01	8.32	0.95	1.20	1.80
	5 g/l	6.02	7.00	7.31	1.21	1.45	2.05
$MgSO_4 \cdot 7H_2O$	0.1 g/l	5.82	7.00	7.11	1.16	1.62	2.00
	0.2 g/l	4.96	6.04	6.55	1.35	1.85	2.56
	0.3 g/l	6.47	7.16	7.67	1.21	1.35	1.98
	0.4 g/l	5.27	6.14	6.79	0.99	1.30	1.91
$ZnSO_4 \cdot 7H_2O$	0.5 mg/l	6.37	7.23	8.08	1.36	1.52	2.20
	1 mg/l	6.16	7.06	8.17	0.94	1.16	1.80
	2 mg/l	5.28	6.47	7.58	1.50	2.15	2.95
	2.5 mg/l	5.24	6.45	6.56	1.34	1.75	2.28
$FeSO_4 \cdot 7H_2O$	2 mg/l	5.36	6.56	6.67	1.92	2.80	3.55
	7 mg/l	5.66	6.80	6.98	1.68	2.10	2.85
	12 mg/l	5.79	7.00	7.92	1.34	1.85	2.30
	17 mg/l	5.78	6.96	7.11	1.19	1.62	2.10
$CuSO_4 \cdot 5H_2O$	50 mg/l	5.62	5.87	6.31	1.49	1.68	1.87
	60 mg/l	6.02	6.26	6.97	1.69	1.76	1.92
	70 mg/l	6.94	7.12	7.53	1.85	2.11	2.25
	80 mg/l	6.23	6.77	7.14	1.79	2.01	2.19

Nitrogen (N_2) is a crucial factor for fungal growth as it helps to maintain the pH in the fermentation medium. **[22]** reported that high N_2 concentrations increase mycelium growth and sugar concentration, but they also note that it decreases CA production. Therefore, it is essential to maintain a balanced N_2 concentration in the medium to ensure an efficient process.

The addition of ammonium nitrate to the medium resulted in an increase in CA production in all experimental variants. The maximum bioproduct level was observed at the addition of 3 g/l after 144 h fermentation. This is explained by the fact that nitrogen is crucial for cell metabolism and a significant component of cellular proteins **[31]**. Ammonium nitrate contributed to reduced vegetative growth, while ammonium sulfate resulted in a longer vegetative growth period; the salt consumption caused a decrease in pH within the medium without producing oxalic acid. The maximum CA yield in the laboratory-stirred fermenter was obtained with an ammonium nitrate concentration of 0.2%, which correlates with the data **[31]**.

The accumulation of CA under phosphate-deficient conditions was reported by Perquin in 1938. This concept was patented by Szucs in 1944. The authors concluded that although phosphate concentration should be low, it should not be a limiting factor for CA accumulation **[32]**, **[33]** researched CA production in a chemostat culture, specifically studying the impact of phosphate-limited growth. According to their report, the citric acid output was lower in phosphate-limited steady-state conditions than in nitrogen-limited steady-state conditions. Their findings suggest that nitrogen excess had a more significant effect than phosphate deficiency.

The work by **[34]** showed that CA production by *A. niger* under phosphate-limited growth conditions is possibly suppressed by nitrogen catabolism. Fed-batch fermentation with double nitrogen and phosphorus limitation is an efficient and cost-effective method of CA production.

So, we have indicated a higher production of citric acid (8.62 g/l) when the phosphate concentration in the medium is reduced to 2 g/l, as depicted in Table 8. They are further increasing the concentration of potassium dihydrophosphate, resulting in a decrease in CA production. Adding phosphate at the maximum experimental concentration (5 g/l) reduced citric acid yield to 7.31 g/l after 144 h of cultivation using *A. niger* R5/4.

The necessity of micromycetes for metallic zinc as a micronutrient cannot be denied. Nevertheless, elevated concentrations of this metallic element show toxicity and can cause mutagenic changes in micromycetes **[35]**. The study suggests that zinc sulfate enhanced the fermentation process for the production of citric acid by *A. niger* R5/4. The highest CA production (8.17 g/l) occurred when the concentration of 1 mg/l $ZnSO_4 \cdot 7H_2O$ was

maintained for 144 h. As shown in Table 8, the use of elevated salt concentrations resulted in a decrease in CA yield during the corresponding incubation period.

Minor amounts or even small traces of copper are crucial for many microbes, such as microfungi. The copper concentration is the determinant factor in its impact on fungal growth, whether it be toxic or stimulating **[36]**. The inclusion of $CuSO_4 \cdot 5H_2O$ positively affected CA production during the 144 h synthesis based on the results obtained. The maximum amount of CA produced (7.53 g/l) was obtained with 70 mg/l CuSO4·5H2O in the fermentation medium. Conversely, a higher copper concentration (80 mg/l) was detrimental to A. niger activity, resulting in a lower CA content of 7.14 g/l.

Magnesium is essential for growth and CA production. The optimum amount of magnesium sulfate falls within the range of 0.02-0.025% **[10]**, **[22]**. According to the results obtained, the most favorable condition for the synthesis of citric acid by A. niger R5/4 is the introduction of 0.3g/l $MgSO_4 \cdot 7H_2O$ (7.67 g/l) into the medium.

One of the less well-understood requirements for cultivating A. niger to overproduce citrate is the limitation of iron concentration growth **[9]**. The direct relationship between citrate-metabolizing iron-dependent enzymes has been explained. Nonetheless, this may not always hold.

In aerobic media, bivalent iron gets converted into trivalent iron oxyhydroxide polymers (FeOOH), which are stable and exhibit low solubility in a medium, particularly at a neutral pH. Thus, despite the ubiquitous presence of iron, it is often biologically unavailable. Excess iron can also have detrimental effects, as Fe (II) can catalyze the formation of reactive oxygen species that impair cells. Consequently, microbes have implemented elaborate mechanisms to regulate iron uptake and storage within cells **[37]**. There are four recognized systems for acquiring iron in fungi: divalent iron uptake, heme decomposition, reduced iron assimilation, and trivalent iron uptake, which is mediated by siderophores. Low bivalent iron uptake is of less importance in iron-deficient media, while recent studies have investigated the haem uptake system of *A. niger*. Siderophores are small molecules microorganisms produce to sequester trivalent iron from the surrounding environment and facilitate its uptake by microbes. Their synthesis and function are crucial in microbial iron metabolism and growth **[10]**, **[37]**, **[38]**, indicating that A. niger's role as a siderophore is supported by the physiological response of increased citrate secretion under iron-limited conditions.

Our results indicate that the iron (II) sulfate addition to the culture medium at a concentration of 12 mg/l leads to a significant increase in CA secretion by *A. niger* R5/4, reaching a concentration of 7.92 g/l. At $FeSO_4$ levels above 12mg/l, CA decreases to 7.11g/l (Table 8).

The fermentation medium for *A. niger* R5/4 has been optimized using corn starch maltodextrins (DE 18-20%) as the basis, with a concentration of 140 g/l of fermentable sugars in the medium. The medium also contains NH_4NO_3 – 3 g/l, KH_2PO_4 – 2 g/l, $MgSO_4 \cdot 7H2O$ – 0.3 g/l, $ZnSO_4 \cdot 7H_2O$ – 1 mg/l, $FeSO_4 \cdot 7H_2O$ – 12 mg/l, and $CuSO_4 \cdot 5H_2O$ – 70 mg/l.

Effect of different alcohol concentrations on CA production

Among the three alcohols studied, CA production was optimized under pilot-scale conditions using maize starch hydrolysate medium with 3% ethanol after 120 h fermentation (Table 9).

Table 9 Effect of alcohol addition on CA production by *Aspergillus niger* R5/4.

Alcohol		Final pH	CA yield (g/l)	Conversion rate, (%)
Name	Concentration, %			
Control	0	3.1	3.4	4.5
Methanol	1.0	3.4	0.4	0.5
Methanol	2.0	3.8	7.1	9.8
Methanol	3.0	3.7	2.4	2.0
Ethanol	1.0	3.9	7.9	11.4
Ethanol	2.0	3.3	13.7	20.3
Ethanol	3.0	3.1	19.6	27.9
Ethanol (6 h)[2]	3.0	3.0	20.9	31.2
Ethanol (18 h)[2]	3.0	3.3	8.4	10.5
Ethanol (24 h)[2]	3.0	3.3	8.7	11.2
Ethanol	4.0	3.2	7.6	10.1
Ethanol	5.0	3.6	4.2	6.1
Isopropanol	1.0	3.9	2.1	3.1
Isopropanol	2.0	3.7	1.4	1.6
Isopropanol	3.0	3.8	0.2	0.0

A concentration of 2% of methanol led to a minor increase in CA yield, but at higher levels, both CA production and growth were significantly reduced. There was a notable reduction in the growth and productivity of citric acid upon the addition of isopropanol. The higher the concentration of alcohol, the less product was synthesized. At a concentration of 3% isopropanol, the synthesis of citric acid was greatly inhibited.

Adding ethanol has been found to enhance the excretion of citric acid, with the maximum level of formation reached at 3%. However, higher doses resulted in a decrease in product yield. Upon addition of ethanol 6 h after inoculation, the sugar conversion to citric acid was 31%. No increase in CA productivity was observed when ethanol was added after 18 and 24 hours of culture growth. Ethanol increased citrate synthase activity twofold but decreased aconitase activity by 75%, according to **[39]**. It can form acetyl-CoA, which is a precursor of citric acid. Additionally, micro-fungi utilize ethanol as a carbon source. Reduced growth and CA production at higher ethanol concentrations may be attributed to its toxic impact. So, contrary to the findings of other researchers who identified methanol as the most potent simulator, our research indicates that ethanol is the most potent inducer of citric acid biosynthesis by *A. niger* R5/4.

Effect of different *Aspergillus niger* R5/4 inoculum concentrations on CA production

Based on the results depicted in Figure 3, it is evident that the highest quantity of citric acid was obtained with a 3% inoculum.

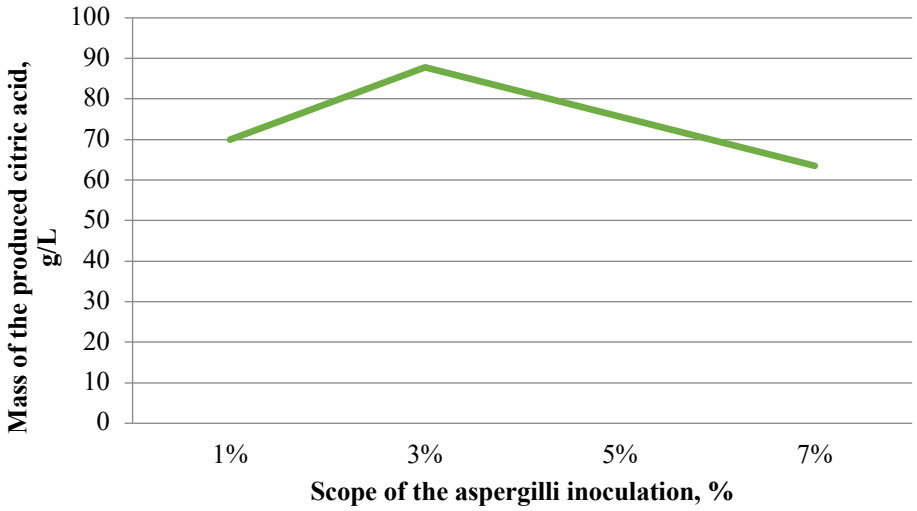

Figure 3 Effect of different inoculum concentrations on CA production.

According to the data presented in Figure 3, it is evident that the optimal CA yield was attained using a 3% inoculum concentration. The CA production peaked at a seed size of 5%, resulting in a maximum output of 83.24 g/l **[40]**. The highest CA amount was obtained with a 1% inoculum (96.86 g/l), as reported by **[41]**.

In our research on enhancing citric acid biosynthesis through *A. niger* R5/4 in a 3.6 l Labfors 5 Infors-HT bioreactor located in Switzerland, the Box-Behnken design of the experiment was used. The effect of medium acidity, incubation time, and fermentation temperature on CA production was optimized in a fermenter using a Box-Behnken design. Critical values of the medium components previously obtained in optimizing the culture medium were adopted. The optimized fermentation medium consists of corn starch maltodextrins with a DE of 18-20, with a fermentable sugar concentration of 140 g/l medium, containing the following components NH_4NO_3 – 3 g/l, KH_2PO_4 – 2 g/l, $MgSO_4 \cdot 7H_2O$ – 0,3 g/l, $ZnSO_4 \cdot 7H_2O$ – 1 mg/l, $FeSO_4 \cdot 7H_2O$ – 12 mg/l, $CuSO_4 \cdot 5H_2O$ – 70 mg/l. Previous studies have also identified the optimum 3% ethanol and 3% inoculum concentrations.

Optimization studies using the Box-Behnken design (Eq. 3) under in vitro conditions in flasks showed that the maximum CA yield occurred at a temperature regime of 30 ±1 °C, a medium acidity of pH 5.0 and an incubation time of 168 h. These physical and chemical parameters provided sufficient reference points. Citric acid yield values were obtained after 168 h of fermentation in 17 experiments (Table 10). Similar findings were published by other authors. They noted that CA production increased proportionally with incubation time until the seventh day, after which the production rate decreased. This tendency can be explained by several parameters, including decreased sugar content, the age and increased biomass of Aspergillus spp., the concentration of dissolved oxygen (important because fungi are aerobes), and the cation level in the cultivation medium **[14]**, **[17]**, **[24]**.

Table 10 Experimental *A. niger* R5/4 sowning for CA production by using a Box-Behnken design.

Experiment number	Factors					
	Coded variables			Real variables		
	X1	X2	X3	X1	X2	X3
1	-1	-1	0	29	4.5	168
2	+1	-1	0	31	4.5	168
3	-1	+1	0	29	5.5	168
4	+1	+1	0	31	5.5	168
5	-1	0	-1	29	5.0	144
6	+1	0	-1	31	5.0	144
7	-1	0	+1	29	5.0	192
8	+1	0	+1	31	5.0	192
9	0	-1	-1	30	4.5	144
10	0	+1	-1	30	5.5	168
11	0	-1	+1	30	4.5	192
12	0	+1	+1	30	5.0	192
13	0	0	0	30	5.0	168
14	0	0	0	30	5.0	168
15	0	0	0	30	5.0	168
16	0	0	0	30	5.0	168
17	0	0	0	30	5.0	168

$$Y = -1529.9 + 1615.1X31 + 70.19X2 + 58.7X3 - 1.25X1X2 - 8.62X1X3 - 0.41X2X3 - 1501.3X2.1 - 1.68X2.2 - 0.87X2.3 \quad (3)$$

Where:

Y – CA yield; *X1*, *X2* and *X3* - coded variables of temperature, medium acidity, *pH* and incubation period, respectively (Table 11).

Table 11 Box-Behnken design: evaluating the effects of medium parameters on CA production in a fermenter.

Medium parameters	Regression	*t*	*p*
Mean value/interaction	−1529.9	−5.46	0.003
(1) Temperature (°C) (L)	1615.1	4.24	0.006
Temperature (°C) (Q)	−1501.3	−5.37	0.003
(2) Medium acidity, pH (L)	70.19	5.396	0.003
Medium acidity, pH (Q)	−1.68	−5.561	0.002
Incubation period (h) (L)	58.7	3.516	0.018
Incubation period (h) (Q)	−0.87	−2.756	0.041
1 L k 2 L	−1.25	−0.133	0.878
1 L k 3 L	−8.6	−0.988	0.375
2 L k 3	−0.41	−1.267	0.259

The regression analysis calculation results in a coefficient of determination (R2 = 0.959), demonstrating that only 3.35% of the total variation remains unexplained by the model. The adjusted coefficient of determination (adj. R2 = 0.899) is similarly high, signifying strong significance of the model.

The statistical design analysis shows high accuracy of the polynomial model, reflecting a high degree of agreement between predicted and experimental data. Response surface plots as a function of two factors simultaneously, with all other factors held at fixed levels (e.g. zero), can be easily obtained by calculating from the model and taking variables for one factor as the other is varied (from -1.0 to +1.0, e.g. a step of 0.5), with this value of Y constrained. The corresponding response surface plots can also predict the productivity values for different variable concentrations (Figure 4).

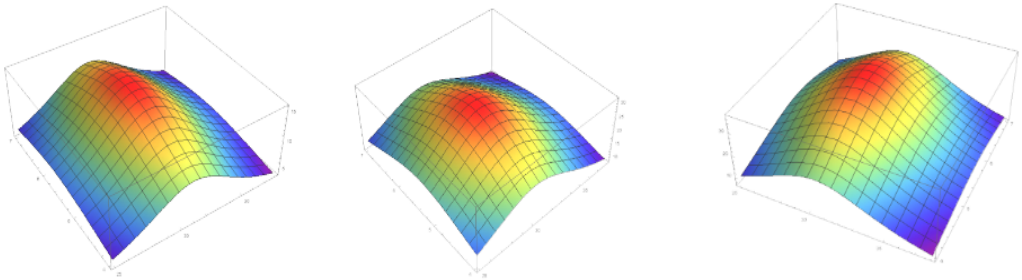

Figure 4 Response surface curve for CA production in the fermenter: A. – represents the interaction between temperature and medium pH; B – represents the interaction between temperature and fermentation period; C – represents the interaction between medium pH and fermentation period.

The surface plot illustrates the separate impact of the variables and their combined effect on the response, stemming from the two test variables in question. Note that the preferred parameters for the optimal process of CA production by *A. niger* R5/4 by deep fermentation for 168 h are: temperature 30.4 °C and initial medium pH 5.2. This information is consistent with previous research but provides a clearer range. Previous studies have shown that a medium pH above 6.0 is not ideal for CA production **[24]**, which is supported by the findings **[22]**. Our experiment provides more detailed data and confirms that a pH of 5.2 is the best for synthesizing CA.

The fermentation process's productivity was assessed by computing fermentation efficiency ratios after 168 h (Table 12).

Table 12 Fermentation process productivity indicators.

Experiment number	Fermentation efficiency coefficients		CA content, g/l	Conversion rate, %
	Product yield from the substrate, $Y_{P/S}$	Specific product formation rate, q_P		
1	0.52 ±0.20	0.07 ±0.5	88.1 ±1.3	62.9
2	0.68 ±0.12	0.08 ±0.2	99.5 ±0.4	71.1
3	0.68 ±1.3	0.04 ±0.5	84.14 ±1.2	60.1
4	0.70 ±0.58	0.06 ±0.5	97.7 ±1.0	69.8
5	0.62 ±0.3	0.07 ±0.10	93.0 ±0.2	66.4
6	0.55 ±0.05	0.05 ±0.26	64.12 ±2.1	45.8
7	0.69 ±0.02	0.09 ±0.04	99.7 ±0.3	71.2
8	0.59 ±0.68	0.08 ±0.61	97.6 ±1.9	69.7
9	0.66 ±0.72	0.05 ±0.02	68.3 ±0.5	48.6
10	0.64 ±0.48	0.07 ±0.98	81.5 ±0.8	58.2
11	0.70 ±0.21	0.08 ±0.16	85.8 ±0.9	61.3
12	0.73 ±0.13	0.09 ±0.07	96.3 ±1.2	68.8
13	0.92 ±0.29	0.11 ±0.03	117.3 ±0.5	83.8
14	1.01 ±0.04	0.12 ±0.05	117.2 ±0.8	83.73
15	0.95 ±0.1	0.11 ±0.1	117.2 ±0.8	83.7
16	1.01 ±0.01	0.12 ±0.02	120.6 ±1.2	86.15
17	1.1 ±0.99	0.11 ±0.01	119.56 ±0.8	85.4

Therefore, a Box-Behnken design with three variables - temperature, pH, and fermentation time - was used to optimize the production of citric acid in a fermenter using an optimized corn hydrolysate culture medium with the following composition: sugars - 140 g/l, NH_4NO_3 – 3 g/l, KH_2PO_4 – 2 g/l, $MgSO_4 \cdot 7H_2O$ – 0.3 g/l, $ZnSO_4 \cdot 7H_2O$ – 1 mg/l, $FeSO_4 \cdot 7H_2O$ – 12 mg/l, $CuSO_4 \cdot 5H_2O$ – 70 mg/l. The micro-mycete *A. niger* R5/4 produces the highest amount of citric acid by deep fermentation at a temperature of 30.4 °C, a pH of 5.2, and an incubation period of 168 h. Accordingly, the specific product formation rate (CA) was 0.12 Qp, and the conversion rate reached 86.15%.

CONCLUSION

The study demonstrated that multi-step mutagenesis yields *A. niger* strains with high levels of CA production. The fermentation medium for *A. niger* R5/4 has been optimized. The medium base consisted of corn starch maltodextrins with DE 18-20 and a fermentable sugars concentration of 140 g/l. The medium contained the following components: NH_4NO_3 – 3 g/l, KH_2PO_4 – 2 g/l, $MgSO_4 \cdot 7H_2O$ – 0.3 g/l, $ZnSO_4 \cdot 7H_2O$ – 1 mg/l, $FeSO_4 \cdot 7H_2O$ – 12 mg/l, $CuSO_4 \cdot 5H_2O$ – 70 mg/l. Adding ethanol increased citric acid excretion, with the highest bioproduct obtained at a concentration of 3%. The sugars were converted into CA at a rate of 31% when ethanol was added 6 h after the inoculation. Additional ethanol supplementation at 18 and 24 h of culture growth did not increase CA production. The conclusion from our study is that the inoculum concentration has a significant effect on the level of external citrate accumulation. The highest amount of citric acid was achieved with a 3% inoculum. A Box-Benken design was used to optimize CA production in a fermenter using an optimized culture medium involving three variables: temperature, pH, and fermentation time. At a temperature of 30.4 °C, with a medium pH level of 5.2 and an incubation period of 168 h, *A. niger* R5/4, a micromycete, produces the highest CA concentration through deep fermentation procedures. These findings also demonstrated that the specific rate of product formation was 0.12 qP, and conversion rates reached 86.15%.

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