REPUBLIC OF TÜRKİYE

ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOENGINEERING

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ECE KAYA

MASTER OF SCIENCE

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I hereby declare that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all information that is not original to this work.

[Signature]

Ece KAYA

ABSTRACT

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Ece KAYA

Department of Bioengineering

Supervisor: Assoc. Prof. Dr. Hatice İmge OKTAY BAŞEĞMEZ

April 2023, 68 pages

Citric acid, which is used as an additive in many industries, including food, beverage, and pharmaceutical, is commercially produced from microorganisms by fermentation. Although many types of bacteria, yeast, and fungi are used in the production of citric acid, the most preferred species in industrial production are *Aspergillus niger* species, also known as the black mold. Carbon sources with high sugar content are preferred in fermentation in the production of citric acid. Therefore, especially fruit wastes are suitable substrates for fermentation. Grape pomace, which is an important waste of wine and fruit juice factories, has a sugar content of 50-80%. For this reason, dried and ground grape pomace was chosen as the substrate in this study. In order to obtain citric acid in high yield, the fermentation parameters must be optimized. However, testing all parameters is a negative situation in terms of time and cost. For this reason, the optimum conditions of the parameters can be determined by some statistical methods. In this study, the optimum conditions of substrate concentration, initial pH, fermentation temperature, and time were investigated using the Response Surface Methodology Box Behnken Design. 30-120 g/L for substrate concentration, 2-8 for pH, 25-35^{0}C for fermentation temperature, and 48-168 hours for fermentation time were determined for minimum and maximum values. Optimum conditions for substrate concentration, initial pH, fermentation temperature, and time were determined as 120 g/L, 5, 30^{0}C, and 168 hours respectively, and the citric acid concentration was found to be 7.57 g/L. The statistical analysis showed that the created model was significant and the R^2 was found to be 0.936.

Keywords: *Aspergillus niger*, Box Behnken Design, citric acid optimization, grape pomace

ÖZET

***Aspergillus niger* TARAFINDAN SİTRİK ASİT ÜRETİMİ İÇİN ÜZÜM SUYU ATIKLARININ DEĞERLENDİRİLMESİ**

Ece KAYA

Biyomühendislik Anabilim Dalı

Danışman: Doç. Dr. Hatice İmge OKTAY BAŞEĞMEZ

Nisan 2023, 68 sayfa

Gıda, içecek ve ilaç başta olmak üzere birçok endüstride katkı maddesi olarak kullanılan sitrik asit, ticari olarak mikroorganizmalardan fermantasyon yoluyla üretilmektedir. Sitrik asit üretiminde birçok bakteri, maya ve mantar türü kullanılsa da endüstriyel üretimde en çok tercih edilen tür siyah küf olarak da bilinen *Aspergillus niger* türleridir. Sitrik asit üretiminde fermantasyonda şeker oranı yüksek karbon kaynakları tercih edilmektedir. Bu nedenle özellikle meyve atıkları fermantasyon için uygun substratlardır. Şarap ve meyve suyu fabrikalarının önemli bir atığı olan üzüm posası %50-80 oranında şeker içermektedir. Bu nedenle bu çalışmada substrat olarak kurutulmuş ve öğütülmüş üzüm posası seçilmiştir. Yüksek verimde sitrik asit elde etmek için fermantasyon parametrelerinin optimize edilmesi gerekmektedir. Ancak tüm parametrelerin test edilmesi zaman ve maliyet açısından olumsuz bir durumdur. Bu nedenle bazı istatistiksel yöntemlerle parametrelerin optimum koşulları belirlenebilir. Bu çalışmada, substrat konsantrasyonu, başlangıç pH'ı, fermantasyon sıcaklığı ve süresinin optimum koşulları, Yanıt Yüzey Metodolojisi Box Behnken Tasarımı kullanılarak incelenmiştir. Minimum ve maksimum aralıklar substrat konsantrasyonu için 30-120 g/L, pH için 2-8, fermantasyon sıcaklığı için 25-35^{0}C ve fermantasyon süresi için 48-168 saat olarak belirlenmiştir. Substrat konsantrasyonu, başlangıç pH'ı, fermantasyon sıcaklığı ve süresi için optimum koşullar sırasıyla 120 g/L, 5, 30^{0}C ve 168 saat olarak belirlenmiş ve sitrik asit konsantrasyonu 7.57 g/L olarak bulunmuştur. İstatistiksel analiz oluşturulan modelin anlamlı olduğunu göstermiş ve R^2 0.936 olarak bulunmuştur.

Anahtar Kelimeler: *Aspergillus niger,* Box Behnken Design, sitrik asit optimizasyonu, üzüm posası

Dedicated to my lovely sister, my gorgeous mother and my precious father

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NOMENCLATURE

A. niger	: *Aspergillus niger*
ANOVA	: Analysis of Variance
BBD	: Box-Behnken Design
CCC	: Circumscribed Design
CCD	: Central Composite Design
CCF	: Face Centered Design
CCI	: Inscribed Design
$FeSO_4.7H_2O$	: Ferrous sulphate heptahydrate or Iron(II) sulphate heptahydrate
H_3PO_4	: Phosphoric acid
HCl	: Hydrochloric acid
HPLC	: High Performance Liquid Chromatography
IUPAC	: International Union of Pure and Applied Chemistry
KCl	: Potassium chloride
KH_2PO_4	: Potassium dihydrogen phosphate
$MgSO_4.7H_2O$	: Magnesium sulphate heptahydrate
$NaNO_3$	: Ammonium nitrate
NaOH	: Sodium hydroxide
PDA	: Potato Dextrose Agar
RSM	: Response Surface Methodology
spp.	: Species (plural)

1. INTRODUCTION

Citric acid, a weak organic acid, is used in many industries. It is used as an additive in the pharmaceutical, cosmetic, and detergent industries, especially in the food and beverage industry. The need for citric acid, which is used as a preservative and acidity regulator, especially in the food and beverage industry, is increasing every year in the world. It is also used as a sweetener in beverages, as a neutralizer and buffer in pharmaceuticals and cosmetics, and as a chelator in metals (Soccol et al., 2006).

There are many production facilities to meet the need for citric acid, which plays an important role in many industrial sectors. China ranks first in citric acid production, meeting about 320 million tons of global citric acid need (Imarc Group, 2022). The United States, Belgium, and England are among the leading countries in the production of citric acid outside of China (Wang et al., 2020). It started to produce citric acid in Türkiye in 2020 and became one of the countries that meet the citric acid needs of both especially Türkiye and European countries.

Citric acid can be produced by chemical means as well as by fermentation of microorganisms. After the discovery of citric acid production from microorganisms, industrial citric acid production is carried out by fermentation methods. In the energy production metabolism of microorganisms, citric acid, which is naturally released in the Krebs cycle, is obtained by the manipulation of the process. Since the production of citric acid from microorganisms is a low-cost and fast process, it is preferred in large-scale industrial production (Max et al., 2010).

Yeasts, bacteria, and molds can be used for the production of citric acid. *Yarrowia lipolytica* (yeast), *Candida* sp. (yeast), and *Aspergillus* sp. (mold) are some of the microorganisms used for the production of citric acid. Especially *Aspergillus niger* (*A. niger*) species are the most preferred microorganisms in terms of citric acid production. *A. niger* is preferred in the industry due to its features such as rapid growth, wide breeding temperature, and high product yield (Soccol et al., 2006).

In the production of citric acid from microorganisms, substrates such as corn pulp, fruit waste, sugar cane, and molasses are used as carbon sources. The production of citric acid from microorganisms is especially important for the evaluation of wastes (Abbas et al., 2016). In this way, both the rapidly increasing citric acid need of the world can be met and the wastes with high environmental damage can be evaluated. Especially fruit waste is a valuable resource due to its high sugar content. Grape wastes generated in the fruit juice and wine industry disrupt the pH balance of nature and adversely affect the quality of soil and water. Therefore, the evaluation of these wastes is very important for the natural environment (Morseletto, 2020). *Aspergillus* sp. are organisms that love sugar very much and consume high sugar in their metabolic activities and used in the evaluation of grape wastes with high sugar content.

Optimizing processes is very important in terms of time and cost in the industry. Thousands of trials may be required to achieve process optimization. However, nowadays, by using computer technology for process optimization, the number of trials reduced considerably, thus saving both time and money. Response Surface Methodology (RSM) is most commonly used for process optimization. An experimental design is created by determining the ranges of parameters to be optimized with RSM. The results of the studies carried out according to the given experimental design are entered into the statistical analyses program used and calculations are made according to the importance of the data, and the optimum conditions of the process are determined. The determined optimum values are then supported by experiments and scaled for industry (Dean et al., 2017).

Grape pomace, which is a valuable waste of fruit juice and wine factories, was used in this study. According to the United Nations Food and Agriculture Organization (FAO) 2013 data, approximately 77 million tons of grapes are produced. According to the Fresh Fruit and Vegetable Industry report (2017) published by the Republic of Türkiye Export General Directorate, Department of Agricultural Products, grape production in Türkiye is approximately 203 thousand tons. Grape juice production is expected to increase by 3% until 2024 (Technavio, 2020). Wine production is expected to increase by approximately 4.3% annually until 2028 (Fortune Business Insights, 2020). This means that the industrial waste rate of grape pomace will increase. These wastes should be evaluated due to both material damage and environmental damage. Due to its high sugar content, it can act as an important substrate in the production of microbial fermentation products.

In this master thesis, optimization and production of citric acid from *A. niger* strain was carried out using grape pomace due to its high sugar content. Box-Behnken Design method, a type of Response Surface Methodology, was preferred for optimization, and the concentration of grape pomace to be added to the medium, initial pH of the medium, fermentation temperature, and time were optimized. This study is a preliminary study and was carried out in 250 mL flasks in 100 mL medium. Scale-up studies with bioreactors are required to improve the study and optimize it for industrial production.

2. LITERATURE REVIEW

2.1. Citric Acid

Citric acid, which has a closed formula of $C_6H_8O_7$ and is named 2-hydroxy-1,2,3-propane tricarboxylic acid according to the IUPAC nomenclature, is derived from the Latin word "citrus" (Cavallo et al., 2017; Vandenberghe et al., 2017). The molecular structure of citric acid is shown in Figure 2.1.1.

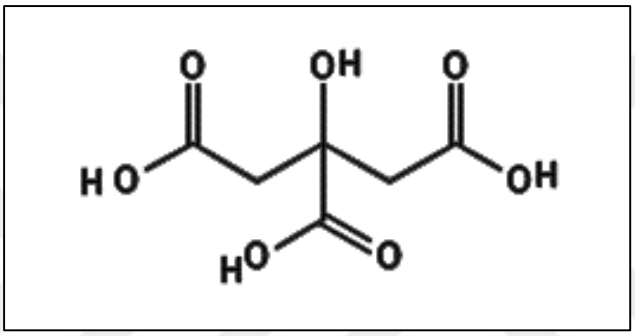


Figure 2.1.1. Citric acid molecular structure
(created on https://pubchem.ncbi.nlm.nih.gov//edit3/index.html)

Citric acid was first discovered by the 8th century alchemist Cabir bin Hayyam. Swedish chemist Carl Wilhelm Scheele obtained it for the first time in crystallized form from lemon juice in 1784 (Vandenberghe et al., 2018; Wang et al., 2019). For industry, the production of citric acid from lemon juice continued until the early 20th century.

Wehmer (1893) discovered that *Penicillium* sp. (old name, *Citromyces*) was capable of producing citric acid in a medium containing sugar and inorganic salts. After this discovery, the ways of citric acid production from different microorganisms began to investigated. Curie (1917) has shown that *Aspergillus niger* continues to grow in environments containing sugar and inorganic salts at low pH and produce citric acid with high efficiency in this process (Max et al., 2010).

Through these historical developments, the production process of citric acid started with fermentation rather than chemical means and pioneered the industry to become what it is today.

Citric acid is found naturally in many plants, especially in citrus products such as oranges and lemons. It is known that there is approximately 8% citric acid in the dry matter of lemons and its varieties (Cavallo et al., 2017; Vandenberghe et al., 2018).

2.1.1. Citric Acid Chemical Properties

The molecular weight (M_W) of citric acid is 192.124 g/mol. Its density is 1665 g/mL. The melting temperature of citric acid is 153°C and its boiling temperature is 310°C. The thermal degradation temperature is known as 175°C (Cavallo et al., 2017; Vandenberghe et al., 2017). Citric acid has three carboxyl groups. Hence, it has been reported that it has three ionization constants, 3.13, 4.76 and 6.39 (Table 2.1.1.1.) (Show et al., 2015).

There are two different solid forms in the commercial production of citric acid: monohydrate and anhydrate. The anhydrate form of citric acid was determined by Bennett and Yuill in 1935, while the monohydrate form was determined by Burns and Iball and Roelofsen and Kanters (Apelblat, 2014). Moreover, the production of liquid citric acid is also involved in the industry. The monohydrate form of citric acid is converted to anhydrate form by removing the water in it at 78°C (Cavallo et al., 2017).

Table 2.1.1.1. Citric acid chemical properties

Formula:	$C_6H_8O_7$
IUPAC name:	2-hydroxy-1,2,3-propane tricarboxylic acid
Molecular weight (M_W):	192.124 g/mol
Density:	1.665 g/mL
Melting temperature:	153°C
Boiling temperature:	310°C
Thermal degradation temperature:	175°C
Ionization constant:	3.13 4.76 6.39
Forms:	Citric Acid Anhydrous (solid) Citric Acid Monohydrous (solid) Liquid citric acid

Citric acid has no colour and odour, its taste is sour. It is soluble in water. Citric acid has the GRAS (Generally Recognized as Safe) status in the USA (Ciriminna et al, 2017). It does not have any negative effects on the environment and animal health. However, it can be irritating in direct contact with skin or eyes.

2.1.2. Citric Acid in Industry

Citric acid ionizes in aqueous solutions and converts sodium, calcium, etc. It forms various salts with ions and these salts are used in various industries (Cavallo et al., 2017). According to the European Union food content code; on food and beverage packaging labels, E330 citric acid, E331 sodium citrate and E332 represents potassium citrate (Ciriminna et al, 2017).

Citric acid is especially used in the food industry and beverage industries. While 51% of the produced citric acid is used in the beverage industry, 18% is used in the food industry. This rate is 16% in detergents and soaps, and 8% in the pharmaceutical and cosmetic industry (Figure 2.1.2.1.) (Kamzolova and Morgunov, 2017; Ciriminna et al., 2017; Wang et al., 2020).

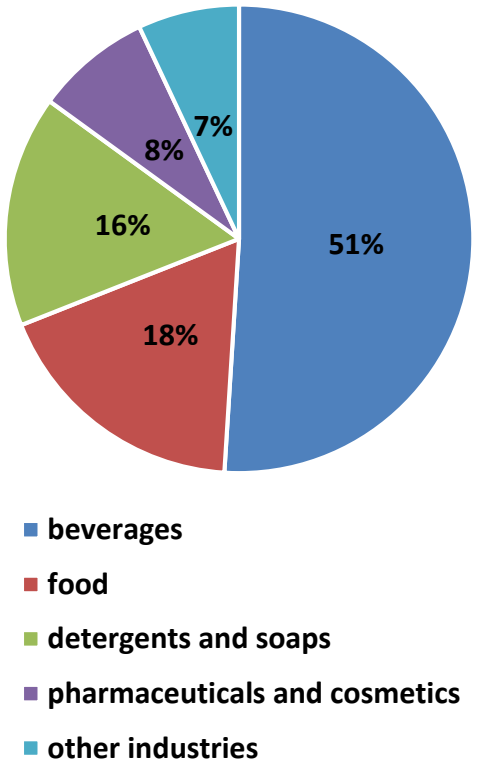


Figure 2.1.2.1. Industrial uses of citric acid

Citric acid is used for various purposes in different industries as given in Table 2.1.2.1. Citric acid is used as acidity regulator and flavor enhancer in foods and beverages, while it is used as a neutralizing buffer in pharmaceutical and cosmetic products. Also, it shows antioxidant and protective properties in food, beverages, and drugs. It has the feature of increasing the acidic taste in soft drinks. At the same time, due to its chelating feature of metal ions, many foods also help inhibit oxidative enzymes and prevent spoilage in foods such as wine, frozen fruits, and dairy products. It also inverts the sucrose in candies, preventing its crystallization and increasing the flavor (Soccol et al., 2006; Max et al., 2010). Furthermore, citric acid is used in many industries from photography to construction (Vandenberghe et al., 2017; Soccol et al., 2006). Recently, harmful chemicals found in detergents have been replaced by compounds that are not harmful to the environment and health, and therefore citric acid is preferred in cleaning products. (Nangare et al., 2021).

Table 2.1.2.1 Industrial applications of citric acid

INDUSTRY	APPLICATIONS	REFERENCES
FOOD AND BEVERAGES	Antioxidant in prepared and tinned food	Vandenberghe et al., 2017; Soccol et al., 2006
	pH stabilizer in jam, gelatins etc.	Vandenberghe et al., 2017; Soccol et al., 2006
	pH adjustment in soft drinks, candies etc.	Vandenberghe et al., 2017; Soccol et al., 2006
	Antimicrobial in fruit juice	Nangare et al., 2021; Ciriminna et al., 2017
	Enhancing the flavors and aromas of fruit juices, ice cream, and marmalades	Behera et al., 2021; Max et al., 2010
	Preserver in food and beverages	Behera et al., 2021; Max et al., 2010
PHARMACEUTICAL, DETERGENTS AND COSMETİCS	Binding agents in biodegradable detergents	Vandenberghe et al., 2017; Soccol et al., 2006
	pH stabilizer in tablets and detergents	Vandenberghe et al., 2017; Soccol et al., 2006
	Effervescent in powders and tablets	Vandenberghe et al., 2017; Vandenberghe et al., 1999
	Improve the taste of syrups	Nangare et al., 2021; Swain et al., 2011
	Disinfectant against several viruses	Nangare et al., 2021
OTHER INDUSTRIES	Remove metal oxides from the surface of objects of ferrous and other metals	Vandenberghe et al., 1999
	Cross-linking of different other materials for biomedical applications	Nangare et al., 2021
	Adhesive for wood	Umemura et al., 2012
	Enhancing drought and thermo tolerence in plants	Xie et al.,2022; Sadak et al., 2015

It is known that the production of citric acid is more than 2 million tons every year, and the increase in demand is about 5% compared to the previous year. Citric acid production reached 2.1 million tons in 2016 (Kirimura & Yoshioka, 2019). According to global market research,

citric acid production reached 2.7 million tons in 2021. This rate is expected to reach 3.2 million tons with an increase of 3.3% until 2027 (Figure 2.1.2.2.) (Imarc Group, 2022).

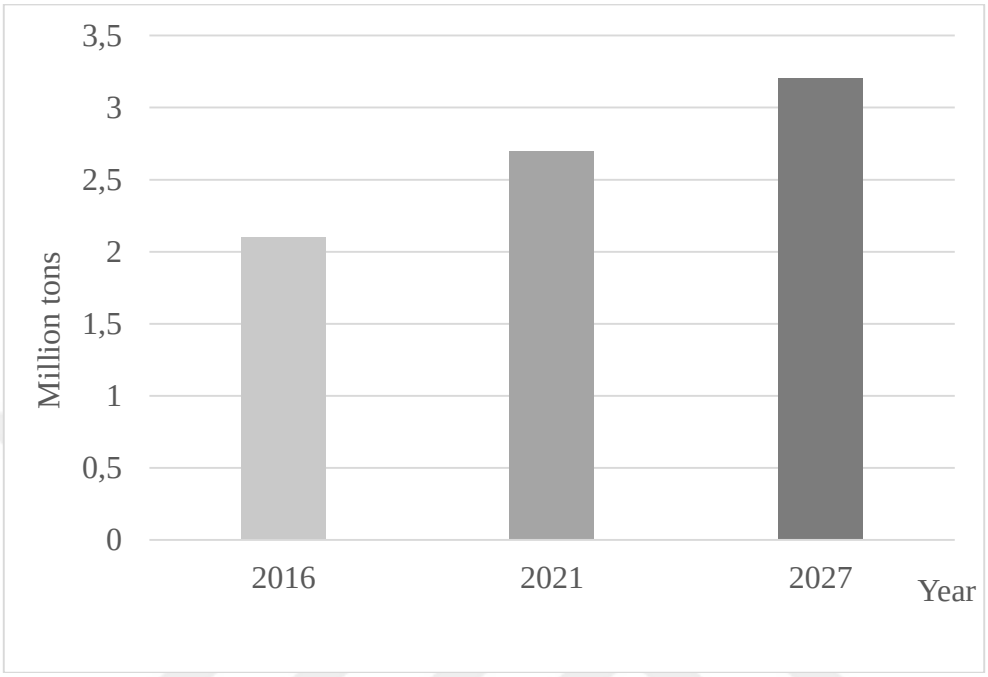


Figure 2.1.2.2. World citric acid production

China, the USA, England, Switzerland, and Belgium are leading countries in the production of citric acid (Wang et al., 2020). With a production of 1.3 million tons, China meets more than 50% of the world's citric acid needs (Zang et al., 2017). In addition to these countries, there are many producing countries. With Türkiye's entry into the citric acid market in 2020, the importance of citric acid production has increased for Türkiye. Its contribution to the country's economy is quite high with its domestic and international exports.

According to the World Commodity Export and Import (HS) report published in November 2022, citric acid imports for China in 2021 are approximately 8,773,972 US dollars, while exports are 1,082,754,354 US dollars. Türkiye's citric acid import is 40,662,708 US dollars (TrendEconomy, 2022). According to the Essential Chemicals Report published in March 2021, citric acid exports increased from US$ 347,196.72 in the January-March 2020 period to US$4,047,327.96 in the January-March 2021 period, increasing by 1065.72%. According to the March 2022 report, it reached US$ 13,226,876.43 in the January-March 2022 period, increasing by 226.8 percent compared to the previous year (Figure 2.1.2.3.).

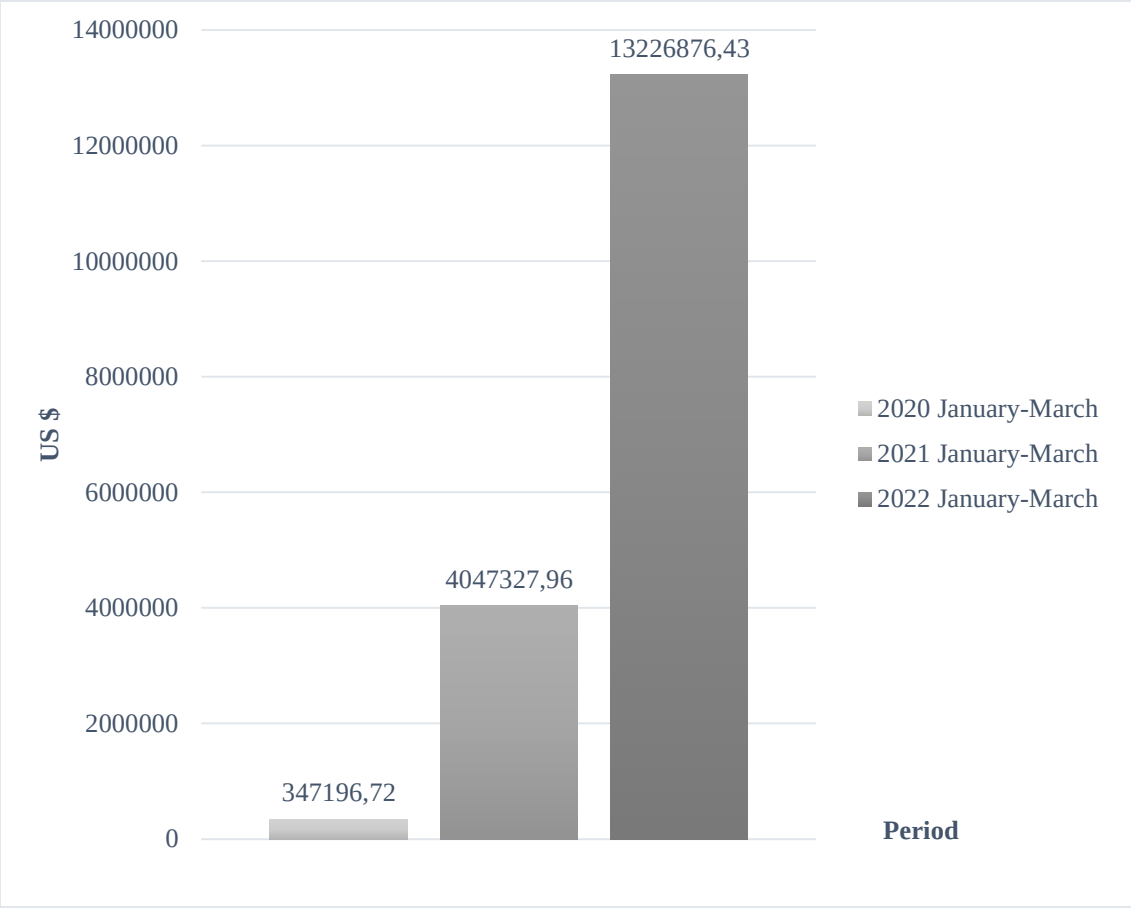


Figure 2.1.2.3. Citric acid Türkiye export revenue 2020, 2021 and 2022 January-March period

Citric acid synthesis can be realized by chemical means as well as by fermentation of microorganisms. Fermentation is generally preferred for the industrial production of citric acid production. Various bacteria, yeasts and molds are used in the production of citric acid. *Candida* sp., yeast species such as *Yarrowia lipolytica*, molds such as *Penicillium* sp., *Aspergillus* sp., some *Bacillus* sp. are some of the microorganisms used in the production of citric acid (Table 2.1.2.2.)

Table 2.1.2.2. List of microorganisms that produce citric acid

Microorganisms		References
Bacteria	*Corynebacterium* spp.	Uzah et al., 2020; Vandenberghe et al., 1999
	Bacillus licheniformis	Uzah et al., 2020; Vandenberghe et al., 1999
	Bacillus subtillis	Uzah et al., 2020
	Arthro- bacter paraffinens	Uzah et al., 2020; Vandenberghe et al., 1999
Fungi	*Aspergillus niger*	de Oliveira et al., 2022; Ajala et al., 2020, Kareem & Rahman, 2013; Imandi et al., 2008; Lotfy et al., 2007; Ikram-Ul, 2004
	Aspergillus foetidus	Cheni 1996
	Trichoderma viride	Makut & Ekeleme, 2018
Yeast	*Yarrowia lipolytica*	Kamzolova & Morgunov, 2017; Rywińska et al., 2010; Papanikolaou et al., 2009; Papanikolaou, 2008
	Candida tropicalis	Afolabi et al., 2018; Max et al., 2010
	Candida lipolytica	Rane & Sims, 1993
	Candida oleophila	Anastassiadis et al., 2002

Aspergillus niger species are the most preferred microorganisms for citric acid production. *A. niger* is preferred in the production of citric acid by fermentation due to its rapid cultivation, its ability to grow even at low pH, its growth in wide temperature ranges, and its low cost (Show et al., 2015; Ali & Daud, 2011; Vandenberghe, 1999).

2.2. Aspergillus niger

Aspergillus species (*Aspergillus* spp.), which are among the most common fungal species in the world, can grow even in wide temperature ranges such as 6-55°C and in environments with low humidity (Williams & Hallsworth, 2009). They are among the fungi that cause food spoilage. Although they generally use polymers from plants as substrates, they feed on many substrates, including human tissue. *Aspergillus* spp. are among the most common airborne fungi, as they can spread their spores over close and long distances (Bennett, 2010). *Aspergillus* spp. range from 260 to 837 anamorph and teleomorph species, some of which are *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus niger* (Krisgsheld et al., 2013).

Among the filamentous fungi, *Aspergillus niger* (*A. niger*) species, also known as black mold, are molds that can produce some organic acids, enzymes, proteins, and secondary metabolites. *A. niger* is widely used in biotechnology studies due to this feature. Many organic acids such as citric acid, malic acid, and gluconic acid are produced with *A. niger* (Timothy et al., 2021).

A. niger spp. has a very wide cultivation temperature between 6-47^{0}C, but the optimum cultivation temperature is between 35-37^{0}C (Semova et al., 2006). They can grow in a wide pH range, from pH 1.5 to 9.8. They can even grow in low-humidity environments, but the optimum humidity is 96-98% (Krisgsheld et al., 2013). *A. niger* with aerobic respiration can reproduce in many environments such as onions and dried fruits (Costa et al.). Since it is a common species in the soil, it is easy to isolate from nature. Since it has low toxicity, it is used safely in the production of food and beverage supplements (Singh and Gaur, 2021). The image of *A. niger* under the light microscope is given in Figure 2.2.1.

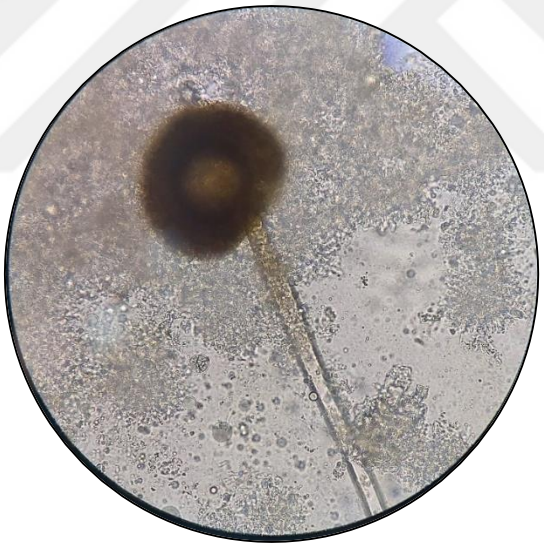


Figure 2.2.1. *A. niger* sp. under the light microscope (40X) (Original)

It is very important to reduce the cost and speed up the process of industrial production. *A. niger* species have many advantages for citric acid production processes. They can be easily isolated from nature. Many species are naturally capable of producing high citric acid. For this reason, they can produce citric acid in high yield without the need for genetic modifications and are considered safe. They can carry out fermentation with low-cost substrates that are easily accessible thanks to their cheap raw material needs. Thanks to their rapid growth, they shorten the process time. Since they continue to multiply at low pH values, they ensure that the process

continues and citric acid is obtained with higher efficiency, even when citric acid begins to form in the fermentation medium. Since they can grow at wide temperatures, they save energy and thus reduce costs by ensuring that the process works even at low temperatures (Show et al., 2015; Ali & Daud, 2011; Vandenberghe, 1999).

Citric acid is formed as an intermediate in the energy production mechanism of aerobic respiration organisms such as *A. niger*. It is formed in the Krebs cycle after the glycolysis phase during aerobic respiration. Acetyl CoA (acetyl coenzyme A), which appears in the last step of glycolysis, participates in the Krebs cycle and induces the formation of citric acid. This cycle is also called the Citric Acid Cycle; as citric acid is formed in the first phase of the Krebs cycle. This cycle is also called the Tricarboxylic Acid Cycle.

The Krebs cycle was described by the German biochemist Hans Adolf Krebs, who lived between 1900 and 1981. In addition to being a metabolic activity that meets the energy needs of living things, the Krebs cycle is also used in the synthesis of molecules involved in important metabolic activities such as metabolites, amino acids, cholesterol, fatty acids and steroids produced during the reaction. The citric acid cycle is the final metabolic pathway in which carbohydrates, proteins, and lipids are oxidized together. Aminoacids, lipids and carbohydrates produce the Acetyl CoA molecule (Akram, 2014). The energy requirement of the Krebs cycle is also met from these molecules. As seen in Figure 2.2.2., Acetyl CoA enters the Krebs cycle, which consists of eight stages, and initiates the series of reactions. The Krebs cycle takes place in the mitochondria and produces NADH and $FADH_2$, which induce the respiratory cycle (Verschueren, 2019).

Acetyl-CoA molecule, a two-carbon compound in the citric acid cycle, and four-carbon oxaloacetate react in the presence of citrate synthetase enzyme to form six-carbon citric acid. It is then converted to isocitrate by reacting with the enzyme aconitase. In the third step of the reaction, five-carbon isocitrate molecules are converted into four-carbon α-ketoglutaric acid molecules by isocitrate dehydrogenase. As a result of this step, the nicotinamide adenine dinucleotide ($NADH+ H^+$) cofactor, which is a reducing molecule, is produced. The α-ketoglutaric acid is then converted to succinyl-CoA by the enzyme α-ketoglutarate dehydrogenase. Succinyl CoA is converted to succinic acid (4C) by succinyl CoA. During this reaction, GDP (guanosine diphosphate) is converted to GTP (guanosine triphosphate) by

phosphorylation. Succinate is converted to fumarate (4C) by the enzyme succinate dehydrogenase. Meanwhile, FAD (flavin adenine dinucleotide) is converted to $FADH_2$ (dihydroflavine adenine dinucleotide). In fumarate, the hydration of the double bond between the fumarase enzyme and carbon atoms occurs and malate (4C) is formed. Malate produces oxaloacetate by malate dehydrogenase. At the end of the reaction, NAD is converted to NADH. The resulting oxaloacetate reacts with Acetyl CoA again and repeats the cycle. A total of 12 ATP (adenosine triphosphate) energy is obtained, 9 ATP from 3 NADH+ H^+, 2 ATP from 1 $FADH_2$, and 1 ATP from 1GTP, which are formed as a result of the citric acid cycle (Akram, 2014).

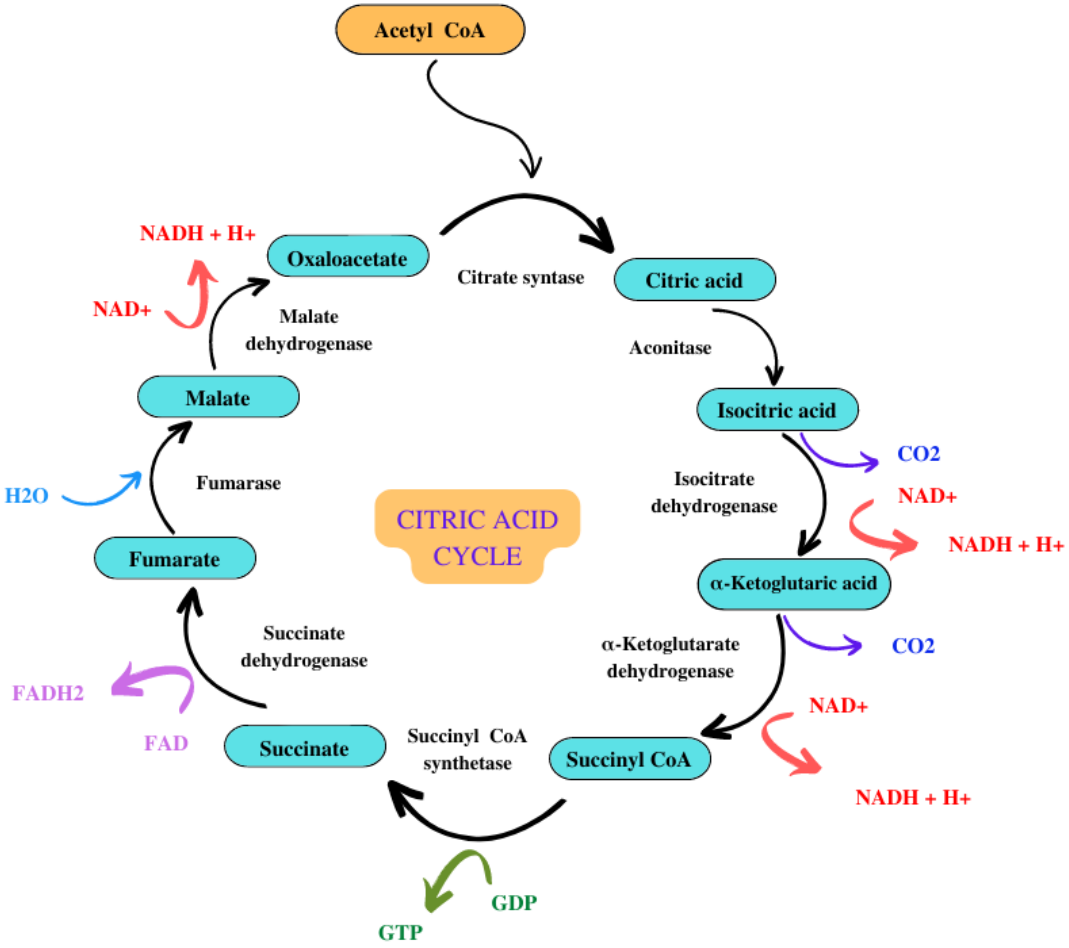


Figure 2.2.2. Krebs Cycle (Citric Acid or TCA Cycle) (designed with Canva)

2.3. Citric Acid Production

Citric acid production is done by fermentation on an industrial scale and *A. niger* species are generally used in these fermentations. Although submerged fermentation is generally preferred in the industry, there are facilities that produce with solid-state fermentation.
A. niger strains are suitable both for the production of citric acid by solid-state fermentation and for the production of citric acid by submerged fermentation. These fermentation types have some advantages and disadvantages compared to each other.

2.3.1. Solid-State Fermentation

Solid-state fermentation is based on the growth of microorganisms in environments with low water content. It mimics the natural habitats of microorganisms (especially fungi). They consume less energy during sterilization due to their low water activity. The risk of bacterial contamination is low. Less waste water is generated at the end of the process and therefore less harmful to the environment. In addition, solid wastes are easier to dispose of. There is no need to add too many chemicals for the solid fermentation medium, thus reducing the toxicity of the product as well as lowering the costs. Fermentation product yields are high, but separation and purification of the product from the process is difficult (Soccol et al., 2017; Kumar et al., 2003).

In some studies, it has been observed that the production of citric acid with solid-state fermentation is more efficient. However, seperation and purification of citric acid in large-scale industrial production is more difficult in solid media. Studies on citric acid production from *A. niger* by solid-state fermentation and the amount of citric acid obtained are given in the Table 2.3.1.1.

Table 2.3.1.1. Citric acid production with solid-state fermentation

Strain	Substrate	Citric acid	References
A. niger B6 – CCT 7717	Cocoa pod husks	978.52 g/kg	de Oliveira et al., 2022
A. niger	Banana peels	97.6 g/L	Odu et al., 2020
A. niger CCT 4355	Sugarcane bagasse	103.45 mg/L	Bastos & Ribeiro, 2020
A. niger PTCC 5010 (ATCC 9142)	Sugarcane bagasse	75.45 g/kg	Yadegari et al., 2013
A. niger sp.	Rice straw	50.23 g/kg	Ali & Daud, 2011
A. niger LPB 21	Cassava bagasse	26.9 g/100g	Prado et al., 2005
A. niger DS1	Sugarcane bagasse	20.2 g/100g	Kumar et al., 2003
A. niger NRRL 2001	Cassava bagasse	88 g/kg	Vandenberghe et al., 2000

2.3.2. Submerged Fermentation

Submerged fermentation is based on the secretion of the fermentation product into the medium by using liquid substrates. In this type of fermentation, the substrates are consumed very quickly. Submerged fermentation is suitable for microorganisms that need high humidity. In submerged fermentation, the process is easier to follow and control. The components required for the medium are homogeneously distributed. Factors that will seriously affect the process, such as temperature, humidity, pH can be kept equal in every part of the medium. Since metabolites secreted in submerged fermentation can be easily taken from the environment, it is preferred over solid-state fermentation in industrial productions (Subramaniyam& Vimala, 2012; Singhania et al., 2010).

In large-scale processes and optimization studies, submerged fermentation is more advantageous. For this reason, submerged fermentation is mostly preferred in industrial citric acid production. Examples of citric acid production by submerged fermentation from *A. niger* strains are given in the Table 2.3.2.1.

Table 2.3.2.1. Citric acid production with submerged fermentation

Strain	Substrate	Citric acid	References
A. niger spp.	Cocoyam	23 g/L	Ezeaa & Ezaka, 2022
A. niger ATCC 16404	Olive Mill Wastewater	16 g/L	Massadeh et al., 2022
A. niger MT-4	Mulberry pomace	120 g/L	Aidynova et al., 2020
A. niger sp.	Pomegranate peel	19.39 g/l	Sutradhar et al., 2020
A. niger EB-12	Sugar beet molasses and chicken feather peptone	46,3 g/L	Ozdal & Kurbanoğlu, 2019
A. niger MH368137	Sugarcane molasses	256.94 mg/mL	Almousa et al., 2018
A. niger NRRL 2001	Hydrolysed Potato Peels	170.22±10.81 mg/L	Makut & Ekeleme, 2018
A. niger EMCC1132	Carob Pod Extract	21.06 g/L	Haider, 2014
A. niger (isolated from the marine soil sample)	Red seaweed Gelidiella acerosa	80 g/l	Ramesh & Kalaiselvam, 2011
A. niger 14/20 *A. niger* 79/20	Pumpkin and cane molasses	14.86 mg/mL 14.44 mg/mL	Majumder et al., 2010
A. niger sp.	Kenana cane molasses	68.21 g/L	El-Hussein et al., 2009
A. niger GCB-75	Molasses	31.1 g/l	Ikram-Ul et al., 2004
A. niger GCBT7	Cane molasses	99.56 g/L	Ali et al., 2002
A. niger ATCC 9142	Spent grain liquor	19 g/L	Roukas & Kotzekidou, 1986

2.4. Factors Affecting Fermentation

After it was discovered that citric acid can be produced by fermentation, the factors affecting fermentation efficiency have been investigated since the 1940s, and many studies have been carried out to determine the optimum values of these conditions.

Experimental studies have found that the factors affecting the fermentation and citric acid yield are carbon source and concentration, nitrogen and phosphate amounts, trace elements, the morphology of the microorganism, pH, and temperature (Max et al., 2010).

2.4.1. Carbon Source

To obtain high efficiency in citric acid production, the selected carbon source must be consumed quickly by the microorganism. Microorganisms naturally have enzymes to hydrolyze the carbon source. Therefore, the carbon source should be selected according to the hydrolytic enzymes produced by the microorganism. For example, since *A. niger* strains, which are most preferred in the production of citric acid, can produce an invertase enzyme that is activated at low pH, substrates containing high sucrose are preferred as carbon sources.

Substrates with high sugar content are preferred as carbon sources in the production of citric acid. Especially molasses, corn, sweet potato, and fruit wastes are known as valuable substrates in citric acid fermentation due to their high sugar content (Max et al., 2010; Soccol et al., 2006).

In the production of citric acid, the concentration of the carbon source is as important as the type. In citric acid production, substrate concentrations of 14-22% are preferred for high yields. At these concentrations, α-ketoglutarate dehydrogenase is suppressed, preventing the continuation of the TCA cycle. At the same time, citric acid yield is affected because low concentrations will reduce the mycelium size of the microorganism and deteriorate its morphology (Max et al., 2010).

2.4.2. Nitrogen and Phosphate Amounts and Trace Elements

Although there is no need to add a nitrogen source in media containing nitrogen-rich substrates, nitrogen sources such as ammonium nitrate, ammonium sulfate, and urea should be added to the nitrogen-poor medium.

Although the amount of phosphate is not limiting, high phosphate concentrations can cause secondary reactions outside the Krebs cycle and affect the biomass increase in the medium.

Microorganisms need some trace elements to grow. However, heavy metals such as copper, iron, zinc, and manganese in high concentrations affect fermentation negatively, so their concentrations should be limited. Manganese reduces the citric acid yield by up to 50% at concentrations as low as 3 mg/L. However, for the growth of *A. niger*, the manganese concentration should be kept at a specific limit. According to the studies, the optimum zinc and iron concentration for citric acid yield should be 0.3 and 1.3 mg/mL, respectively. High iron concentrations are known to suppress the growth of *A. niger* (Max et al., 2010).

2.4.3. pH

In citric acid fermentation, pH is very important in the growth of microorganisms and the realization of citric acid production. The pH must be greater than 5 and 5 for the growth of the microorganism. At these pH values, the spores of the microorganism germinate, and these germinated spores consume ammonia, causing proton release, and the pH of the medium reduces to 2 and below, initiating the production of citric acid. Also, lowering the pH below 2 reduces the risk of contamination and prevents the synthesis of organic acids other than citric acid (Max et al., 2010).

2.4.4. Temperature

The effect of temperature in fermentation depends on the cultivation temperature of the microorganism used. Hence, fermentation temperature should be preferred according to the microorganism's optimum growth temperature. Optimum growth and fermentation temperatures are determined by optimization studies. Optimum fermentation temperatures in citric acid production by *A. niger* are between 30-37°C. It has a negative effect on citric acid production due to the limitation of enzymatic activities at low fermentation temperatures. However, at very high temperatures (>40°C), the yield of citric acid decreases (Sidal, 2022).

2.5. Citric Acid Optimization

The parameters that affect the fermentation efficiency need to be optimized to ensure maximum efficiency from fermentation. Optimization studies can be done by trying all parameters one by one and finding the optimum values, but this causes time loss and high costs. For this reason, statistical experimental design models have been developed for the analysis of processes, which

do not need to test all parameters one by one. In this way, the time and costs to be spent on experiments can be reduced.

2.5.1. Statistical Experimental Design

In order for the results of an experiment to be efficient, the experimental conditions must be optimized. Testing the variables in the experiment with the "one factor at a time" optimization method has left its place to statistical experiment design methods due to the prolongation of the experiment process and increased costs (Veljković et al., 2019).

An experiment has three basic components: the test unit, the administration, and the responses. The experimental unit refers to the independent variables in the experiment. An experiment can have many independent variables. In experimental design, hundreds of experiments may be required for the answers to be generated by these independent experiments. However, with some statistical applications, this number of experiments can be reduced, many variables can be tested at the same time, and optimum conditions for the experiment can be determined (Easterling, 2015).

Especially the most preferred method for statistical experiment design of optimization studies is "Response Surface Methodology".

2.5.1.1. Response Surface Methodology

The “Response Surface Method” developed by Box and Wilson in 1951 under the name “On the Experimental Attainment of Optimum Conditions” was first applied in the chemical industry. The Response Surface Method was defined by Myers and Montgomery as a method in which statistical and mathematical techniques necessary for the development and optimization of processes are used together (Myers & Montgomery, 1995; Box & Wilson, 1951).

In RSM, the optimum conditions of the experiment are determined, in addition to this, experimental analysis data and statistical data are compared, the accuracy of the experiment is checked, and the relationships of the independent variables with each other and their effects in the experiment are determined (Dean et al., 2017).

For optimization studies with RSM, different methods can be preferred according to the data of the experiment and the expected response. These methods are “Central Composite Design” and “Box-Behnken Design”.

2.5.1.1.1. Central Composite Design

The model developed by Box and Wilson, also known as Box-Wilson Center Based Composite Design, includes a full or partial 2^k factorial distribution with a center point. The design always includes the star point as a factor twice, in which case these points represent the lowest and highest values. There are three types of Central Composite Design: Circumscribed Design (CCC), Inscribed Design (CCI), and Face Centered Design (CCF).

Circumscribed Design (CCC), the original form of Central Composite Design (CCD), provides high prediction in the entire experimental field. Inscribed Design (CCI), which is formed by dividing each factor level of CCC by α, creates a full or partial factorial design at certain factor limits. While five levels are required for each factor in CCC and CCI, three levels are required in CCF. While CCC and CCI have the feature of rotation, CCF does not (Figure 2.5.1.1.1.) (Değirmencioğlu & Yazgı, 2006).

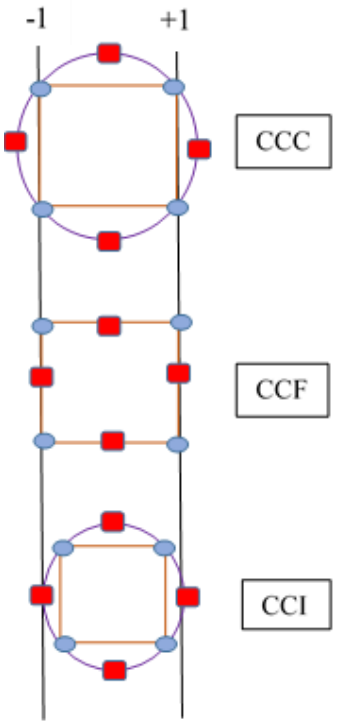


Figure 2.5.1.1.1. Comparison of the Three Types of Central Composite Designs

2.5.1.1.2. Box-Behnken Design

Box Behnken Design (BBD), which is used to determine the quadratic response surfaces and is made to require only three levels coded as -1, 0 and +1, produces a quadratic polynomial model, allowing optimization with less run. Number of experimental studies;

$$N=k^2+k+cp \quad (1)$$

calculated with the equation (1). k is the number of factors and cp is the number of repetitions at the center point (Amenaghawon & Aisien, 2012; Box & Behnken, 1960).

Although CCD provides the same mathematical model as the BBD, the localizations of the experimental points are different. CCD contains high factor combinations. Examination of extreme combinations may be important for some studies. BBD does not examine border areas and generates fewer test runs than CCD. The formation of fewer runs is advantageous in terms of time but also means fewer degrees of freedom (Rakić, 2014)

2.6. Evaluation of Fruit Wastes as Substrate

Fruit wastes serve as an important raw material with high sugars and valuable compounds. Many fruit wastes such as pomegranate peels, mango waste, apple pomace, orange and banana peels were evaluated in citric acid production studies (Abbas et al., 2016).

In the study conducted Sutradhar, Saha, and Chakravarty (2020), pomegranate peels were used as a carbon source for citric acid production. In this study, citric acid production from pomegranate peels by submerged fermentation by *A. niger* strain was investigated and the maximum citric acid production was found to be 19.39 g/L by titrimetric analysis. The study showed that waste pomegranate peels can be used as a substrate in the production of citric acid.

Aidynova, Arslan, and Aydoğan (2020) investigated the production of citric acid from mulberry pomace, which is waste from the wine industry, and pekmez production in Türkiye. *A. niger* MT-4 strain was used in the study. In the study, mulberry pomace, $MgSO_4$, and $(NH_4)_2SO_4$ concentrations were optimized. At the same time, optimum values were determined for pH and incubation time. For the study, 120 g/L mulberry pomace, 1 g/L $MgSO_4$, 2 g/L $(NH_4)_2SO_4$

nutrient content is 5 days' incubation period at pH 7. As a result of the fermentation carried out under these conditions, 24.6 g/L citric acid was obtained.

In the study by Adeoye, Lateef and Gueguim-Kana (2015), cassava peels were used as a substrate for citric acid production. In the study, citric acid was produced by fermentation of mutant *A. niger* strains in the medium created with a mixture of cassava peel-malted sorghum. 88.73 g/L citric acid was produced under optimum fermentation conditions (0.50 g/100 mL substrate concentration, 84 hours fermentation time, pH 4.5 and 6% inoculum size).
In the study by Hamdy (2013), orange peel medium supplemented with sugar cane molasses was used as a substrate for the production of citric acid. In the study, *A. niger* van Tieghem 1867 strain was used, and the fermentation conditions were optimized. The amount of citric acid was found to be 640 g/kg of orange peel by strengthening the medium with molasses at a final sugar concentration of 14% in the presence of 3.5% methanol, pH 5, 30^0C temperature, 250 rpm shaking speed.

In the study conducted by Kumar, Verma and Bhalla (2010), citric acid production from apple pomace was investigated with *A. niger* van Tieghem MTCC 281 strain. Under optimum fermentation conditions (4% (v/w) methanol after 5 days of incubation at 30^0C) 4.6 g/100g apple pomace citric acid was obtained.

According to Majumder et al. (2010), citric acid was produced from *A. niger* 14/20 and 79/20 strains in the fermentation medium containing a mixture of pumpkin and cane molasses, and the amount of citric acid was found 14.86 mg/mL and 14.44 mg/mL, respectively.

2.6.1. Grape Pomace

When tons of waste generated in industries are not evaluated, it poses serious threats to the environment and human health. It is aimed to prevent waste and environmental pollution with various projects in the world due to increasing costs, a decrease in raw materials, and negative environmental effects. For example, "Circular Economy" and "Zero Waste" projects are some of the studies carried out to evaluate the waste generated in industries and to minimize the negative effects. In these projects, raw materials used in industries can be selected from

recyclable products, and almost all by-products produced in the process are either re-processed or used as raw materials for another process or as animal feed and fertilizer (Morseletto, 2020).

Every year, 1.6 billion tons of waste is produced in the world and 89 million tons of this waste is food waste produced in Europe (Freitas et al., 2021; Ravindran et al., 2018). It is very important to evaluate these wastes correctly. Wastes contribute to the climate crisis by increasing the greenhouse gas effect. They also disrupt the balance in habitats (Yaashikaa et al., 2022).

Grapes are an important raw material, especially in wine and fruit juice production. 88% of the grapes collected in Italy (7.231,542 tons) are used in wine production, resulting in 1,807,886 tons of pomace (Perra et al., 2022). Grape pomace consists of peels, stems, and seeds. It is a promising raw material as a carbon source in the production of biotechnology products thanks to its high sugar content and high lignin content of 38-45% (Madadian et al., 2022).

Grape pomace content; varies according to the type of process, the soil in which it is grown, and the grape variety (Dwyer et al., 2014). The moisture content of grape pomace is 50-82% (Iqbal et al., 2021). Its pH varies between 3.34-3.94 (Bender et al., 2020; El Achkar et al., 2016). 100 g grape pomace contains 5-14 g protein (Kandylis et al., 2021; Bender et al., 2020) and 1-13 g lipid (Kandylis et al. 2021). There are 7-9 g cellulose and 6-22 g hemicellulose in 100 g of grape pomace (Bender et al., 2020; El Achkar et al., 2016). White grape pomace contains 60-80% sugar on a dry mass basis, while red grape pomace has 50-70% lower sugar (Papadaki & Mantzouridou, 2019).

Grape pomace is very difficult to dispose of, but this industrial waste is quite harmful to the environment. Since it has an acidic pH, if it mixes with water and soil, it lowers the pH of the water and soil, posing a risk for the creatures living there.

Grape pomace is used as animal feed. However, high woody fibers negatively affect their digestion due to lignin and high secondary metabolites (Perra et al., 2022). At the same time, pure alcohol is obtained by distillation, and the pure alcohol obtained is used in the production of spirits (Badauard, 2021; Ilyas et al., 2021). However, the distillation of grape pomace and the high cost and carbon dioxide emissions cause problems in the production of spirits. At the

same time, the dark, acidic wastewater generated in the process harms the environment (Perra et al., 2022; Strong & Burgess, 2008). If the grape pomace cannot be used for alcohol production, it can be thrown into the soil. However, this changes the pH and chemical properties of the soil and may delay or prevent germination in the soil. Also, other living things such as animals, fungi, and bacteria living in the soil are damaged (Ilyas et al., 2021; Ahmad et al., 2020; Nayak et al., 2018)

Various solutions are suggested for the evaluation of grape pomace, such as obtaining phenolic compounds, producing enzymes, and using them in cosmetic products. However, grape pomace is a unique carbon source that can also be used in fermentation products with higher potential.

In the study conducted by Papadaki and Mantzouridou (2019), citric acid was produced from white grape pomace and green olive processing wastewater from the *A. niger* B60 strain. Green olive processing wastewater was enriched in sugar content by adding white grape pomace. There is a total of 115 g/L carbon source together with glucose and fructose in the mixture. As a conclusion of this study, 85 g/L citric acid was produced with a yield of 0.56 g/g. In this study, an industrial product was produced by fermentation by evaluating both white grape pomace, which is an important waste of the wine industry, and green olive processing wastewater.

2.7. Citric Acid Analyses

Titrimetric analysis, spectrophotometric analysis and chromatographic methods are used for citric acid analysis.

Titration is an analysis method performed by calculating the acidity with the total amount of base spent until the color change is seen when an acid is saturated with a base and reaches pH 8.1. Citric acid analysis by titration is based on the calculation of total acidity in terms of citric acid. The percentage of total acidity is calculated by calculating the ratio of the coefficient of equality and the factor value of the base used in the titration and the amount of base consumed to the initial sample volume. The equivalent of total acidity in terms of citric acid is found by multiplying it by a factor of 0.006404 in the titration formula (Kudzai et al., 2016; Hotha et al., 2014).

Marier & Boulet (1958) carried out the analysis of citric acid in milk with the spectrophotometric analysis method they developed. In the study, skimmed milk solutions containing citric acid at concentrations in the range of 25-200 μg were used. In the method, first of all, 1 mL of citric acid-containing sample or citric acid standard or distilled water is added to the calorimeter tube to form a blank, and 1.30 mL of pyridine is added and mixed by vortexing. Similarly, for the analysis of the samples, 5.7 mL of acetic anhydrate is added to the sample containing 1 mL of citric acid and 1.30 mL of pyridine, and the sample is left in a hot water bath (32^0C) for 30 minutes until the color of the sample changes. For citric acid analysis, the measurement is made by reading the samples one by one after zeroing with the blank at 420 nm in the spectrophotometer.

Penniston et al. (2008) determined the amount of citric acid in lemon juice, lime juice, and various fruit juices by ion chromatography. Analyses were performed on a system consisting of a Dionex ED50 conductivity detector, Dionex AS11-HC 2 250 mm ion column with a 30°C controlled lift shield column, and Dionex ASRS-ULTRA 2 mm pressure shield, using a gradient of 20 to 35 mM sodium hydroxide over 10 minutes, and the citrate was eluted at 7.6 minutes.

High-pressure liquid chromatography (HPLC) is based on the principle that the sample to be analyzed and the mobile phase are separated into its components along the stationary phase (column) under high pressure, moving at different speeds and reaching the exit at different times. Components that reach the output at different times are read through a detector, and then the results are converted into graphs with computer programs (Burcu & Dinçel, 2018). In HPLC systems equipped with ultraviolet (UV) detectors, citric acid analysis is performed by reading the absorbance value at 210 nm (Hotha et al., 2014; Scherer et al., 2012).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Devices and Chemicals

The devices used in the study, along with the brands and models, are given in Table 3.1.1, and the chemicals used in the study are given in Table 3.1.2.

Table 3.1.1. Devices used in the study

Device	Brand/ Model
Biosafety cabinet	Demair Microbiological Safety Cabinet Class II- Tip A
Autoclave	HIRAYAMA-HMC 50L
Incubator	MiproLab MCİ 1208
Shaking Incubator	IKA® KS 4000 ic control
Juicer	Bosch
Coffee grinder	Sinbo Scm-2934
Lyophilizer	Biobase BK-FD10P
Vortex	Dathan Scientific MaXshake™
pH meter	Hanna Edge pH
Precision laboratory scale	Shimadzu ATX224
Light microscope	Leica DM500
HPLC	Shimadzu LC-2030C 3D Plus
HPLC Column	GL Sciences C18 5µ x 4,6 x 250 mm (UP)
Distilled water device	Millipore Direct-Q 3UV
Refrigerator	Vestel NF520 A++

Table 3.1.2. Chemicals used in the study

Chemical	Brand
Potato Dextrose Agar	Conda Lab
Tween 80	SigmaAldrich
HCl	Isolab
NaOH	Isolab
Glucose	Isolab
KH_2PO_4	Sigma Aldrich
KCl	Isolab
$NANO_3$	Isolab
$MgSO_4.7H_2O$	Sigma Aldrich
$FeSO_4.7H_2O$	Sigma Aldrich

3.1.2. Microorganism

In this study, *A. niger* ATCC 10549 strain obtained from the American Type Cell Collection was used. The optimum growth temperature of *A. niger* ATCC 10549 is between 24-26℃. Figure 3.2.1. shows the growth of stock *A. niger* 10549 strain on Potato Dextrose Agar (PDA).

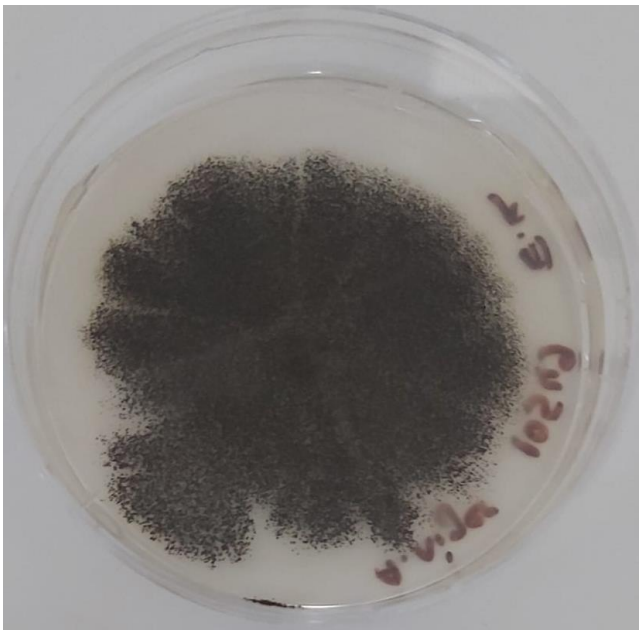


Figure 3.2.1. Stock *A. niger* ATCC 10549 on PDA (Original)

3.2. Methods

3.2.1. Potato Dextrose Agar (PDA)

Potato Dextrose Agar (PDA) was used to growth of *A. niger* strain. 39 g of the ready-to-use medium was weighed, completed with 1 L of distilled water, and transferred to test tubes after thawing by heating. The media in the tubes were sterilized by autoclaving at 121°C for 15 minutes. Slant PDA prepared in test tubes is shown in Figure 3.2.1.1.

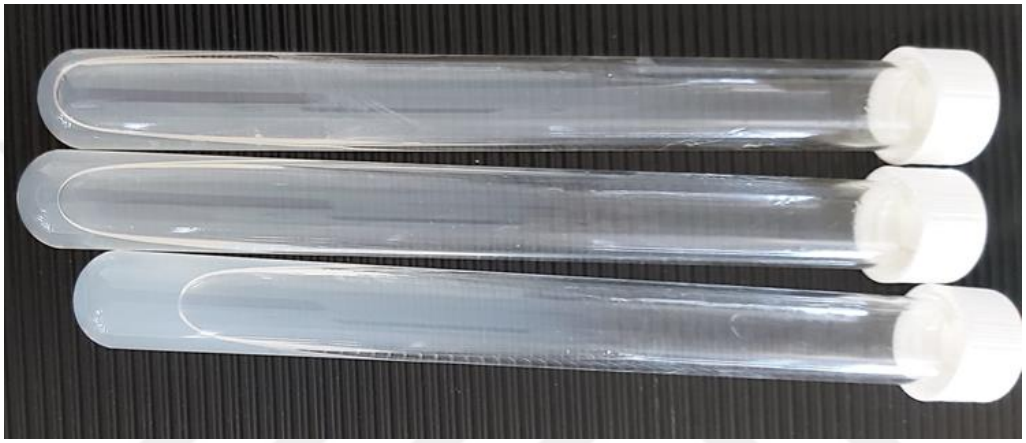


Figure 3.2.1.1. Slant PDA (Original)

3.2.2. Inoculation and Incubation of *A. niger*

Spores taken from the stock *A. niger* 10549 strain were inoculated on PDA media prepared in the form of slant agar. It was incubated at 25°C for 7 days. *A. niger* growth at the end of the incubation is shown in Figure 3.2.2.1.

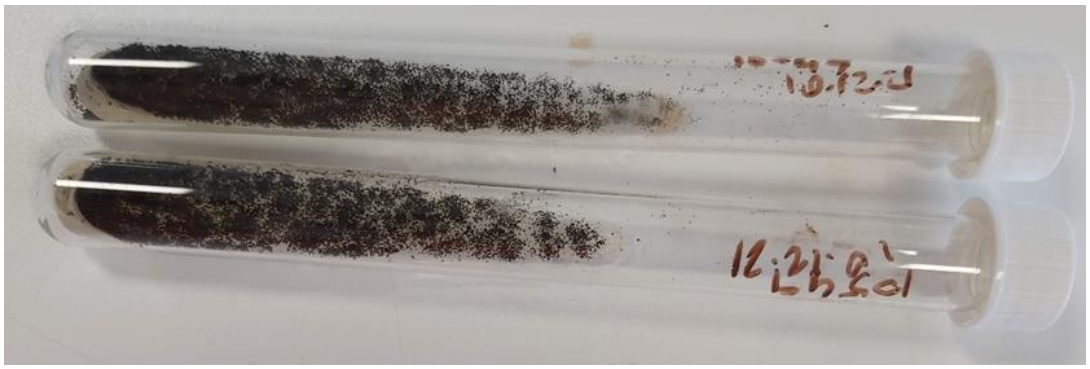


Figure 3.2.2.1. *A. niger* on slant PDA (Original)

3.2.3. Preparation of 0.01 % (v/v) Tween 80 Solution

In order to prepare 0.1% (v/v) Tween 80, dissolve 1000 µL of Tween 80 in 1000 mL of distilled water. Then, some of it is divided into tubes with 10 mL in each tube. These 10 mL solutions were used to harvest the spores of the molds from the agar. Some of the 0.1% (v/v) Tween 80 solutions were divided into tubes with 9 mL in each tube and these were used in the dilution step in the preparation of spore suspension. Prepared 10 mL and 9 mL Tween 80 solutions were sterilized by autoclaving at 121^0C for 15 minutes and used in the study.

3.2.4. Preparation of Spore Suspension and Spore Count

After 7 days of incubation, *A. niger* 10549 strain on slant agar was harvested with 10 mL of 0.1% (v/v) Tween 80 solution, spores were collected and filtered through sterile gauze. 1 mL of the filtrate (stock solution) was taken and transferred to 9 mL of 0.1% Tween 80 solution and diluted to 10^1. 1 mL of 10^1 dilutions was taken and transferred to 9 mL of 0.1% Tween 80 solution to obtain 10^2 diluted spore suspension.

Spore counting was performed with a neubauer slide. 10 µL of the sample taken from the spore suspension was placed on the slide and the spore count was performed under the light microscope. (Figure 3.2.4.1.)

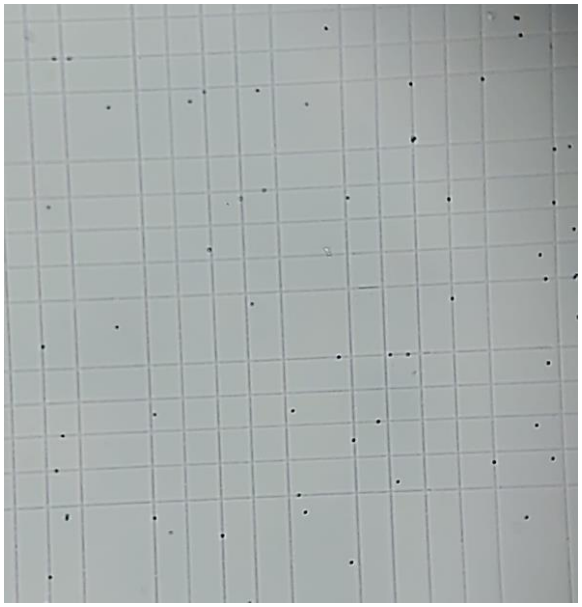


Figure 3.2.4.1 Spore counting on thoma glass (under light microscope 10X) (Original)

The number of spores in the sample was calculated by equation (2).

$$number\ of\ spores = \frac{Average\ viable\ cell\ count\ per\ square \times dilution\ factor \times 10^4}{Volume\ of\ media} \quad (2)$$

Average viable cells count per square = Total numberof viable cells in 4 squares / 4

3.2.5. Preparation of Grape Pomace

In the study, white sultana type grapes purchased from the local public markets of Adana province were used. The grapes were squeezed with a juicer, the juice was discarded and the pulp was dried in a lyophilizer (Fig. 3.2.5.1). The dried grape pomace was ground with a coffee grinder and then sieved and turned into powder completely (Fig. 3.2.5.2). It was stored in a refrigerator at -18^{0}C to be used in the experiments.

Figure 3.2.5.1. Lyophilized grape pomace (Original)

Figure 3.2.5.2. Ground dry grape pomace (Original)

3.2.6. Creating the Experiment Design

Design-Expert® Software 13 Trial Version 13 (State-Ease, Inc., Minneapolis, MN, USA) was used in the study. Box-Behnken Design is preferred. The experimental design was created by entering the desired intervals given in Table 3.2.6.1. into the software. The experimental design is given in Table 3.2.6.2. The coded values for the experimental design are given in Table 3.2.6.3; In the table, A represents the temperature, B represents the nutrient amount, C represents the pH value and D represents the time. -1; for minimum values, 0; for medium values, +1; for maximum values are the codes given. The results obtained as a result of the experiments were entered and the data were analyzed.

Table 3.2.6.1. Independent variables, minimum and maximum values

Independent variable	Unit	Minimum value	Maximum value
Temperature	^{0}C	25	35
Nutrient	g/L	30	120
pH		2	8
Time	Hour (h)	48	168

Table 3.2.6.2. Experimental design model

Std	Run	Factor 1 A: Temperature ^{o}C	Factor 2 B: Nutrient g/L	Factor 3 C: pH	Factor 4 D: Time hour	Response 1 Citric acid concentration (g/L)
26	1	30	75	5	108	
22	2	30	120	5	48	
17	3	25	75	2	108	
16	4	30	120	8	108	
13	5	30	30	2	108	
20	6	35	75	8	108	
23	7	30	30	5	168	
25	8	30	75	5	108	
1	9	25	30	5	108	
7	10	30	75	2	168	
10	11	35	75	5	48	
12	12	35	75	5	168	
5	13	30	75	2	48	
19	14	25	75	8	108	
6	15	30	75	8	48	
18	16	35	75	2	108	
9	17	25	75	5	48	
15	18	30	30	8	108	
27	19	30	75	5	108	
30	20	30	75	5	108	
14	21	30	120	2	108	
11	22	25	75	5	168	
28	23	30	75	5	108	
3	24	25	120	5	108	
2	25	35	30	5	108	
8	26	30	75	8	168	
24	27	30	120	5	168	
29	28	30	75	5	108	
4	29	35	120	5	108	
21	30	30	30	5	48	

Table 3.2.6.3. Coded experimental design model

Std	Run	Factor 1 A: ^{0}C	Factor 2 B: g/L	Factor 3 C	Factor 4 D: hour
26	1	0	0	0	0
22	2	0	+1	0	-1
17	3	-1	0	-1	0
16	4	0	+1	+1	0
13	5	0	-1	-1	0
20	6	+1	0	+1	0
23	7	0	-1	0	+1
25	8	0	0	0	0
1	9	-1	-1	0	0
7	10	0	0	-1	+1
10	11	+1	0	0	-1
12	12	+1	0	0	+1
5	13	0	0	-1	-1
19	14	-1	0	+1	0
6	15	0	0	+1	-1
18	16	+1	0	-1	0
9	17	-1	0	0	-1
15	18	0	-1	+1	0
27	19	0	0	0	0
30	20	0	0	0	0
14	21	0	+1	-1	0
11	22	-1	0	0	+1
28	23	0	0	0	0
3	24	-1	+1	0	0
2	25	+1	-1	0	0
8	26	0	0	+1	+1
24	27	0	+1	0	+1
29	28	0	0	0	0
4	29	+1	+1	0	0
21	30	0	-1	0	-1

The quadratic polynomial constructed to estimate the dependent variable response and the optimum point according to the determined parameters is given in equation (3). In the equation, Y represents the predicted response, A, B, C, and D independent variables, β_0 offset term, linear effects of β_1, β_2, β_3 and β_4, interaction terms β_{11}, β_{22}, β_{13}, β_{14} etc.

(3)

$$Y = \beta_0 + \beta_1 A + \beta_2 B - \beta_3 C + \beta_4 D + \beta_{1\,2} AB + \beta_{1\,3} AC + \beta_{1\,4} AD - \beta_{2\,3} BC + \beta_{2\,4} BD + \beta_{3\,4} CD - \beta_{1\,1} A^2 - \beta_{2\,2} B^2 - \beta_{3\,3} C^2 - \beta_{4\,4} D^2$$

3.2.7. Fermentation Medium

Czapek dox is one of the commonly used media for the production of citric acid from *A. niger*. Because this medium contains all the components needed by the microorganism for the production of citric acid. The contents of the Czapek Dox medium are given in the Table 3.2.7.1.(a). Grape pomace was used as a carbon source in the study. For this reason, dried and ground grape pomace was used instead of glucose according to the amounts given in the experimental design. The media contents for each quantity (30 g/L, 75 g/L, 120 g/L) are given in Table 3.2.7.1.(b). According to the data in the experimental design, the components in the medium were dissolved in a liter of distilled water, the pH values were measured with a pH meter and to adjust the basic pH values (pH: 5 and pH: 8) 1 N NaOH, to adjust the acidic pH values (pH: 2), 1 M pH values were adjusted with HCl. The study was carried out in 250 mL flasks and 100 mL medium (Figure 3.2.7.1.).

Table 3.2.7.1. Czapek Dox Medium and Modified Czapek Dox Medium content with three different grape pomace concentration (30/75/120 g/L grape pomace)

a)

CZAPEK DOX	
CONTENT	AMOUNT (g/L)
Grape pomace	50
KH_2PO_4	1
KCl	0,5
$NANO_3$	2
$MgSO_4.7H_2O$	0,5
$FeSO_4.7H_2O$	0,01

b)

MODIFIED CZAPEK DOX	
CONTENT	AMOUNT (g/L)
Grape pomace	30/75/120
KH_2PO_4	1
KCl	0,5
$NANO_3$	2
$MgSO_4.7H_2O$	0,5
$FeSO_4.7H_2O$	0,01

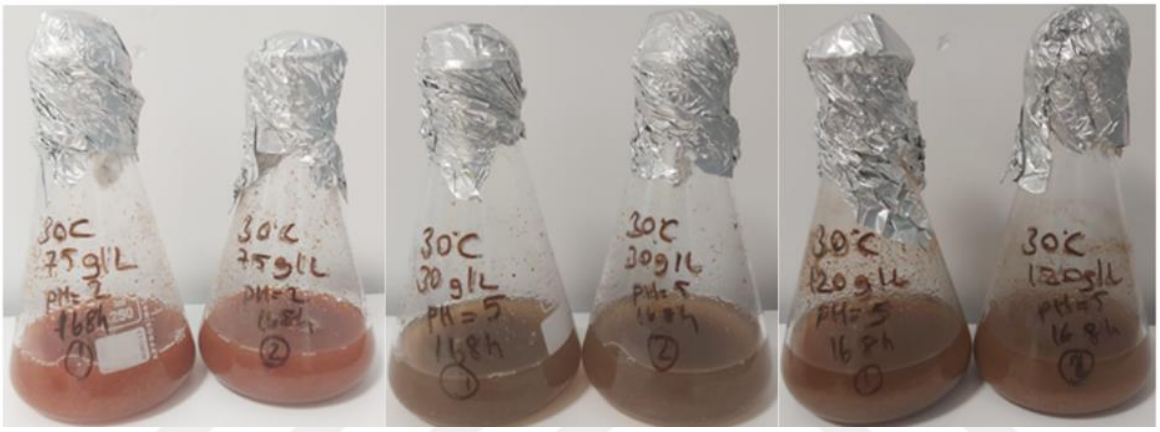


Figure 3.2.7.1. Autoclaved grape pomace medium (Original)

3.2.8. Inoculation and Incubation

After the mediums were adjusted according to the experimental design created for each parameter (nutrient and pH) in the BBD, 1 x 10^6 spores/mL of spore suspension containing *A. niger* ATCC 10549 strain were inoculated in each flask and the same value was used in all experiments.

The incubation times given in the experimental design (Table 3.2.6.2.) were taken into account. IKA® KS 4000 ic control brand shaking incubator was used in the study (Figure 3.2.8.1.). The mixing speed was determined as 200 rpm and the same value was used in all experiments.

Figure 3.2.8.1. Samples in shaking incubator (Original)

3.2.9. Preparation of Chemicals Used in HPLC Analysis

3.2.9.1. Preparation of mobile phase

Dissolve 27.2 g of KH_2PO_4 in 950 mL of ultrapure distilled water. After adjusting the pH to 2.4 with 85% H_3PO_4, the volume is made up to 1 L with ultrapure distilled water and kept in an ultrasonic water bath for five minutes.

3.2.9.2. Preparation of standard citric acid solutions

A 50 g/L stock citric acid solution is prepared. By diluting this solution, citric acid solutions are prepared at concentrations of 0.1, 0.5, 1, 2.5, 5, 10, and 25 g/L, respectively, to form the calibration curve.

3.2.9.3. Preparation of samples

After the fermentation is completed, the samples are filtered with filter paper, passed through 0.22 µm syringe filters, and put into HPLC vials.

3.2.10. Citric Acid Analysis

HPLC was used for citric acid analysis. In the study, 0.2 M KH_2PO_4 mobile phase and C18 25 cm x 4 mm x 5µm column were used. Working conditions were determined as 20 minutes'

analysis time, temperature 25^{0}C, flow rate 0.8 mL/minute, injection volume 10 µl, and detector absorbance 214 nm.

3.2.10.1. Calibration curve

To create the calibration curve, citric acid solutions of different concentrations prepared are analyzed under the determined operating conditions, and a linear graph is created with the graph areas obtained. The citric acid concentrations of the samples are determined by the equation obtained from this graph.

3.2.10.2. Analyses of samples

All samples were analyzed under specified working conditions. The citric acid concentrations produced as a result of fermentation were calculated by substituting the areas given in the graphics as a result of the HPLC analysis in the formula obtained from the standardization curve (AOAC Official Methods of Analysis, 1995).

4. RESULTS AND DISCUSSIONS

4.1. Results

The standard citric acid solutions prepared at concentrations of 0.1, 0.5, 1, 2.5, 5, 10, and 25 g/L were analyzed in HPLC. Chromatograms of standard citric acid samples are given in Figures 4.1.1-4.1.7. As can be seen from the chromatograms, the retention time is approximately 8.7th minutes.

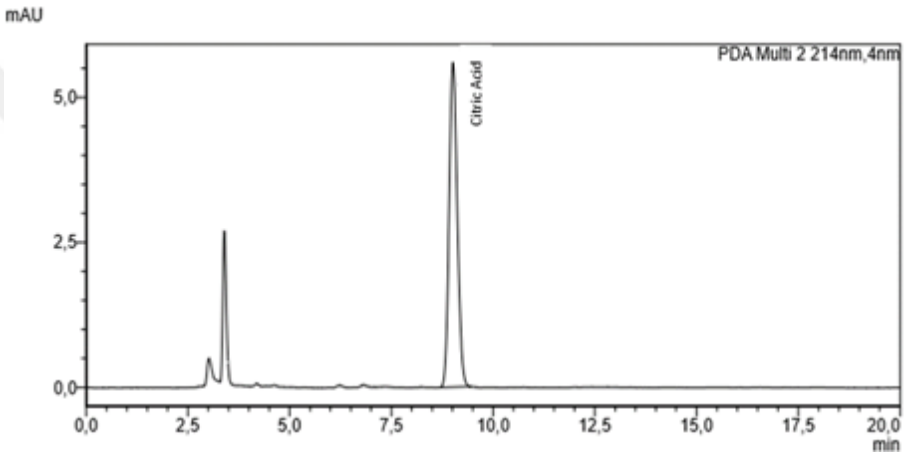


Figure 4.1.1. 0.1 g/L standard chromatogram

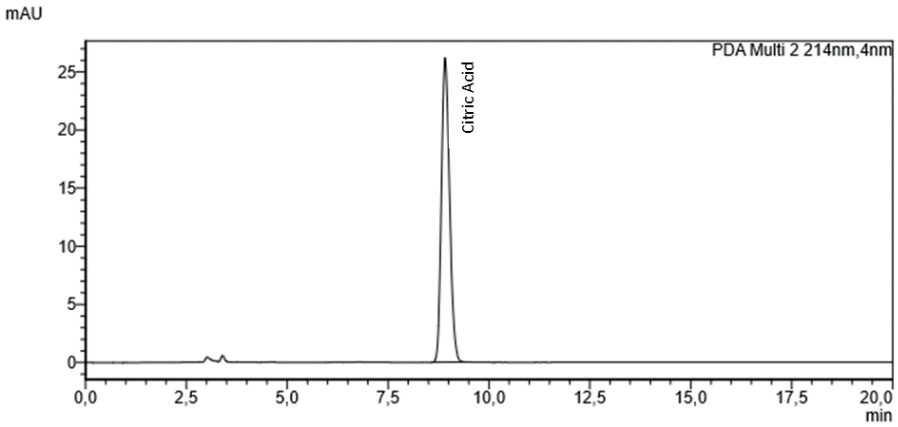


Figure 4.1.2. 0.5 g/L standard chromatogram

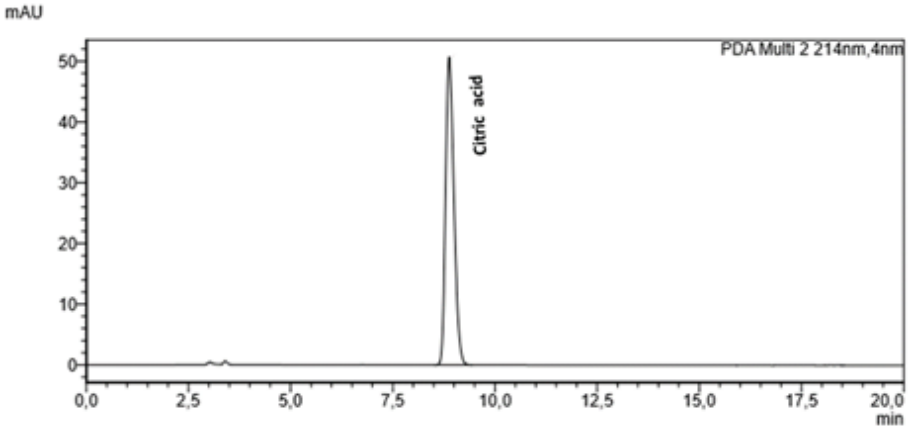


Figure 4.1.3. 1 g/L standard chromatogram

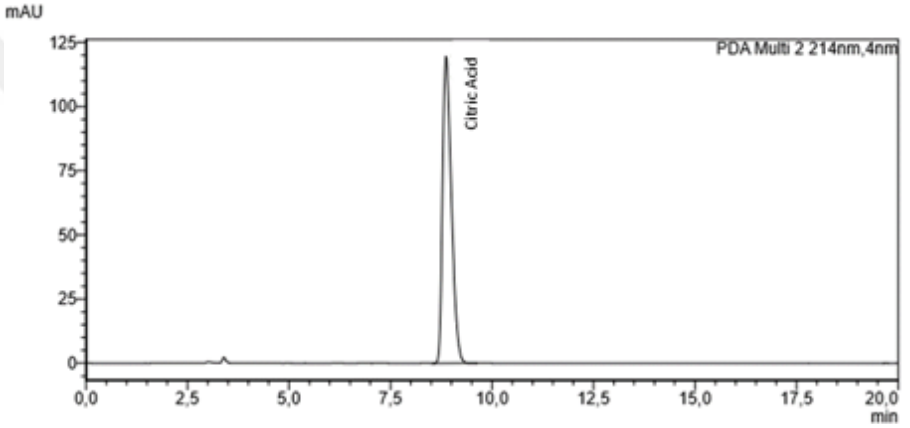


Figure 4.1.4. 2.5 g/L standard chromatogram

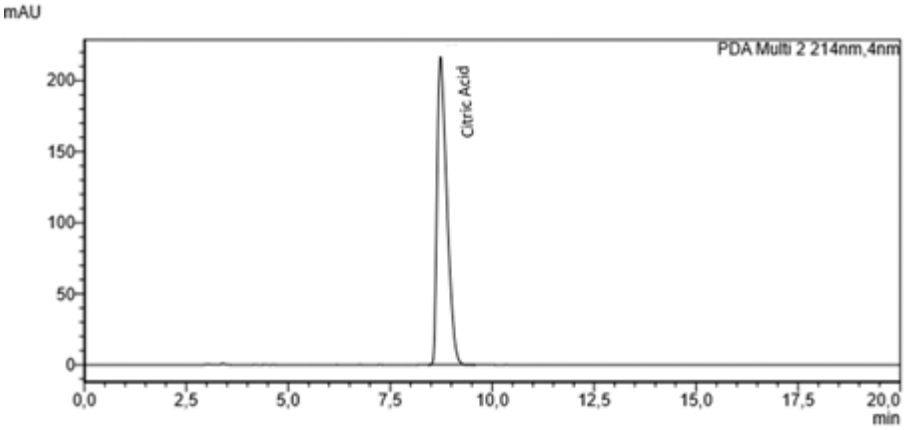


Figure 4.1.5. 5 g/L standard chromatogram

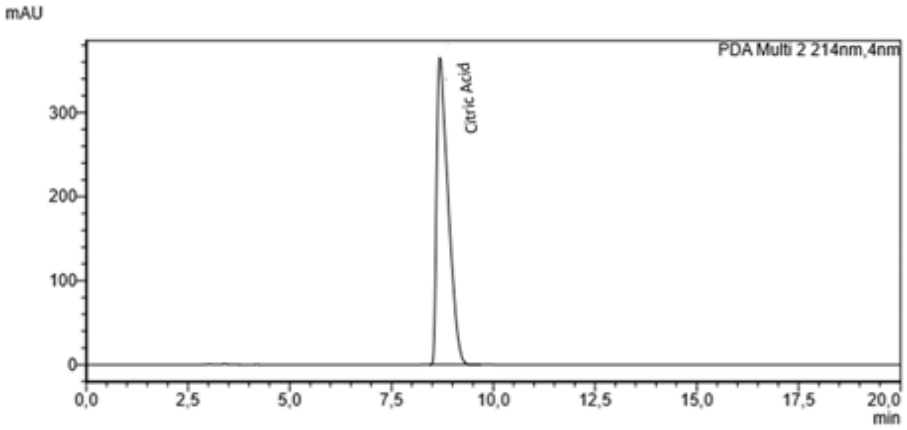


Figure 4.1.6. 10 g/L standard chromatogram

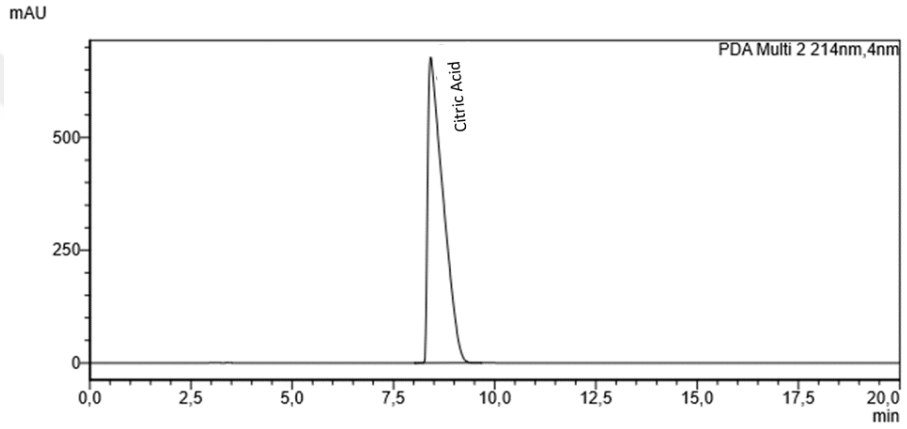
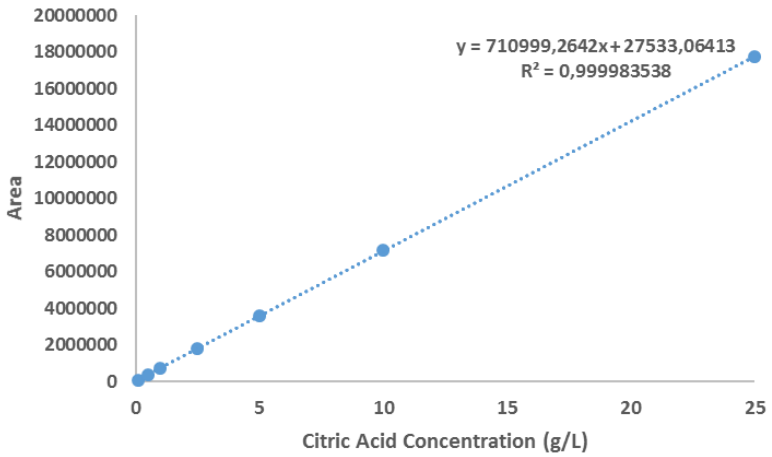


Figure 4.1.7. 25 g/L standard chromatogram

The R^2 value of the calibration curve was calculated as 0.999 and the equation given in Figure 4.1.8 was obtained. In this formula, the y value represents the peak area obtained in 8.7th minutes, and the x value represents the citric acid concentration.



C.A concentration (g/L)	Area
0,1	77805
0,5	367883,5
1	721171
2,5	1814012
5	3604309
10	7182112
25	17780507

Figure 4.1.8. Calibration curve

Chromatograms of samples with concentrations of 0.11, 1.68, 4.76 and 7.57 g/L are given in Figures 4.1.9, 4.1.10, 4.1.11, and 4.1.12, respectively. Citric acid concentrations were calculated by substituting the areas given in the chromatograms of all samples in the equation obtained from the standard curve. Calculated experimental results data are given in Table 4.1.1.

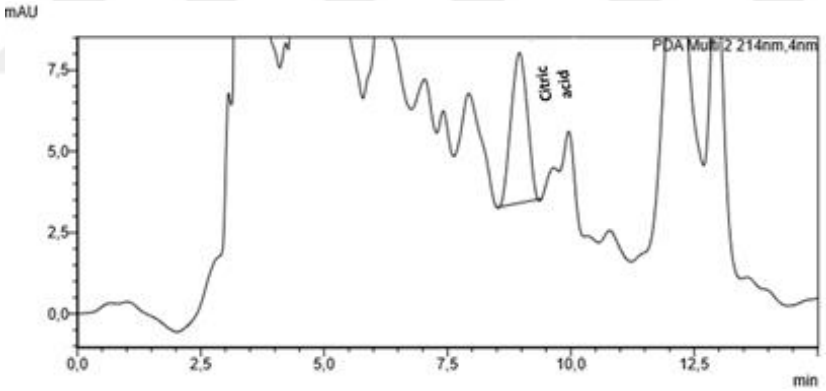


Figure 4.1.9. Chromatogram for 0.11 g/L citric acid

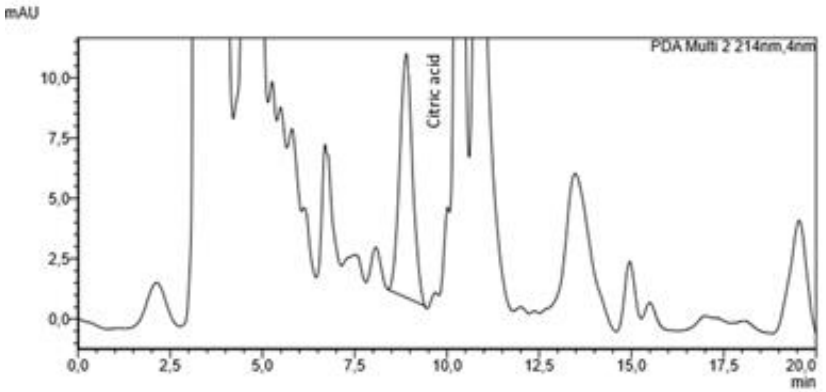


Figure 4.1.10. Chromatogram for 1.68 g/L citric acid

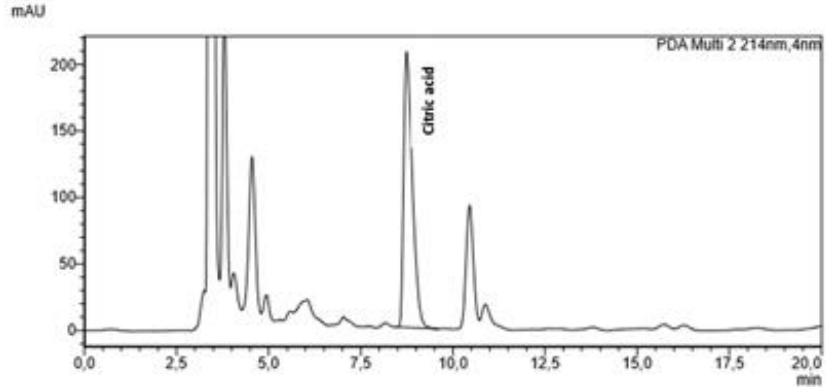


Figure 4.1.11. Chromatogram for 4.76 g/L citric acid

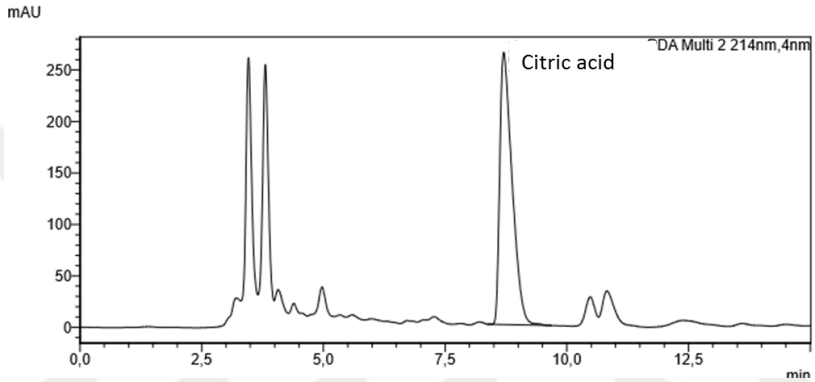


Figure 4.1.12. Chromatogram for 7.57 g/L citric acid

In the model created with BBD, the optimum conditions were chosen as 30°C, 120 g/L substrate concentration, pH 5, and 168 hours, and the predicted citric acid concentration was given as 7.204 (Figure 4.1.13.). As a result of the experimental studies, the citric acid concentration was calculated as 7.57 g/L on selected conditions (Table 4.1.1.). The fact that the amount of citric acid obtained as a result of experimental studies is higher than the predicted amount proves the accuracy of the model.

30,000	*120,000*	*2,000*	*108,000*	7,850	1,000	
30,000	***120,000***	***5,000***	***168,000***	**7,204**	**1,000**	**Selected**
30,000	*75,000*	*8,000*	*168,000*	4,902	1,000	

Figure 4.1.13. Selected point according to RSF

Table 4.1.1. Experimental results

Std	Run	Factor 1 A: Temperature (^{0}C)	Factor 2 B: Substrat concentration (g/L)	Factor 3 C: pH	Factor 4 D: Time (hours)	Response 1 Citric acid concentration (g/L)
26	1	30	75	5	108	4.76
22	2	30	120	5	48	1.68
17	3	25	75	2	108	2.03
16	4	30	120	8	108	2.26
13	5	30	30	2	108	0.11
20	6	35	75	8	108	3.63
23	7	30	30	5	168	0.70
25	8	30	75	5	108	4.76
1	9	25	30	5	108	0.87
7	10	30	75	2	168	2.73
10	11	35	75	5	48	2.34
12	12	35	75	5	168	3.34
5	13	30	75	2	48	5.02
19	14	25	75	8	108	0.69
6	15	30	75	8	48	1.02
18	16	35	75	2	108	4.76
9	17	25	75	5	48	1.68
15	18	30	30	8	108	1.15
27	19	30	75	5	108	5.19
30	20	30	75	5	108	6.50
14	21	30	120	2	108	6.54
11	22	25	75	5	168	1.68
28	23	30	75	5	108	5.38
3	24	25	120	5	108	1.15
2	25	35	30	5	108	0.30
8	26	30	75	8	168	6.34
24	**27**	**30**	**120**	**5**	**168**	**7.57**
29	28	30	75	5	108	5.38
4	29	35	120	5	108	7.57
21	30	30	30	5	48	0.83

Points, where the p-value is less than <0.05, are considered significant. As can be seen in the ANOVA table given in Table 4.1.2., all parameters and their interactions with each other are stated as significant, except the interactions between temperature and pH (AC), temperature and time (AD). The importance of the model is also shown in the Table 4.1.2. The value of lack of fit isnt significant it is show that the model is fit. The coefficient of determination (R^2) values also prove the suitability of the model. R^2 value of 0.936 means that the model will reach the

expected citric acid concentration with 93.6% probability. The difference between the predicted R^2 value and the adjusted R^2 value is less than 0.2, indicating that these values are in reasonable agreement. It is desirable that the Adeq Precision value, which measures the signal-to-noise ratio, is greater than 4.0. In this study calculated the Adeq precision as 12.6, indicating an adequate signal.

Table 4.1.2. ANOVA table of response surface quadratic model

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	148.97	14	10.64	15.74	< 0.0001	**significant**
A- Temperature	15.96	1	15.96	23.61	0.0002	**significant**
B-Nutrient	43.36	1	43.36	64.13	< 0.0001	**significant**
C- pH	3.10	1	3.10	4.59	0.0491	**significant**
D-Time	7.99	1	7.99	11.81	0.0037	**significant**
AB	12.22	1	12.22	18.07	0.0007	**significant**
AC	0.0110	1	0.0110	0.0163	0.9001*	
AD	0.2500	1	0.2500	0.3698	0.5522*	
BC	7.08	1	7.08	10.46	0.0036	**significant**
BD	9.06	1	9.06	13.40	0.0023	**significant**
CD	14.48	1	14.48	21.41	0.0003	**significant**
A^2	18.87	1	18.87	27.90	< 0.0001	**significant**
B^2	16.96	1	16.96	25.08	0.0002	**significant**
C^2	5.30	1	5.30	7.83	0.0135	**significant**
D^2	7.52	1	7.52	11.13	0.0045	**significant**
Residual	10.14	15	0.6761			
Lack of Fit	8.10	10	0.8099	1.98	0.2331	**not significant**
Pure Error	2.04	5	0.4067			
Core Total	152.11	29				

Table 4.1.3. Coefficients of parameters

CA concentration	=	=
-29,64108		
+2,48300	* A	* Temperature
-0,099275	* B	* Nutrient
-0832741	* C	* pH
-0,017769	* D	* Time
+0,007867	* AB	* Temperature * Nutrient
+0,045500	* AC	* Temperature * pH
+0,0011250	* AD	* Temperature * Time
-0,011259	* BC	* Nutrient * pH
+0,000558	* BD	* Nutrient * Time
+0,007194	* CD	* pH * Time
-0,054483	* A^2	* $Temperature^2$
-0,000611	* B^2	* $Nutrient^2$
-0,064398	* C^2	* pH^2
-0,000394	* D^2	* $Time^2$

According to the results, the calculated values for each parameter are given in the Table 4.1.3. Substituting these values in equation (3), the following equation (4) is obtained.

(4)

$$CA\ concentration = -29{,}64108 + 2{,}48300 * A - 0{,}099275 * B + 0832741 * C - 0{,}017769 * D + 0{,}007867 * AB + 0{,}045500 * AC + 0{,}0011250 * AD + 0{,}011259 * BC + 0{,}000558 * BD + 0{,}007194 * CD + 0{,}054483 * A^2 + 0{,}000611 * B^2 + 0{,}064398 * C^2 + 0{,}000394 * D^2$$

According to Figure 4.1.14., the expected and actual values of citric acid concentration are proportional to each other. This proves that the model is significant and the results are accurate.

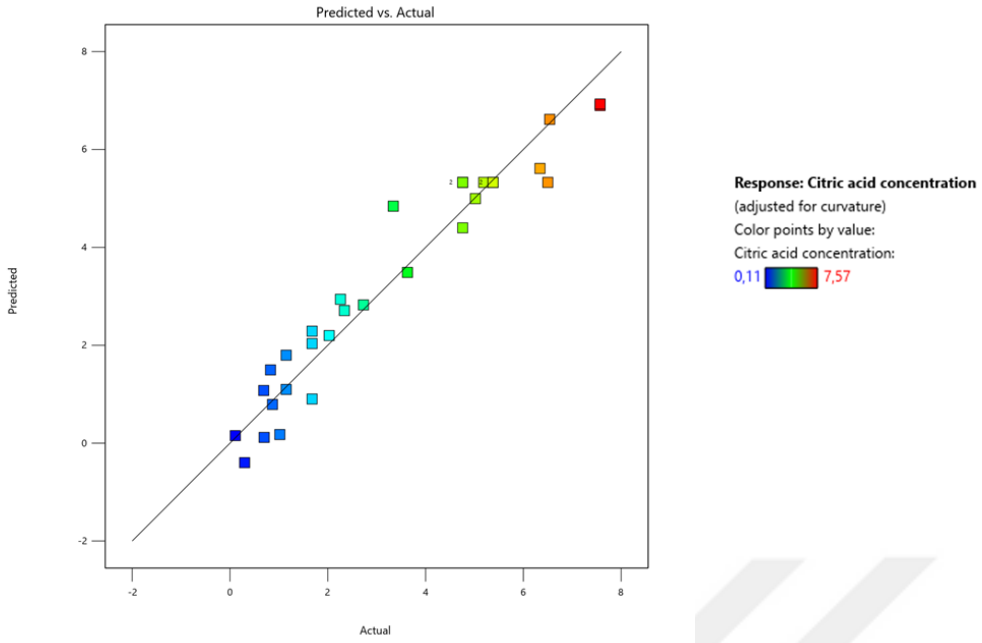


Figure 4.1.14. Comparison of predicted and actual citric acid concentration

The pH-temperature response surface graph shown in Figure 4.1.15 shows the effect of the interaction of pH and fermentation temperature on citric acid concentration. At low temperatures and acidity, citric acid production decreases. Maximum citric acid production occurs at 30°C at pH 5, although it increases the concentration of citric acid at higher temperatures and decreasing pH values.

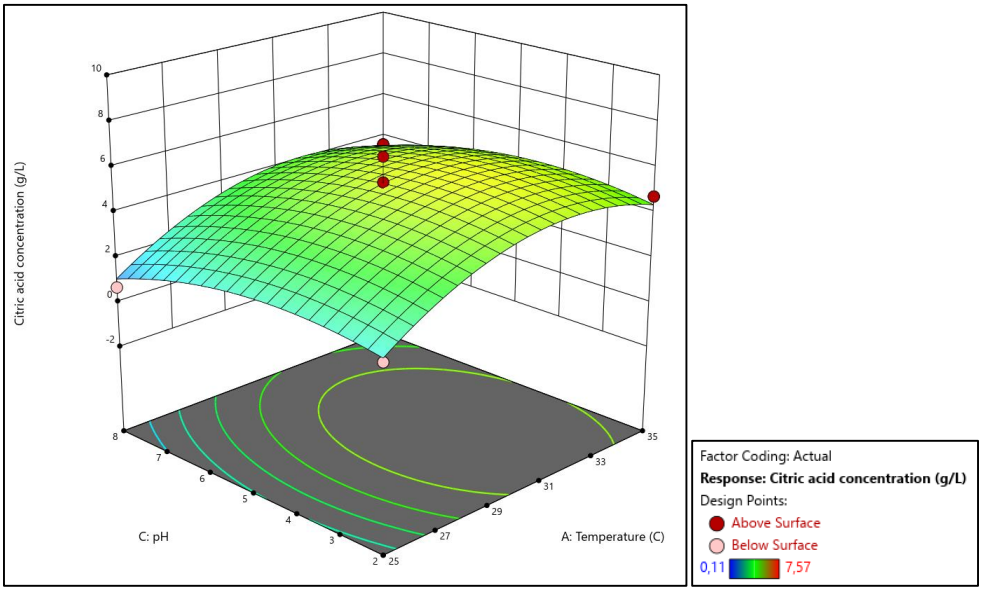


Figure 4.1.15. pH- temperature response surface graphs

The temperature-time response surface graph given in Figure 4.1.16 shows the effect of temperature and time relationship on citric acid concentration. As the fermentation temperature and time increase, the citric acid concentration increases. However, the maximum production of citric acid takes place at 30°C during 108 hours of fermentation.

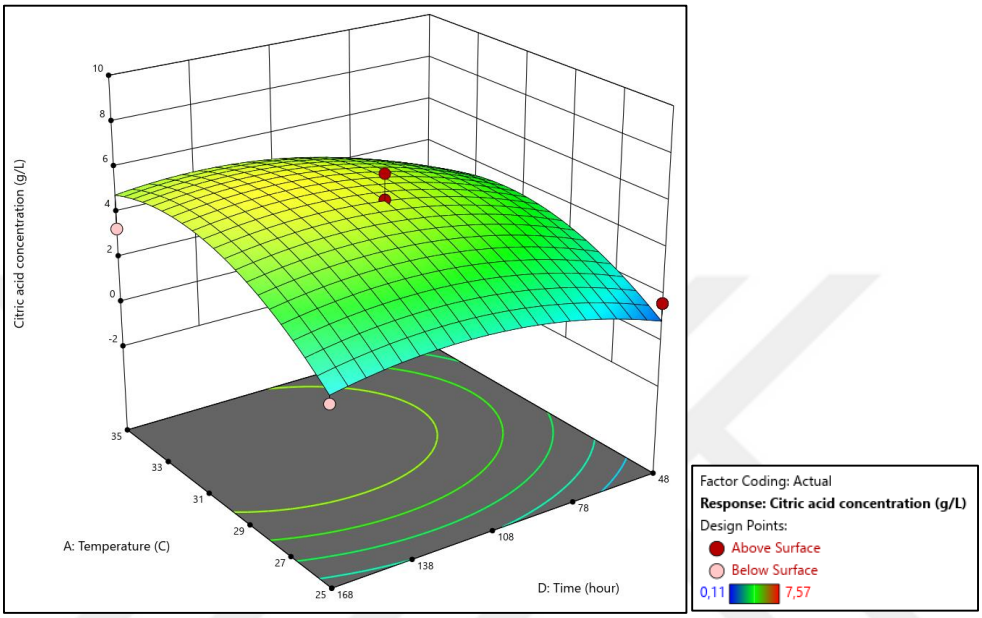


Figure 4.1.16. Temperature – time response surface graph

The pH-substrate concentration response surface graph given in Figure 4.1.17 shows the effect of the interaction between the initial pH and the substrate concentration on the citric acid concentration. The maximum citric acid concentration is obtained at a substrate concentration of 120 g/L and pH 5. At lower substrate concentrations, citric acid production is reduced.

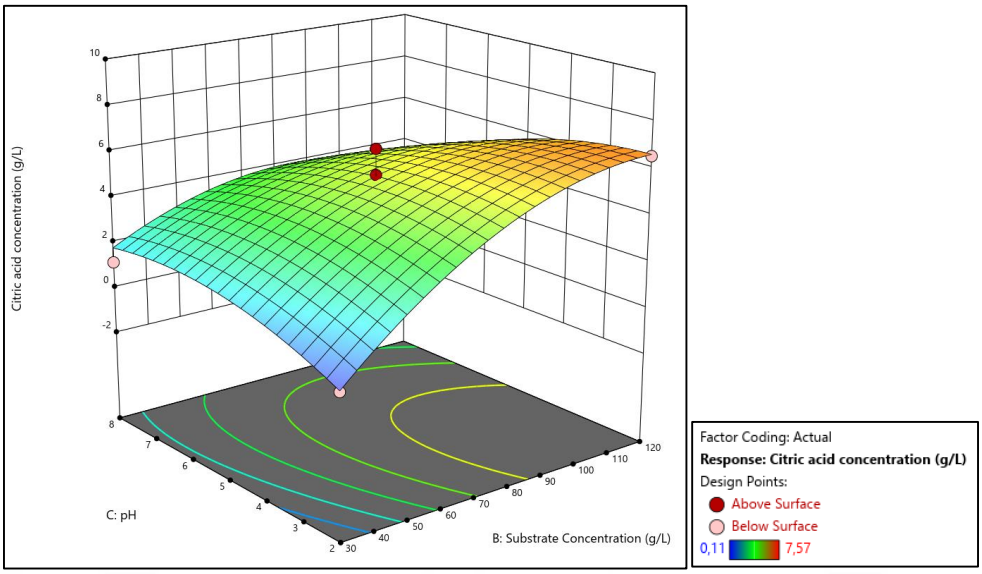


Figure 4.1.17. pH – substrate concentration response surface graph

In the time-substrate concentration response surface graph given in Figure 4.1.18, it is seen that there is a correlation between the fermentation time and the substrate concentration in citric acid production. Continuing the fermentation period for 168 hours reduces citric acid production, especially at low substrate concentrations. It is seen that the citric acid concentration is maximum in the 108 hours of fermentation period in the medium prepared with a substrate concentration of 120 g/L.

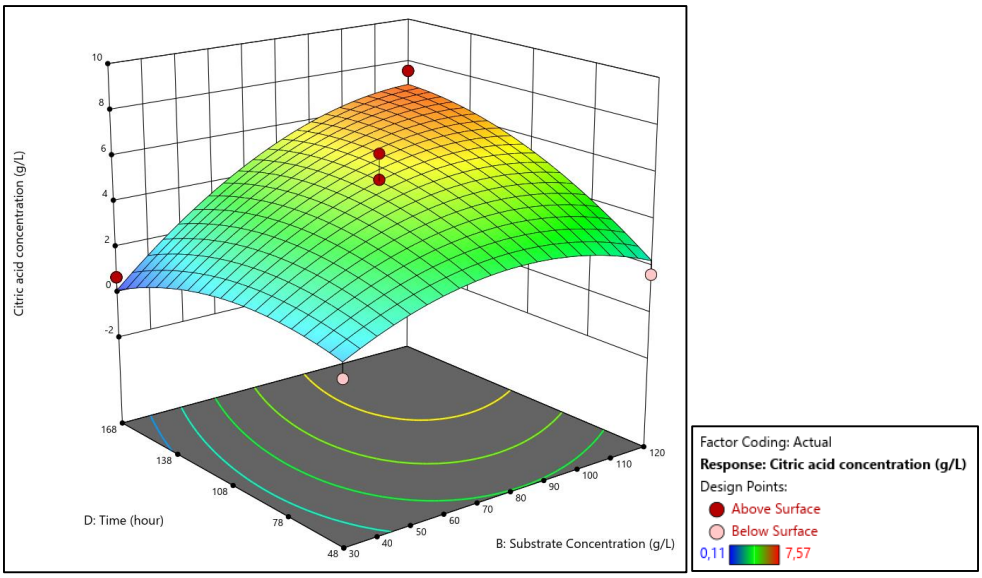


Figure 4.1.18. Time – substrate concentration response surface graph

In the time-pH response surface graph given in Figure 4.1.19, it is seen that the citric acid concentration is adversely affected by the increase in the initial medium pH and the decrease in the fermentation time. The maximum citric acid value was obtained at pH 5 during the fermentation time of 108 hours.

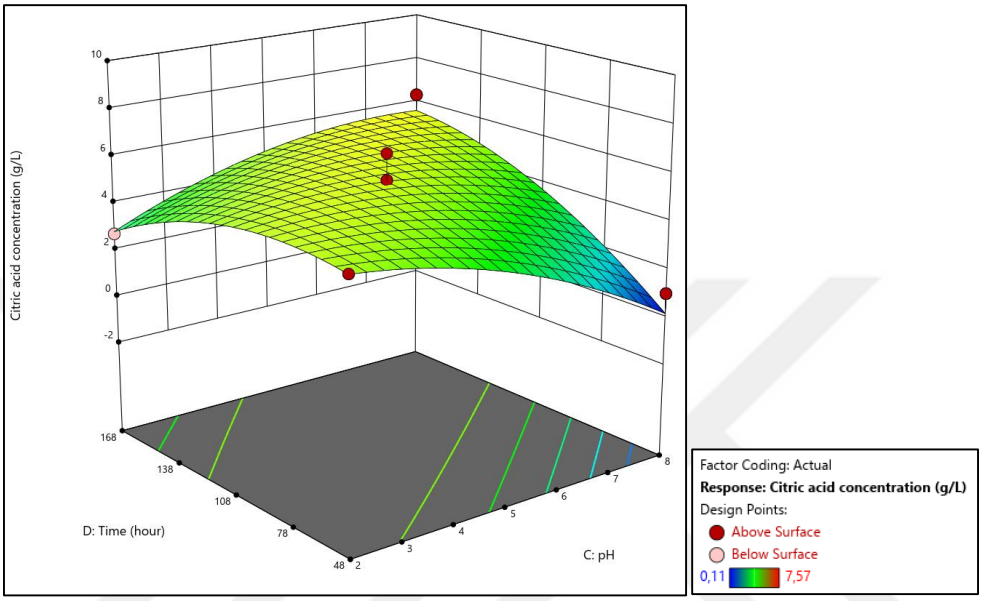


Figure 4.1.19. Time- pH response surface graph

According to the substrate concentration-temperature response surface graph given in Figure 4.1.20, it is seen that the maximum citric acid concentration is obtained in the fermentation at 120 g/L substrate concentration and 30°C temperature.

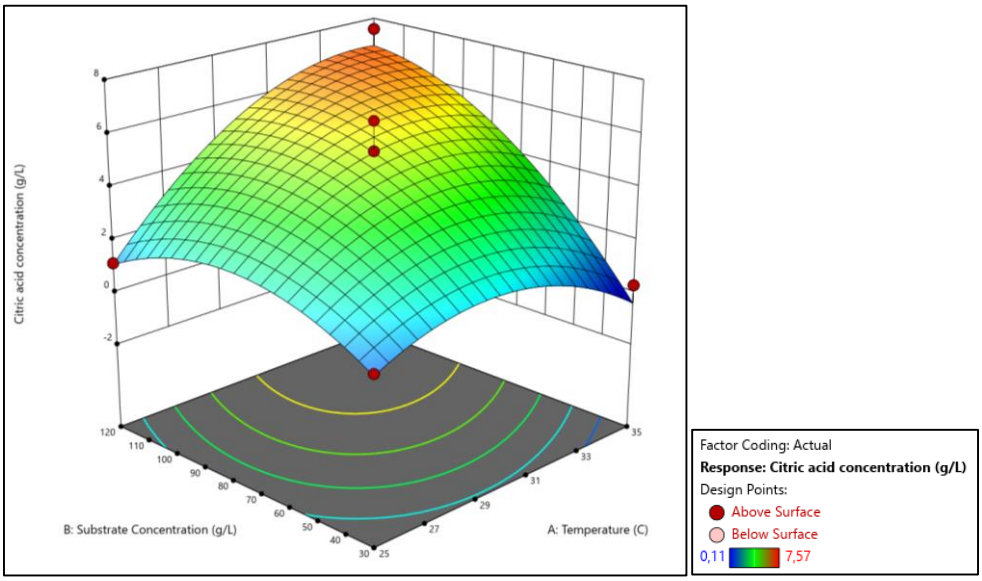


Figure 4.1.20. Substrate concentration – temperature surface graph

In Figure 4.1.21, the correlations of temperature, substrate concentration, pH, and time parameters optimized in the study with citric acid concentration are given. According to the graphics, although the value ranges determined for the temperature are sufficient for this study, it states that the pH, time, and substrate concentration values can be studied in a wider range. Optimum values of the parameters determined for maximum citric acid production were determined in these working intervals.

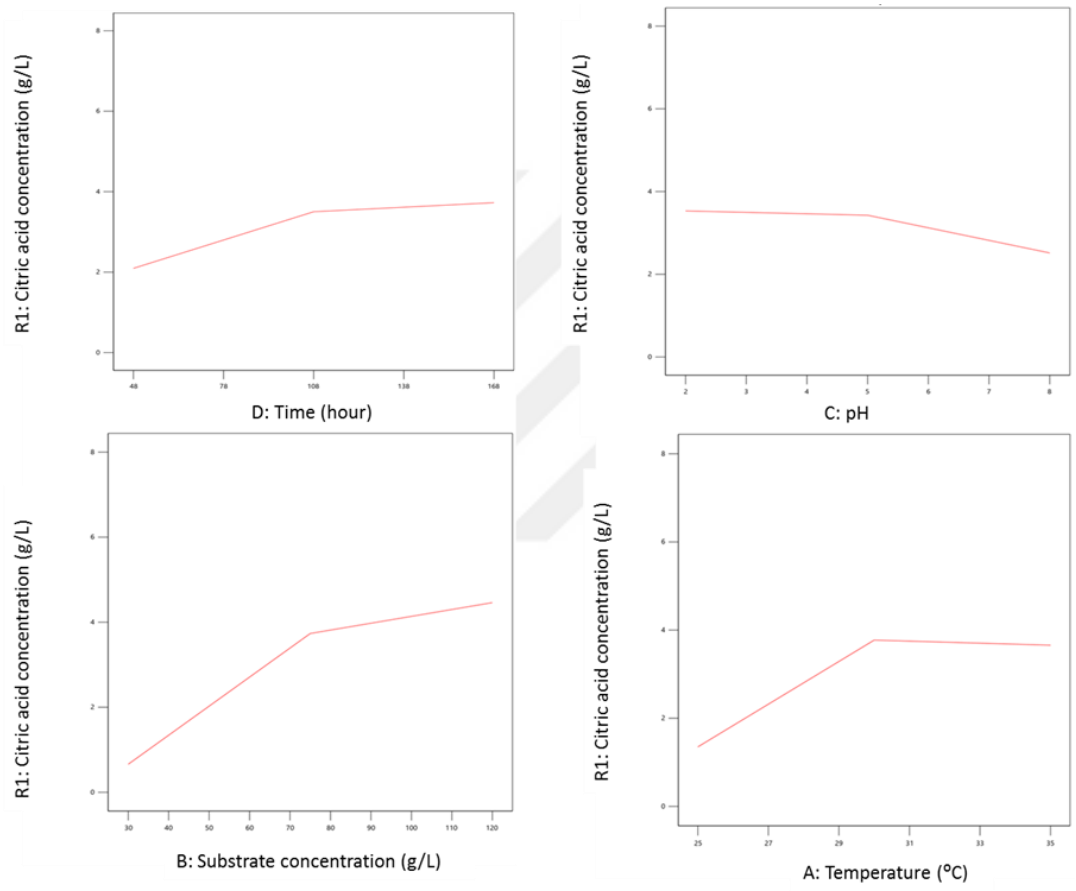


Figure 4.1.21. Correlation between parameters and citric acid concentration

4.2. Discussion

It is known that molasses and corn hydrolyzate are more preferred in the commercial production of citric acid. As a result of the studies, it was demonstrated that the wastes have the potential to be used as a substrate in the production of citric acid. Especially due to the negative effects of industrial wastes on the environment, the use of them in the production of a wide range of products provides significant gains. As seen in the Table 4.2.1., many studies have investigated the use of wastes such as orange, banana and apple as substrate in citric acid fermentation.

Table 4.2.1. Citric acid production using wastes

Microorganism	Substrate	Citric Acid Concentration	Reference
A. niger LCFS 5	Cashew apple juice	92.8 g/L	Adoeye and Lateef (2021)
A.niger sp.	Tangerine and pineapple peels	137.2 g/kg	Kumar et al. (2020)
A. niger sp.	Cassava peels	4.9 g/L	Ajala et al. (2020)
A. niger sp.	Pomegranate peels	19.39 g/L	Sutradhar, Saha, and Chakravarty (2020)
A. niger MT-4	Mulberry pomace	24.6 g/L	Aidynova, Arslan, and Aydoğan (2020)
A. niger sp.	Pineapple waste	15.51 g/L	Ayeni et al. (2019)
A. niger sp.	Hydrolysed potato peels	170.22 mg/L	Makut and Ekeleme (2018)
A. niger sp.	Cassava peels-malted sorghum	88.73 g/L	Adeoye, Lateef and Gueguim-Kana (2015)
A. niger van Tieghem 1867	Orange peels with sugar cane molasses	640 g/kg	Hamdy (2013)
A. niger UABN 210	Banana peels	82 g/kg	Kareem and Rahman (2013)
A. niger ATCC 9142	Valencia orange peels	193 mg/g	Torrado et al. (2011)
A. niger van Tieghem MTCC 281	Apple pomace	4.6 g/100g	Kumar, Verma and Bhalla (2010)
A. niger 14/20 *A. niger* 79/20	Mixture of pumpkin and cane molasses	14.86 mg/mL 14.44 mg/mL	Majumder et al. (2010)

The production of citric acid by cassava peels was investigated by Ajala et al (2020). The study was carried out with two different methods, solid-state and submerged fermentation. As a result of solid-state fermentation, 4.1 g/L citric acid was obtained, while 1.8 g/L citric acid was obtained with submerged fermentation.

In particular, apple pomace is frequently preferred as a substrate in the production of citric acid. In a study, the citric acid production potential of hazelnut pomace was investigated by using solid state fermentation method. As a result of the study, 4.6 g/100 g citric acid was obtained from *A. niger* strain and apple pomace under optimum conditions (Kumar et al., 2010).

Sutradhar et al. In the study by al (2020), pomegranate peels were selected as substrate and citric acid production by submerged fermentation was investigated. In the study, 19.39 g/L citric acid concentration was reached and contributed to the evaluation of an industrial waste in citric acid production.

Table 4.2.2 includes some citric acid optimization studies found in the literature. These studies show that optimizing the parameters affecting fermentation in citric acid production is important for obtaining high-yield citric acid. As can be seen in the table, different methods were preferred for optimization, and the coefficient of determination was determined as > 0.7. The R^2 value gives the percent probability of citric acid production under optimum conditions.

Table 4.2.2. Citric acid optimization studies

Strain	Medium	Parameters	Statistical Method	Citric Acid Concentration	Reference
Aspergillus brasiliensis ATCC 16404	Banana pseudostem pretreated with paper wastewater	Desorbent (acetone) concentration (1 v/v), ammonium nitrate concentration (0.50 w/v), temperature (35^{0}C)	BBD 17 runs	14,408 g/L	Laltha, Sewsynker-Sukai, and Kana (2022)
A. niger ATCC 16888	Algeria dates GHARS and MECH DAGLA	Methanol amount (4%), initial pH (3), fermentation time (8 days), temperature (30^{0}C)		42.7 g/L: GHARS 39.35 g/L: MECH DAGLA	Chergui et al. (2021)
A. niger sp.	Sweet potato peel starch	Carbon source (97.25%), nitrogen concentration (1.25% w/v), pH (6.5), fermentation time (7 days)	Simplex mixture design 22 runs R^2 = 0.948	4.36 mg/mL	Aboyeji et al. (2020)
A. niger	Apple pomace and corn steep liquor	Apple pomace concentration (33,81 g/L) Corn steep liquor concentration (42,5 g/L) Methanol (2.05 % v/v) Methanol addition time (33 h) pH (4.54) Temperature (32.88^{0}C)	BBD 54 runs R^2 = 0.92	68.26 g/L	Sekoai et al. (2018)
A. niger sp.	Cassava peels	Substrate concentration (0.50 g/100 mL), Fermentation time (84 h), Inoculum size (6 %), Initial pH (5)	CCD 29 runs R^2 = 0,75	88.73 g/L	Adeoye, Lateef, and Gueguim-Kana (2015)
A. niger	Coffee husk and lemon peel pomace	Coffee husk (6%) Lemon peel pomace (10%) pH (5) Time (120 h)	BBD 29 runs R^2 = 0.7034	103 g/kg	Selvankumar et al. (2014)
A. niger 9142	Corn starch	Substrate concentration (50 g/L), pH (2), Temperature (25^{0}C)	BBD 15 runs R^2 = 0.976	31.96 g/L	Amenaghawon et al. (2013)
A. niger NRRL 567	Apple pomace	Moisture (75% v/w) Inducer: Methanol (3 % v/w) and ethanol (3% v/w)	CCD 13 runs R^2 = 0,91	342.41 g/kg	Dhillon et al. (2011)
A. niger NRRL 567	Peat moss	Phytate (19 g/kg) Olive oil (49 g/kg) Methanol (37 g/kg)	CCD 13 runs R^2 = 0.997	354.8 g/kg	Barrington and Kim (2008)

In the citric acid optimization study conducted by Adeoye, Lateef, and Gueguim-Kana (2015), wild *A. niger* species were mutated by UV using cassava peels as substrate and optimum parameters for citric acid production of these species were investigated using RSM's Central Composite Design. *A. niger* strain exposed to UV for 10 minutes was found to be the most productive strain with 9.4 g/L citric acid and the study was carried out with this strain. The parameters to be optimized were determined as substrate concentration, fermentation time, inoculum size, and initial pH. Optimum conditions in the study were found to be 0.50 g/100 mL substrate concentration, pH 5, 6% inoculum size, and 84 hours of fermentation time. In the model created with CCD, a process model with 0.75 R^2, and 88.73 g/L citric acid yield is given. According to Analyses of Variance (ANOVA) data, substrate concentration, pH, and inoculum size were found to be important. In the study, the relationships between substrate concentration and fermentation time, pH and substrate concentration, substrate concentration and inoculum size, and inoculum size and fermentation time were shown with response surface graphics.

Amenaghawon et al. (2013) optimized citric acid production conditions from corn starch with *A. niger* 9142 strain. In the study conducted with Box-Behnken Design (BBD), the effects of substrate concentration, medium pH, and fermentation temperature on citric acid yield were investigated simultaneously. A three-variable and three-level model consisting of 15 runs was designed with BBD. As a result of the experiments and the data obtained with BBD, the optimum conditions for the substrate concentration were 50 g/L, pH 2, fermentation temperature 25^0C, and the maximum amount of citric acid was calculated as 31.96 g/L. According to ANOVA data, R^2 was calculated as 0.976. According to the ANOVA table created for the quadratic model of citric acid concentration, it was found that the model designed for the study, all the parameters determined for optimization, and the interactions of the parameters except the substrate concentration and temperature relationship were significant.

Aboyeji et al. (2020) investigated the optimization of media components and fermentation conditions in the production of citric acid from *A. niger* spp. using sweet potato peel starch as a carbon source. The study was carried out under submerged fermentation conditions and two sets of optimization studies were carried out, including low resolution of two factorials, and 3-level design algorithms. The study showed that the effects of carbon source, nitrogen concentration, pH, and fermentation time on citric acid production were significant and optimum conditions were 97.25%, 1.25% w/v, 6.5 and 7 days, respectively. The R^2 of the model

was calculated as 0.948. While the expected amount of citric acid according to the determined optimum conditions was 3.22 mg/mL, as a result of experimental studies, citric acid production was found to be 4.36 mg/mL.

According to Chergui et al. (2021) optimized citric acid production conditions from *A. niger* ATCC 16888 strain using downgrade Algeria dates GHARS and MECH DAGLA as substrates. The parameters to be optimized were determined as methanol amount, initial pH, fermentation time, and temperature. The maximum citric acid yield for both types of dates was obtained after eight days of incubation in 4% methanol at pH 3 at 30^0C, and the yield was calculated as 0.28 g/g for GHARS and 0.32 g/g for MECH DAGLA. Under optimum conditions, 42.7 g/L citric acid was produced in the medium containing GHARS and 39.35 g/L in the medium containing MECH DAGLA. Optimum conditions were repeated three times for both date species, and the results were calculated with an ANOVA. According to the ANOVA values, it was concluded that pH, methanol concentration, and temperature did not have a statistically significant effect on citric acid for both substrates, but the fermentation time was significant.

Optimization of citric acid production conditions with banana pseudostem pretreated with paper wastewater and *Aspergillus brasiliensis* ATCC 16404 strain was investigated by Laltha, Sewsynker-Sukai, and Kana (2022). In the study, the experimental design created with Box-Behnken Design includes 17 runs. Optimization parameters are desorbent (acetone) concentration, ammonium nitrate concentration, and temperature. The suitability of the model was evaluated with ANOVA, and it was found that the model was important and the most important parameter was the acetone concentration. When optimized conditions (1 v/v acetone, 0.50 w/v ammonium nitrate, 35^0C) were met, 14.408 g/L citric acid was obtained.

This study, it was aimed to produce citric acid from grape pomace. It was studied by submerged fermentation in 100 mL mediums in 250 mL flasks. With Box-Behnken Design, optimum conditions were determined as 120 g/L grape pomace concentration, pH 5, 30ºC, and 168 hours and 7.57 g/L citric acid concentration were reached. The fact that the coefficient of determination (R^2) is 0.936 has proven that the model is significant and that citric acid production will be realized by submerged fermentation from the grape pomace under the determined optimum conditions.

5. CONCLUSIONS

In this study, optimum conditions for nutrient, temperature, pH, and time parameters were determined for citric acid production from A. niger in grape pomace fermentation medium using the Response Surface Methodology Box-Behnken Design.

Experimental studies and statistical analyzes according to the model created have shown that substrate concentration, pH, fermentation time, and temperature directly affect citric acid production.

In the study, it was shown that the concentration of citric acid produced during fermentation was correlated with substrate concentration, pH, fermentation temperature, and time with a validated quadratic regression model.

As a result, a model that represent citric acid production was obtained. The model was linear with R^2 value of 0.94 indicating a reasonable fit of the model. The quadratic regression model was able to predict with a high level of confidence the concentration of citric acid produced during fermentation by *A. niger*.

The combination of optimum fermentation conditions is 120 g/L substrate concentration, initial pH 5.00, 30^0C fermentation temperature, and 168 hours of fermentation time. Under these conditions, the maximum citric acid concentration was 7.57 g/L. As an overall conclusion, it has been shown that grape pomace could be a valuable substrate in the production of citric acid.

6. RECOMMENDATIONS

When the optimization studies in the literature are examined, it is seen that the citric acid concentrations obtained are higher than in this study. However, this difference may be due to fermentation types, microorganisms, and working volume. Although the citric acid concentration obtained in the study was lower, the fact that the study was carried out with submerged fermentation provides convenience for its application in industrial production. In addition, since the study was conducted with a medium volume of 100 mL in 250 mL flasks, the amount of citric acid that can be obtained is not expected to be very high. In further studies, it is expected that the citric acid concentration to be obtained will be higher when fermentation is carried out in bioreactors by scaling up and providing the same conditions. In addition, with the realization of bioreactor studies, more consistent results will be obtained as all parameters will be more controllable compared to the study carried out in the flask.

In this study, microorganisms were taken directly to the fermentation medium, which prolonged the adaptation process of microorganisms to the fermentation medium, thus the fermentation process was prolonged. If a 24-36 hours seed fermentation with the same fermentation medium is added to the study before the citric acid fermentation, the microorganism will adapt to the fermentation conditions faster and thus the fermentation time will be shortened. In addition, it is expected that the citric acid concentration obtained will be higher since the microorganism that gets used to the environment will consume citric acid less to maintain its energy metabolism.

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