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# Influences of phenolic compounds on citric acid productivity by *Aspergillus niger* in stirred fermentor

**Bekir Sıtkı Çevrimli[1] · Ergin Kariptaş[2] ✉ · Ahmet Yaşar[3] · Mustafa Yiğitoğlu[4]**

1 Department of Chemistry, Atatürk Vocational School, Gazi University, Ankara, Turkey

2 Department of Biology, Faculty of Sciences and Arts, Ahi Evran University, Kirsehir, Turkey

3 Department of Chemistry, Faculty of Sciences and Arts, Gazi University, Ankara, Turkey

4 Department of Chemistry, Faculty of Sciences and Arts, Kirikkale University, Kirikkale, Turkey

✉ Corresponding author: ekariptas@ahievran.edu.tr
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**Abstract** Influences of phenol, α-naphtol and β-naphtol, which are toxic chemicals, on citric acid biosynthesis and biomass in the artificial culture setting of *Aspergillus niger* using batch fermenter are examined in the most favorable fermentation conditions, and a model is proposed. Addition of certain concentrations of phenol, α-naphtol and β-naphtol to the culture increases the citric acid production. According to this model, maximum citric acid concentration is 48.3 g $L^{-1}$ in a culture that does not contain any toxic chemicals, whereas the maximum concentrations obtained in cultures containing 25 mg $L^{-1}$ phenol, 25 mg $L^{-1}$ alpha-naphtol and 15 mg $L^{-1}$ beta-naphtol are 62.5 g $L^{-1}$, 78.1 g $L^{-1}$ and 86.0 g $L^{-1}$, respectively. Moreover, addition of toxic chemicals to the culture reduces fermentation time by 24 hrs.

**Keywords:** *A. niger*, citric acid, fermenter, toxic chemicals (phenols)

## INTRODUCTION

Citric acid is one of the organic acids of which world market in growing every year and is extensively used in food and pharmaceutical industries. It is produced mainly by submerged, surface and solid state fermentation using *Aspergillus niger* from different sources of carbohydrates. For commercial reasons, the use of molasses, sucrose or glucose syrups are favoured (Ali et al. 2002; Roukas and Kyriakides, 2002; Haq et al. 2004; Moataza, 2006; Papagianni, 2007). Although conventional citric acid production by submerged culture of high-yielding strains of *Aspergillus niger* has been optimized, there is still interest in redesigning the traditional manufacturing process to increase yield and subsequently minimize overall operating cost (Pazouki et al. 2000; Ali et al. 2002; Çevrimli et al. 2009; Çevrimli et al. 2010).

It has been shown that citric acid production by *A. niger* is markedly influenced by a number of culture parameters such as the nutritional composition of the media, trace elements, temperature, pH, dissolved oxygen tension, aeration, deficiency of manganese, alcohols, lipids, amino acids and toxic chemicals (Pera and Callieri, 1999; Adham, 2002; El-Holi and Al-Delaimy, 2003; Khosravi-Darani and Zoghi, 2008; Khosravi-Darani et al. 2008). Several researchers have tried to improve the production of citric acid by various additives. The trace elements such as iron, zinc, copper and manganese present a critical problem during submerged fermentation of crude substrates. Copper and cadmium inhibited the growth, as well as citric acid production (depending on the heavy metal concentrations) of citric acid producing *Aspergillus niger* (Tsekova et al. 2000). The concentration of these heavy metals, therefore, should be decreased well below that required for optimal growth, as well as maximum citric acid production (Haq et al. 2002; Moataza, 2006). There are various literature reports showing that the production of citric acid by *A. niger* can be increased by the presence of alcohol in the fermentation medium (Hang and Woodams, 1985; Yaykaşlı et al. 2005).

As yet, few attempts have been made to examine the production of citric acid with toxic chemicals. Qureshi and Qadeer (1987) observed that the increase in citric acid production may be due to either the direct effect of phenols on the growth process or to the inhibition of enzymes involved in further metabolism of citric acid.

The present study is undertaken mainly to determine the effect of addition of toxic chemicals such as phenol, α-naphtol and β-naphtol on citric acid synthesis by *A. niger* in a stirred fermenter.

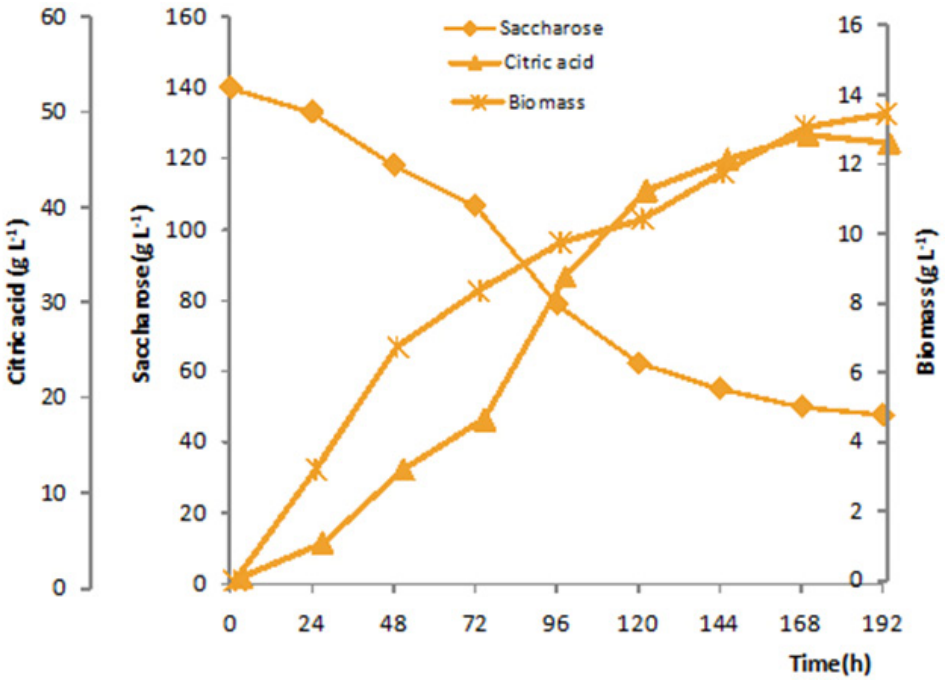


**Fig. 1 Citric acid production without toxic chemicals in batch culture.**

## MATERIALS AND METHODS

### Organism and culture maintenance

*A. niger* ATCC 16404 was used throughout this study. The cultures of *A. niger* was maintained on potato dextrose agar (PDA, Difco) slants at 4ºC and sub-cultured at intervals ranging between 15-30 days. The cultures were incubated on PDA petri dishes at 30ºC for 7 days. The sporulated culture was scraped off and suspended in 5.0 mL of sterile-distilled water to prepare the inoculum. All culture media were sterilized by autoclaving at 121ºC for 20 min.

### Fermentation media and culture conditions

Fermentation procedure carried out in the present study was developed according to the method of Ali et al. (2002). The fermentation was carried out in a 5 L glass bioreactor (Unises 1 Model) with a working volume of 4 L. The medium having initial pH 3.0 was autoclaved at 121ºC for 20 min. After cooling, the fermentation medium composed of (g $L^{-1}$) *saccharose*, 140; $(NH_4)_2SO_4$, 3.0; $KH_2PO_4$, 3.0; $MgSO_4.7H_2O$, 0.5; $NH_4Fe(SO_4)_2.12H_2O$, 0.86; $ZnSO_4.7H_2O$, 0.44; and $CuSO_4.5H_2O$, 0.24 was added to the fermenter. The medium was inoculated with 5 ml of vegetative inoculum under aseptic conditions. The incubation temperature was kept at 30 ± 1ºC throughout the fermentation period of 168 hrs (7 days). After the aeration rate in the fermenter medium was adjusted to maximum, the experiments were carried out with a Maria Model 9071 type probe to adjust dissolved oxygen at 8,2

mg/L. Agitation speed of the stirrer was 200 rpm. Sterilized silicone oil was used to control foaming during fermentation. At appropriate time intervals, fermentation broth (25 mL) was removed from the reactor and transferred to a weighed Whatman filter paper (no. 541) to remove mycelium. The filtrate was washed three times with distilled water, dried at 105ºC to a constant mass and weighed as the biomass.

After the fermenter was induced to produce citric acid, phenol was added to fermenter medium so that its concentration would be 5 mg $L^{-1}$ and the experiment which was to continue for 7 days was initiated. These procedures were repeated for each concentration, after phenol concentration in the batch fermenter medium was set to 15, 25, 35, 45, 55 and 65 mg $L^{-1}$. All experiments conducted for phenol were then carried out in the same way for α-naphtol and β-naphtol.

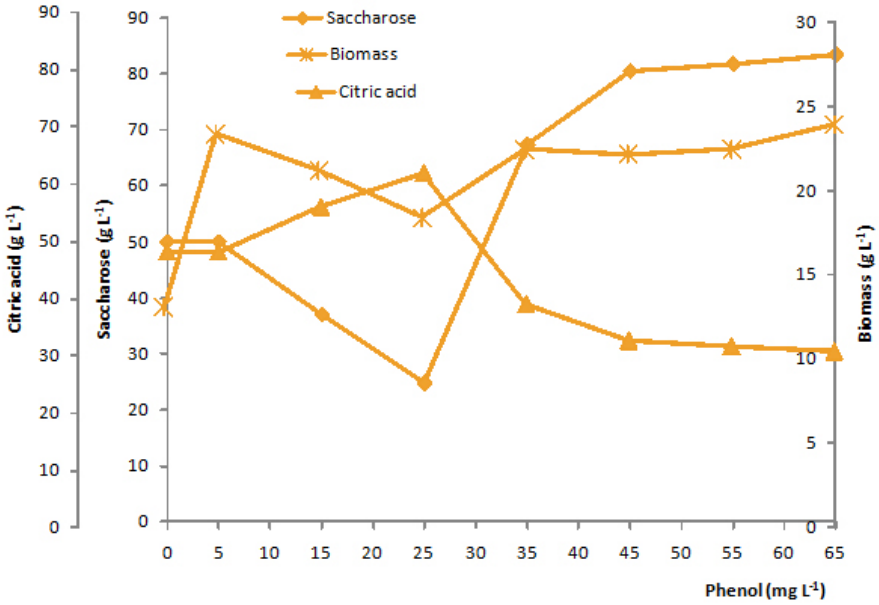


**Fig. 2 Effect of phenol concentration on citric acid, saccharose and biomass concentrations.**

## Analytical methods

The citric acid and sucrose concentration were assayed by the method of Marier and Boulet, (1958) and Dubois et al. (1956) respectively. Ammonium was determined by the method of Weichselbaum et al. (1969).

## RESULTS AND DISCUSSION

Figure 1 shows the results obtained using the standard medium and operating conditions. Following a 30 to 35 hrs period of biomass formation, citric acid production started and reached the maximum level of 48.3 g $L^{-1}$ in 7 days. Biomass concentration was 13.08 g $L^{-1}$. Saccharose was consumed depending on the amount of synthesized citric acid and biomass amount, and 50 g $L^{-1}$ of saccharose was left in the medium after fermentation.

Several concentrations of phenol, α-naphtol and β-naphtol were added to optimum citric acid production media to investigate their effect on the mould morphology and citric acid production. We observed that the morphology of *A. niger* was little affected by the addition of toxic chemicals. It remained the same as that of control culture.

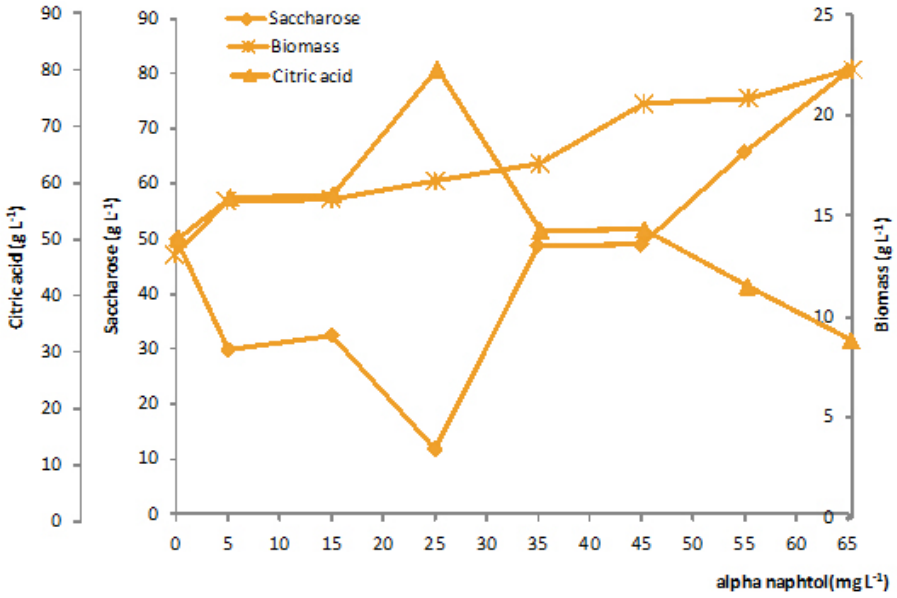


**Fig. 3 Effect of α-naphtol concentration on citric acid, saccharose and biomass concentrations.**

### Influences of toxic chemicals on citric acid biosynthesis

An overall evaluation of results shows that maximum concentrations of citric acid obtained from a fermenter medium containing toxic chemicals are higher than those obtained from fermenter media without toxic chemicals. Furthermore, it was observed that the time that lapsed for maximum citric acid concentrations to form in the fermenter medium with toxic chemicals was 24 hrs shorter than that passed in the fermenter medium without toxic chemicals.

**Phenol.** Effects of various concentrations phenol (15 to 65 mg $L^{-1}$) on citric acid synthesis, saccharose and biomass amounts are presented in Figure 2. Significant increase in citric acid production was recorded at concentration 20-25 mg $L^{-1}$. Addition of 25 mg $L^{-1}$ phenol to the culture elevated citric acid production to 62.5 g $L^{-1}$. Addition of more than 25 mg $L^{-1}$ of phenol to the fermentation medium was seen to reduce citric acid production. Addition of a mean of 65 mg $L^{-1}$ phenol reduced citric acid production to the level of 30 g $L^{-1}$. The value obtained 62.5 g $L^{-1}$ is higher than 48,3 g $L^{-1}$ which is the maximum citric acid concentration of a fermenter medium that does not contain phenol.

**α-naphtol.** Effects of α-naphtol concentration on citric acid, saccharose and biomass concentrations are presented in Figure 3. Addition of 25 mg $L^{-1}$ of α-naphtol to the culture elevated citric acid production to 78.1 g $L^{-1}$. This value is higher than the maximum citric acid concentration of 48,3 g $L^{-1}$ obtained in the fermenter medium without α-naphtol. Addition of more than 65 mg $L^{-1}$ of α-naphtol was observed to reduce citric acid production to the level of 35 g $L^{-1}$.

**β-naphtol.** Figure 4 presents the effects of β-naphtol concentration on amounts of citric acid, saccharose and biomass. Citric acid fermentation greatly increased by the addition of β-naphtol. The highest citric acid concentration of 86 g $L^{-1}$ obtained in the experiments where β-naphtol was used as the toxic chemical was reached with 15 mg $L^{-1}$ α-naphtol. Addition of a mean of 65 mg $L^{-1}$ β-naphtol reduced citric acid production to the level of 30 g $L^{-1}$.

It was found that all concentrations of phenol**,** α-naphtol and β-naphtol in the studied interval added to the fermentation media led to an increase in biomass amount. It is obvious that newly formed cells will synthesize more citric acid. A previous study showed that addition of ammonium and saccharose

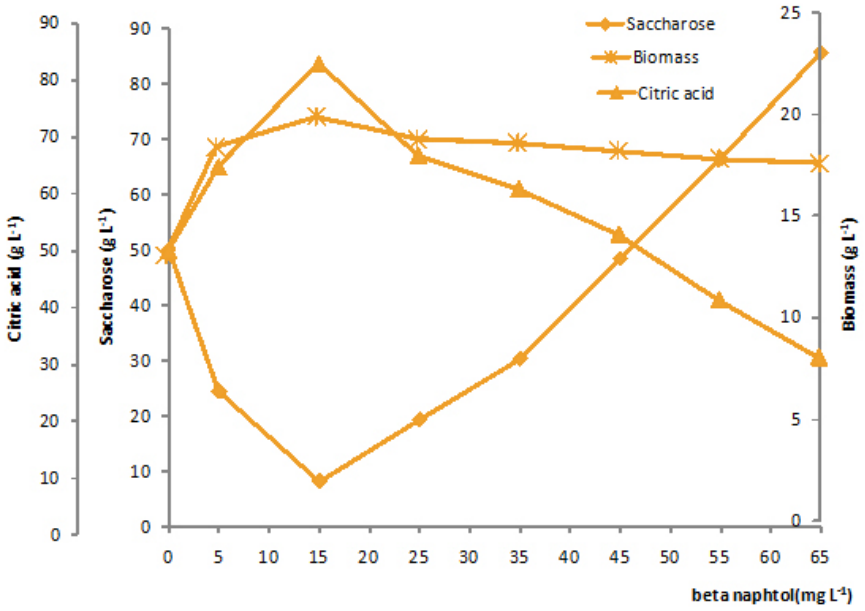


**Fig. 4 Effect of β-naphtol concentration on citric acid, saccharose and biomass concentrations.**

(Yiğitoğlu and McNeil, 1994) to the fermentation media caused synthesis of new cells, thus increasing citric acid production.

Similarly, acid production was increased 3 folds in another study with the addition of methyl alcohol to the culture (Hang and Woodams, 1985). Although the mechanism by which addition of alcohol affects citric acid production in this manner is unknown, it was assumed that methanol addition caused the microorganism tolerate $Fe^{+2}$, $Zn^{+2}$ and $Mn^{+2}$ (Marison, 1988). Phenol, α-naphtol and β-naphtol used in this study may form weak covalent bonds with $Fe^{+2}$, $Zn^{+2}$ and $Mn^{+2}$ found in the solution media. Unshared electrons found on the oxygen of these molecules may interact with $Fe^{+2}$, $Zn^{+2}$ and $Mn^{+2}$ ions. Acting as a sort of adsorbent, these molecules arrest these elements, which are superfluous than the microorganism needs. Thus, the maximum level in acid production is reached. However, as a considerable amount of trace elements needed by the microorganism is arrested when the amount of toxic chemicals increase too much, citric acid production declines markedly, dropping below the amount of citric acid produced in a culture that does not contain toxic chemicals.

Qureshi and Qadeer (1987) have investigated the influence of addition of toxic chemicals (range 0 to 60 mg $L^{-1}$) such as phenol, α-naphtol and β-naphtol on spore germination, mycelial dry weight and citric acid production in a based glucose medium using *A. niger* EU-1. It has been reported slight increase in citric acid formation in the presence of phenol (20 mg $L^{-1}$) and β-naphtol (20 mg $L^{-1}$). They have concluded that the increase in citric acid production might be due to either the direct effect of these phenols on the growth process *i.e.*, metabolism of *A. niger*, or to the inhibition of ezymes involved in further metobolism of citric acid. In the present study, which used *A. niger* in a batch fermenter medium as the microorganism, effect of toxic chemicals like phenol**,** α-naphtol and β-naphtol on citric acid production was tested, it was established that the values obtained were higher than those obtained in similar studies carried out according to erlen culture method. It is seen that the results obtained are consistent with those of other studies (Qureshi and Qadeer, 1987; Haq et al. 2001). Another reason why citric acid yield is higher than that in other studies is that $DO_2$ saturation in the fermenter medium was not allowed to drop below 80% throughout the experiment (Jianlong, 2000).

Nitrogen concentration's [as $(NH_4)_2SO_4$] being kept at 3 g $L^{-1}$, instead of 2 g $L^{-1}$, increased citric acid yield. The results of this study are similar to the observations of Yiğitoğlu and McNeil (1994). Lastly, by the use of fermenter, instead of erlen culture method, it is assumed that mutation of toxic chemicals increases citric acid concentration and shortens the duration of fermentation, thereby leading to acceleration in substrate consumption (Haq et al. 2001).

Summarizing the previously mentioned results, the addition of certain concentrations of phenol, α-naphtol and β-naphtol to the culture positively affects the citric acid process.

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