Product Concentration, Yield Percentage and Productivity of Citric Acid Formation Using *Aspergillus niger* Isolated from Palm Dates

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ABSTRACT

Aspergillus niger isolated from palm dates was examined to produce citric acid. The isolates from zahdi dates (*A. niger* S11) had the best yield of citric acid with a straightforward and efficient isolation technique without the need for purification of the isolates. The best results of citric acid obtained in the static air, using Czapek dox liquid medium, were 37.116 g/l, 33.742% yield, and 0.0859 g/L·h productivity, and in the 1 vvm air were 47.248 g/L, 42.953% yield, and 0.1094 g/L·h productivity, under temperature 30 °C, mixing speed 150 rpm, pH 4, sugar concentration 11%, and inoculum size 5%. Yield and concentration were 23.139% and 25.453 g/L using Date syrup.

Keywords: *A. niger*, palm dates, submerged fermentation, citric acid.

INTRODUCTION

Citric acid serves various purposes and actively participates in various industrial applications because of its advantageous properties of high solubility and low toxicity. It is extensively used in the production of cosmetics, drinks, pharmaceuticals, food, detergents, and several chemical and metallurgical products (Mattey, 1992; Ates et al., 2002; Soccol et al., 2006; Karthikeyan and Sivakumar, 2010; Ali et al., 2016; Zhang et al., 2017; Campanhol et al., 2019).

According to Currie J. (1917), who adjusted the factors to achieve an increasing amount of citric concentrations in a sugar media as a carbon source, commercial citric acid manufacturing was started. Currie discovered that growing *Aspergillus niger* strains using a surface fermentation method allowed for accumulating large amounts of citric acid (Vandenberghe et al., 2016). Since then, this method, useful in the commercial production of citric acid developed through different carbohydrates, primarily molasses and cane sugar, and particular *Aspergillus niger* strains, has undergone several advancements. The first

organization to use sugar fermentation was carried out in 1923 by Pfizer, an American pharmaceutical corporation, to commercialize citric acid by *A. niger* (Dashen et al., 2014; Show et al., 2015). A Belgian collection called "Citrique Belge" used this project in 1929. (Roehr et al., 1996; Vandenberghe et al., 2016, 2018). Molasses was utilized in 1948 as a cheap feedstock and a substitutional to produce citric acid instead of sucrose. Citric acid was manufactured from normal alkanes (Ethane, Butane, and Propane) using the appropriate species of yeasts and bacteria during the 1960s and 1970s, due to the relatively low price of hydrocarbons at the time (Anastassiadis et al., 2008; Sauer et al., 2013). Nowadays, employing carbohydrates and submerged fermentation with fungus, yeast, and bacteria is the best commercial method for producing citric acid.

Species of fungi, for example: *A. fonsecaeus*, *A. foetidus*, *A. awamori*, *A. phoenicis*, *A. wenti*, *A. carbonaries*, *A. aculeatus*, *Mucor pyriformis*, and *Trichoderma viride*, were investigated their capability to produce citric acid (Dezam et al., 2017). Citric acid can also be produced by bacteria, including *Bacillus subtilis*, *B. licheniformis*,

Corynebacterium spp. (Kapoor et al., 1982) and *Arthrobacter paraffinens* (Vandenberghe et al., 2016). Additionally, several yeast species, including *Candida tropiclaus* (Käppeli et al., 1978), *Candida guilermondi* (Angumeenal et al., 2003), *Candida oleophilis* (Anastassiadis et al., 2006), and *Yarrowia lipolytica* (Förster et al., 2007; Da-Silva et al., 2012; Angumeenal and Venkappayya, 2013) are also producing citric acid (Dhillon et al., 2011; Lee et al., 2015). One drawback of using yeast in citric acid production is that yeast produces extremely large amounts of iso-citric acid, an unfavorable by-product, so mutant strains with a low aconitase activity are selected (Lee et al., 2015). However, *A. niger* is currently the most well-known microorganism for producing citric acid from waste materials due to its easy and secure processing, ability to consume several inexpensive stuff, and high yields (Grewal and Kalra, 1995; Kareem et al., 2010; Nadeem et al., 2010; Dhillon et al., 2011; Mostafa and Alamri, 2012; Angumeenal and Venkappayya, 2013; Rao and Reddy, 2013; Vandenberghe et al., 2016; Dunya and Safaa 2020).

Improving microbial strain is one of the promising approaches to increase the yield of commercially beneficial product results by microbes. Several investigators revealed that mutation "radiation and/or chemical mutagens" along with "selection technique" can be applied to *Aspergillus niger* to enhance citric acid yield (Ikram-ul et al., 2004; Soccol et al., 2006; Rodrigues et al., 2009; Adeoye et al., 2015). Rohr (1983), Kubicek et al. (1985), and Mattey (1992) are reviewed many previously published articles, but part of these articles and screening methodologies are still secret because they carried out by industry and inter in intellectual rights (Kristiansen et al., 2002).

In the current study, wild species of *Aspergillus niger* from local sources, including palm dates, were examined to identify the species and fermentation conditions that led to a high output of citric acid. Sucrose and date syrup were the two carbon sources chosen for the fermentation. The presence, and low prices, of some kinds of palm dates, and waste from palm packaging factories, make it a suitable raw material for producing citric acid in significant amounts in our country and encourage sustainability by increasing lands cultivated by palm trees in an available wide area.

MATERIALS AND MATHODS

Materials

Bromocresol-green, sucrose, Czapek dox agar, and potato dextrose agar (PDA) are return to Himedia. Czapek dox liquid media provided by OXOID. Merck Chemicals and CDH contributed other substances.

Isolation and identification of fungi strains

Aspergillus niger was isolated from soil, window glass dust, and dates. A serial dilution technique was used for the isolation of the fungi. The fungi isolates were cultivated on PDA and kept at 30 °C for seven days in an incubator (Memmert, Germany). Lactophenol cotton blue was used to assist in microscopically observing the generated colonies – identification of *Aspergillus niger* based on their dominance and distinctive physical characteristics. If necessary, recurrent subculturing was utilized to select and purify the isolate strains of fungi.

Primary screening of wild culture

By inoculating the strain on the Czapek-Dox agar medium with the addition of 5% bromocresol-green indicator, *Aspergillus niger* was subjected to a qualitative screening procedure. After being sterilized at 121 °C for 15 minutes, the medium was cooled, inoculated, and incubated for five days at 30 °C then labeled.

Secondary screening

The larger yellow zone on the Czapek-Dox agar medium served as the key indicator of the isolates during the primary screening step, which were chosen for secondary screening. A liquid Czapek dox medium was employed in submerged fermentation for culturing *A. niger* by incubating in a shaking incubator (JSR, Korea) at 150 rpm and 30 °C using 100 ml of this medium (pH=6.8±0.2), sterilized at 121 °C for 15 minutes. The best conditions of the fermentation process were experimented with the submerged method for the perfect strains of higher citric acid yield.

Inoculum preparation

10 ml of distilled water that has been sterilized was added to the 7-day-old culture to create the

spore suspension. Clusters were scraped cleanly with a wire loop under aseptic circumstances to remove the spores. The suspension was then put into a sterilized glass tube. Spores were counted by using a hemocytometer (Neubauer chamber) and adjusting the reading to 5–25×10⁶ spores/mL.

Analysis and measurements

Sugar and acid concentration

The pyridine-acetic anhydride method was used to measure the citric acid concentration (Marier and Boulet, 1958). A UV-Visible spectrophotometer was used to measure the fermentation mixture's absorbance at 427 nm (Shimadzu UV-1800, Japan). The DNS approach was used to measure residual sugar spectro-photometrically (Tasun et al., 1970).

Gravimetric method of mycelia

Filtration and weighing were used to determine Mycelia's wet weight. Mycelia mat was dried overnight at 90 °C using an oven (HYSC, Germany) to calculate the dry weight gravimetrically by a sensitive balance (Haq and Daud, 1995).

Fermentation conditions

Multiple experiments were conducted to investigate the fermentation conditions. The precise study settings were inoculum size (1%, 5%, and 10% (v/v)), sugar content (3%, 7%, 11%, 15%, 18, and 21% (w/v)), and pH (2, 3, 4, 5, 6, and 7). At 30 °C and 150 rpm, the fermentation was carried out in 500 mL Erlenmeyer flasks containing 100 mL of Czapek dox media. Every 24 hours, samples were examined to determine the amounts of citric acid (g/L), residual sugar (g/L), yield, and productivity.

Effect of oxygen on the citric acid formation

The influence of dissolved oxygen on the yield of citric acid was studied by using the batch fermentation system. Air was purified by polypropylene-carbon filters and sterilized by a UV light. Sparger was used for dispersing air bubbles into the fermenter. Glass fermenter (500 mL) equipped with a stone sparger fixed by Teflon pipe was used. Fermentation medium of 300 mL Czapek-dox medium was fed into the fermenter and inoculated by spores of *A. niger* (5–25×10⁶ spores/mL) at 30 °C, 1 vvm, and for 432 h by

submerged fermentation. All equipment's and media were sterilized at 121 °C for 15 minutes befor fermentation.

Fermentation using date syrup

Date syrup is employed as a fermentation medium for forming citric acid. Zahdi dates were used (addition of 3 fold of distilled water to one fold of dates followed by heating to 65 °C for 30 min, filtration, and ultrafiltration). The best strain of *A. niger* for citric acid production, selected from the secondary screening step, was cultivated in an Erlenmeyer flask (500 ml) with 11% palm dates syrup cultured by 5% inoculum (5–25×10⁶ spores/mL). The incubation period was 20 days using a shaking incubator at 150 rpm and 30 ˚C. Citric acid was tested every two days. All equipment's and media were sterilized at 121 °C for 15 minutes before cultivation.

RESULTS AND DISCUSSION

Isolation

The identification process was completed using the colonies' observed features and dependent on their morphological traits for the three local sources soil, window glass dust, and palm dates (Campbell et al., 2013). Figure 1a and 1b shows the *A. niger* appearance and its microscopic conidia isolated from palm dates for the first time of isolation without purification by re-culturing, rather than *A. niger* isolated from other sources by culturing and purification, which is shown in Figure 1 (c and d). The isolation of pure *A. niger* from palm dates without the need for a purification step may be due to the high sugar content of palm dates which is harmful to other microorganisms.

Qualitative screening

Czapek-Dox agar, containing bromocresol green, was used to indicate the most efficient strain in producing citric acid. Yellow zones on the blue color media refer to citric acid formation, and larger zones indicate the most formation of citric acid by the strain. This color exchange from blue to yellow is due to the pH reduction of the media of less than four caused by citric acid formation. Figure 2 illustrates the organic acids detection technique using bromocresol green (including citric acid). Czapek dox agar with bromcrecol green
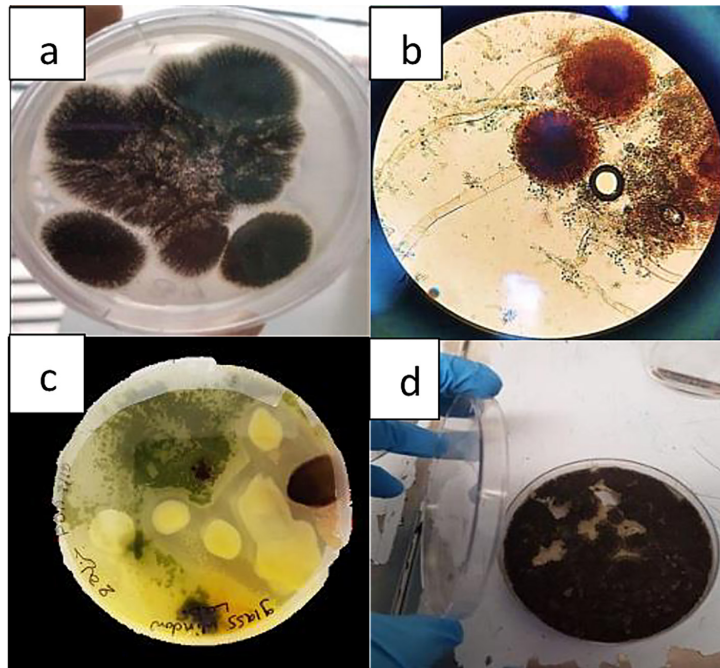
Figure 1. (a) *A. niger* appearance isolated from palm dates, (b) microscopic conidia of *A. niger*,
(c) *A. niger* appearance isolated from soil and window's glass dust before purification,
(d) *A. niger* appearance isolated from soil and window's glass dust after purification
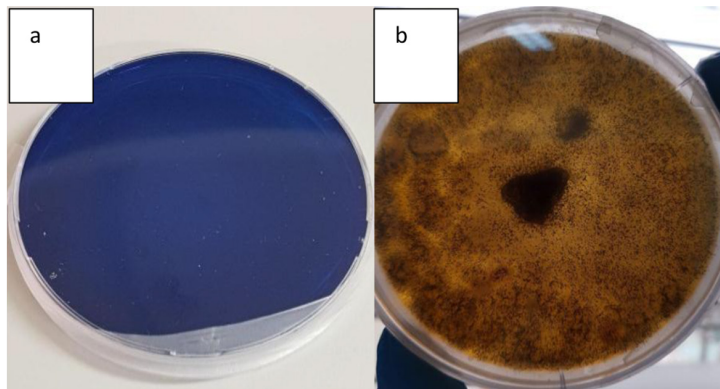
Figure 2. (a) Bromocresol green dye − Czapek dox agar, (b) citric acid formation on Czapek dox agar

was also employed by Almousa et al. (2018) to identify the best strain for citric acid formation.

Quantitative screening

The four isolates of *A.niger* represented by strains 1, 10, 11, and 12 that secluded from the ground, window's dust, "Zahdi" palm dates, and "khstawi" palm dates, respectively, which represent the best production of citric acid, were further screened by using submerged fermentation in Czapek dox liquid medium to obtain maximum citric acid concentration. Figure 3 shows citric acid concentration produced from these isolates using sugar of 3% (30 g/L) including in Czapek medium, PH 6.8±0.2, 150 rpm, 30 °C, and inoculum (1/100 ml) with 5–25×10^6 spores/mL.

Figure 3 shows that strains S12 (from Khstawi dates) and S11 (from Zahdi dates) of *A. niger* obtained the maximum concentrations of citric acid of 6.312 and 7.002 g/l, respectively, at 240 hours, whereas strains from other sources obtained lower citric acid concentrations within 0.252 to 4.2 g/l at fermentation time up to 14 days. The current finding on variations in citric acid synthesis due to various sources of microorganisms was supported by Auta et al. (2014). Figure 4 shows the yield of citric acid for strains
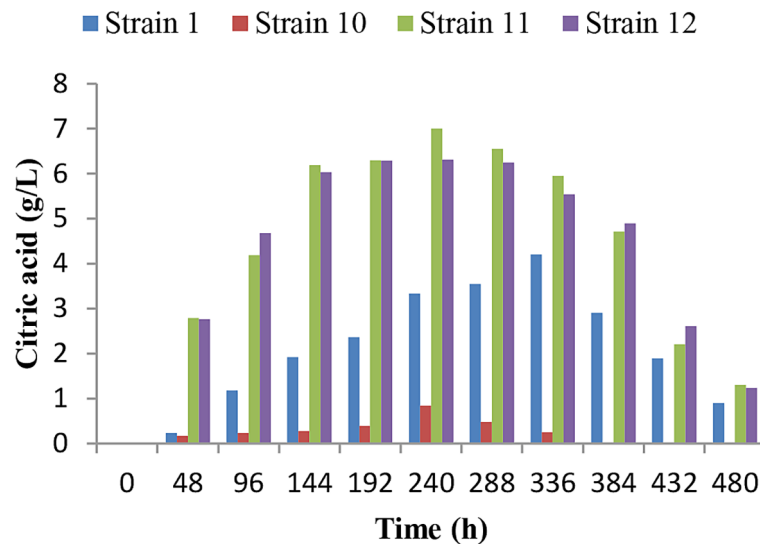
Figure 3. Citric acid concentration; Strain1 from soil, Strain10 from window's glass, and Strain11 and Strain12 from zahdi and khstawi palm dates, respectively

11 and 12. After reaching its peak, the value of citric acid decreased to a low level. The consuming of citric acid by the *A. niger* may be the cause of it, as the concentration of sugar in the broth falls to a level that may not be sufficient for fungi to survive. The palm dates represent an excellent source of *A. niger* isolates due to their simplicity of isolation without contamination by other microorganisms. *A. niger*'s prolonged exposure to the sun's UV rays when perched on palm dates before harvesting may have improved its ability to make citric acid through mutagenesis (Ikram-Ul et al., 2004; Adeoye et al., 2015; Vasanthabharathi et al., 2013).

Study of the fermentation conditions

Inoculum size

Culture media of 1, 5, and 10 ml/100 ml of *A. niger* S11 was investigated to find the effect of inoculum size ($5–25 \times 10^6$ spores/ml) on yield of citric acid (Figure 5). The fermentation conditions were 3% (30 g/L) of sugar, temperature of 30 °C, shaking speed of 150 rpm, and pH 7. Produced citric acid (Figure 5) was increased with elapsed time until it reached 23.34% (7.002 g/L) and 27.09% (8.127 g/L) for inoculum size 1% and 5%, respectively, at incubation time of 10 days, while, it reached 24.96% (7.488 g/L) for inoculum size 10% (v/v) at incubation time of 8 days. After reaching its maximum value, citric acid synthesis decreased as a result of the declining sugar concentration, which caused citric acid to be digested.
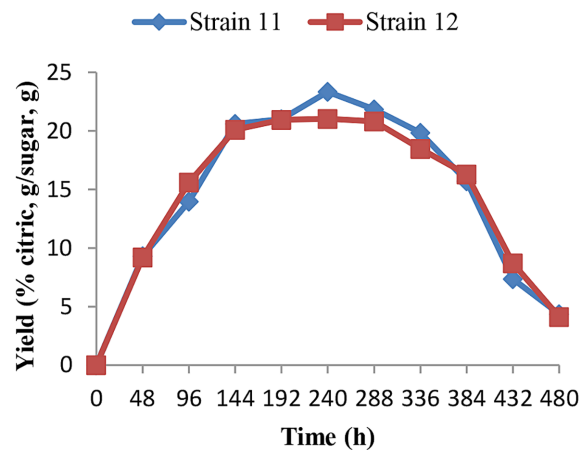
Figure 4. Citric acid yield for the strains S11 and S12 isolated from "zahdi" and "khstawi" palm dates, respectively

From these data, the preferred value of inoculum was 5%, which represents the maximum citric acid formation of 27.09% (8.127 g/L), at a fermentation period of 10 days, rather than inoculum size of 10% (v/v), which gave citric acid close to the former (24.96% or 7.488 g/L), although it was at a lower incubation time of 8 days. That is because of two trends, the first one is the lower quantity of inoculum can be used, which reduces the exertion and cost of inoculum preparation, and the second is the less sensitivity of elapsed time after reaching the maximum yield, where the yield of citric acid decreases only a little 23.58% (7.074 g/L) after 12 days for 5% (v/v) inoculum, while it declined a lot 2.63% (0.789 g/L) after ten
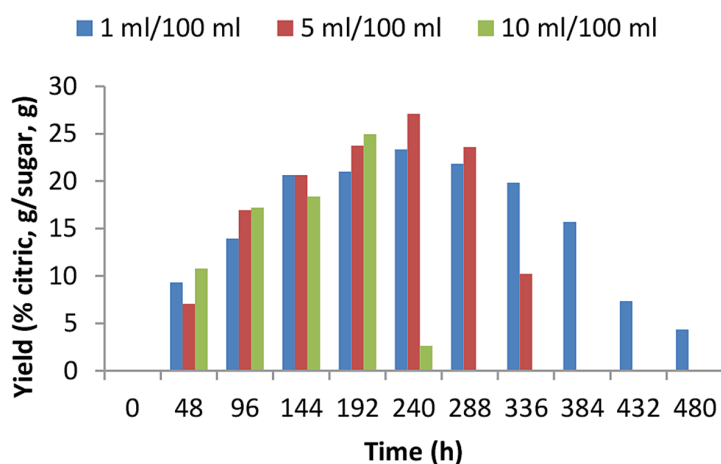
Figure 5. Influence of inoculum size on yield of citric acid

days for 10% (v/v) inoculum, so it is preferred to use non-extreme values of inoculum size that affect citric acid yield.

This finding agrees with others who reported that the 5% size of the inoculum of *A. niger* was the best for the maximum citric acid formation (Almousa et al., 2018). The accumulative productivity of citric acid formation, calculated in the range up to maximum concentration, was plotted against incubation time for the three-inoculum size 1, 5, and 10 mL/100 mL, as shown in Figure 6. Within the incubation time of 2 days, the productivity reached its maximum value at the early stages of fermentation for inoculum size 1% and 10% (v/v), which were (0.058 g/L·h) and (0.067 g/L·h), while, it was less than the maximum (0.053 g/L·h) for incubation size of 5%. The productivity decreased gently at the other stages of fermentation, but generally, it was close to each other at the final stages (at maximum citric acid concentration), where they were 0.039 g/L. h for inoculum size of 5% and 10% at incubation times 10 and 8 days, respectively, while it was 0.029 g/L·h for an inoculum size of 1% at an incubation time 10 days.

Substrate

Different initial concentrations of sucrose, at 3, 7, 11, 15, 18, and 21% w/v (equivalent to 30, 70, 110, 150, 180, and 210 g/L) were examined for their effect on citric acid production as shown in Figure 7. Initial pH were 7, *A. niger* 5% (v/v), temperature 30 °C, and 150 rpm.

Citric acid concentrations were increased as incubation time progressed till they approached maximum values of 8.127, 8.775, 12.609, 12.495, 12.5, and 12.3 g/L at 10,12, 18, 20, and 22 days for sugar concentrations of 3, 7, 11, 15, 18 and 21%
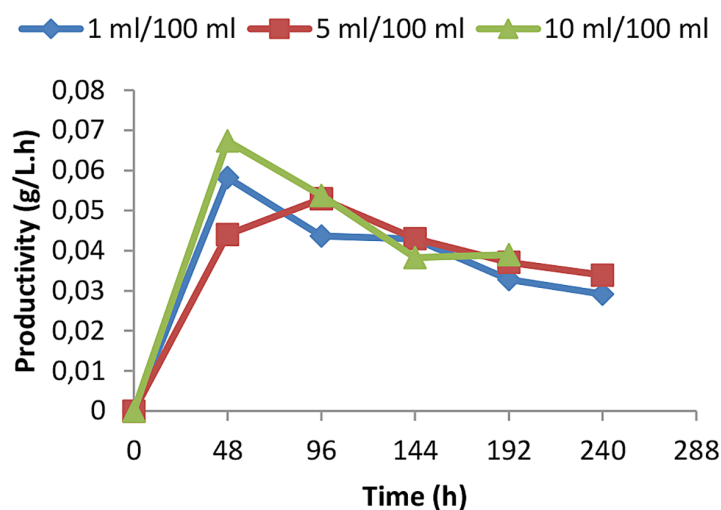

Figure 6. Influence of inoculum size on accumulative productivity of cirtic acid

(w/v), respectively. It is seen from Figure 7 that the most favorable initial sugar concentration was 11% (w/v) which was produced at an incubation time of 18 days, rather than other sugar concentrations, because of the maximum citric acid concentration gained (12.609 g/L), although the incubation time (18 days) was higher than the incubation time at sugar concentrations 3 and 7 % w/v which is 10 and 12 days, respectively, the later gave appreciable lower citric acid concentration. The lower initial concentrations of sugar cause lower citric acid concentrations because of the formation of oxalic acid in the fermentation broth (Ikram-ul et al., 2002). On the other hand, the citric acid yield reached its maximum value of 27.09% (8.127 g/L)

at a sugar concentration of 3%, which was higher than that obtained by a sugar concentration of 11%, which was 11.46% (12.609 g/L), although the opposite effect in citric acid concentration, as shown in Figure 8. A study by Ali et al. (2001) stated that weak mycelia growth under a low sugar concentration causes low citric acid. It prefers (in our opinion) to study the effect of other conditions (pH and aeration) on citric acid production using sugar concentration that obtains the higher concentration of citric acid.

A sugar concentration of 15% gives a maximum citric acid concentration (12.495 g/L) close to that obtained by a sugar concentration of 11% (12.609 g/L) but at a higher incubation time (20
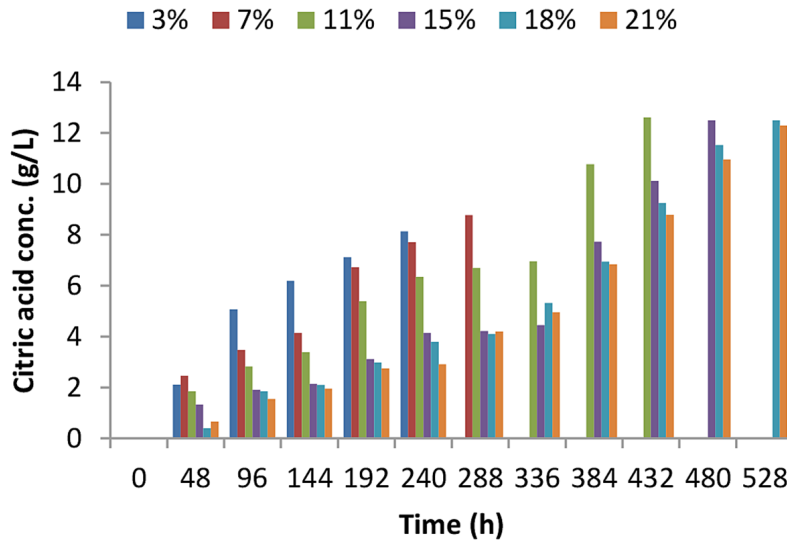

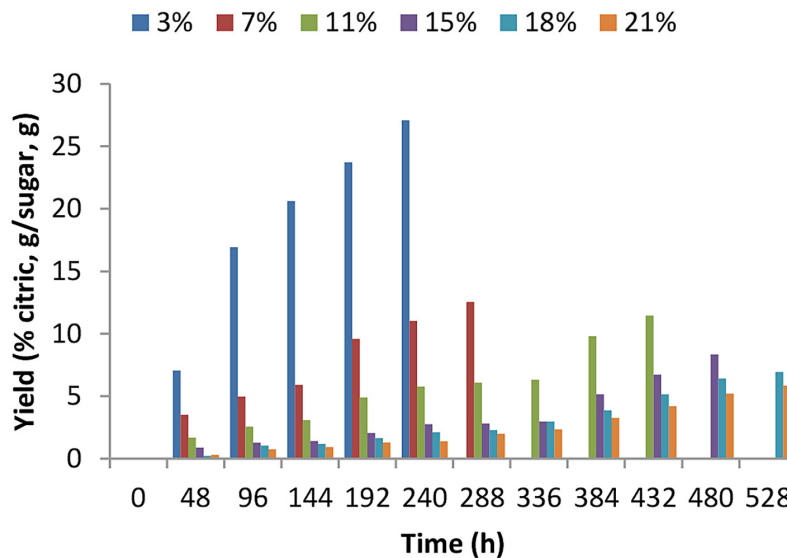**Figure 7.** Influence of sugar on citric acid concentration

Figure 8. Influence of sugar on citric acid yield

days). 18 and 21% (w/v) of sucrose give concentrations of 12.5 and 12.3 g/L, respectively, at 22 days. Accumulation of citric acid and sugar concentration are directly related. Xu et al. (1989) and Show et al. (2015) mentioned that the amount of citric acid produced increases as carbon concentration increases. However, previous research showed that a sugar range of 14–22% obtained the maximum concentration of citric acid (Show et al., 2015; Max et al., 2010). Xu et al. (1989) reported that maximum yield was obtained, by using a sugar concentration of 10% (w/v) of sucrose, fructose, mannose, and maltose,

while for glucose, a sugar concentration of 7.5% (w/v) produced the maximum yield of citric acid.

Figure 9 shows the productivity of citric acid to get maximum yield at 3, 7, 11, 15, 18, and 21% w/v. The productivity had only slight changes in the range of sugar concentration variations, which were 0.0338, 0.0304, 0.0291, 0.0260, 0.0236, and 0.0232 g/L.h at 3, 7, 11, 15, 18, and 21% w/v, respectively. A sugar concentration of 11% is preferred because it obtains the maximum concentration of citric acid in a less period of fermentation.
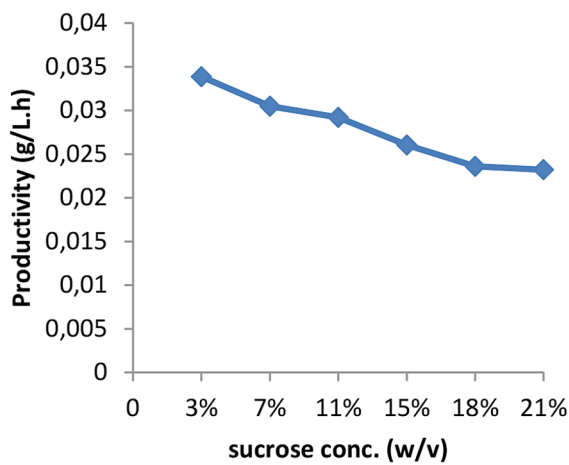
Incubation pH

The effect of pH on the formation of citric acid was explored at pH values of 2, 3, 4, 5, 6, and 7 and shown in Figure 10. The fermentation conditions used in these sets of experiments were: initial sugar concentration of 11% (w/v), inoculum size ($5\text{--}25 \cdot 10^6$ spore/mL) of 5% (v/v), 30 °C, and 150 rpm. Figure 10 describes the variation of citric acid yield at different time intervals. Citric acid yield appeared to have a good response with the variation in pH values. Citric acid yield increased gently at the beginning of the fermentation process and attained 5.16 to 7.25% (5.6745 to 7.975 g/L) at pH 3 to 6 after ten days of incubation. Citric acid yield increased appreciably to higher values by increasing the incubation time and recorded yields of 27.56, 33.74, 28.91, and 21.03% (30.312,
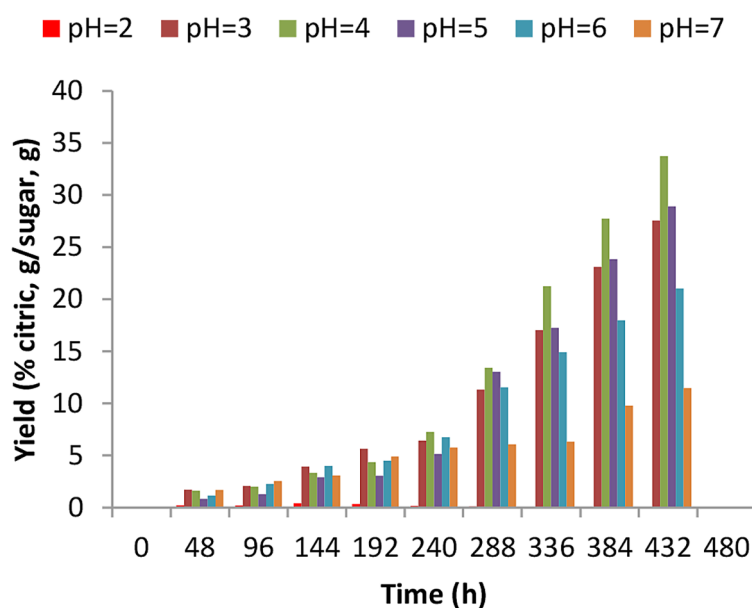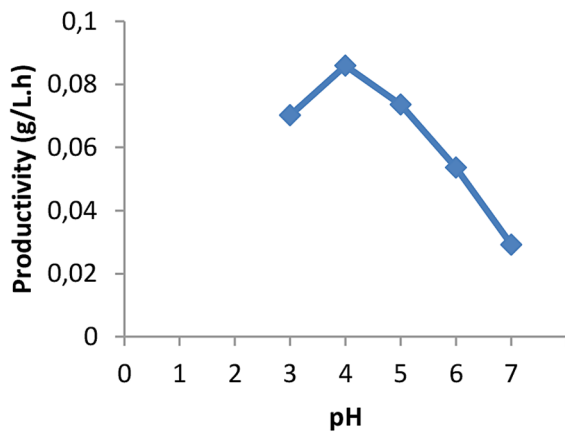

Figure 9. Influence of inoculum size on accumulative productivity of citric acid

Figure 10. Citric acid yield for different initial pH of fermentation process at *A. niger* 5 mL/100 mL, sucrose of 11%, 30 °C and 150 rpm

37.116, 31.806, and 23.13 g/L) at pH 3, 4, 5, and 6, respectively, within 18 days. For pH 7, the increase in citric acid yield happened gradually and stopped with a higher value of 11.46% (12.609 g/L) after 18 days. Only a little citric acid yield was obtained of 0.43% (0.468 g/L) at pH 2 after 6 days of incubation. The inability of *A. niger* to thrive at low pH during the early stages of fermentation may be the cause of the lower citric acid generation at pH 2. (Ikram-ul et al., 2002). From these results, the best initial pH of 4 was required to obtain a maximum yield of citric acid of 33.74% (37.116 g/L) in 18 days. Ali et al.(2001) and Lotfy et al.(2007) found that

the best citric acid formation was at pH values of 3.5 and 4, respectively. According to Shadafza et al. (1976), a higher pH of the fermentation leads to oxalic acid accumulation.

According to the pH track during incubation, the media's pH dropped from the investigation site's starting pH values to low values (2.83 to 1.98) after just two days. Citric acid, which, even at low concentrations, has a substantial acid action, causes this decrease in pH readings. Show et al. (2015) noted that the pH could drop to below 3 due to the metabolic activities of microorganisms like Rhizopus, Penicillium, and Aspergillus. Citric acid productivity is seen in Figure 11 for various beginning pH values. These data concluded that pH 4, which provided the highest productivity and concentration of citric acid, was the most beneficial.

Relationship between concentrations of mycelia, sugar, and citric acid

For an initial sugar concentration of 11% w/v, Figure 12 depicts the progression of the fermentation process of citric acid, sugar, and mycelia concentrations at inoculum 5% (v/v), pH 4, 30 °C, 150 rpm, and 18 days incubation. This figure showed that the citric acid formation was during the exponential phase of *A. niger* pellets (mycelia) generation. The mycelia reached 26.4 g/L with the maximum citric acid formation of 37.116 g/L and sugar reduction to 2.1 g/L. In

Figure 11. The productivity of citric acid for a range of pH at inoculum size 5 mL/100 mL, sucrose of 11%, temperature of 30 °C and 150 rpm
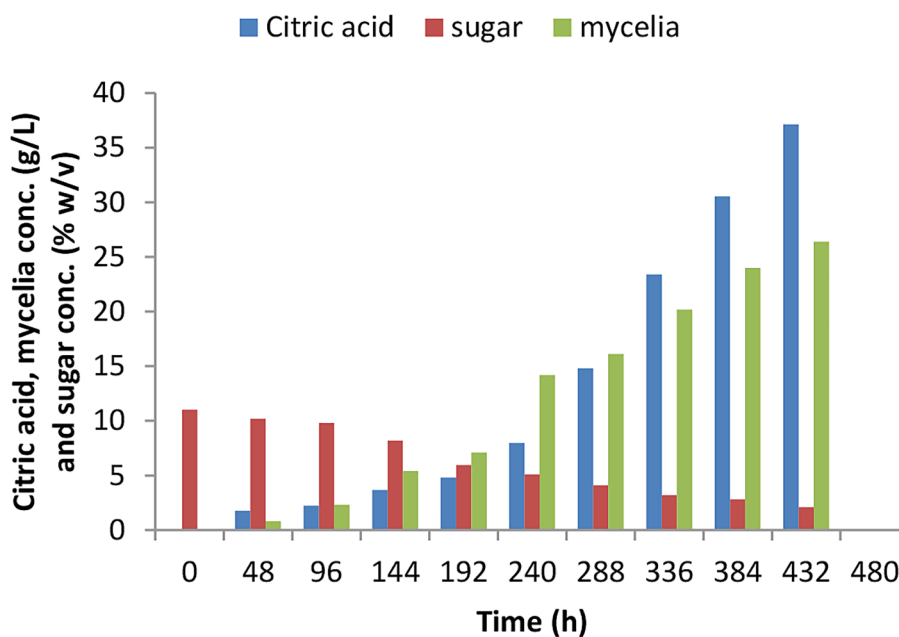
Figure 12. Relationship of mycelia, sugar, and citric acid

various fermentation settings, Lee and colleagues reported a comparable impact (Lee et al., 2015).

Effect of oxygen

Citric acid production by fermentation is aerobic, so aeration is a significant factor affecting citric acid formation. Various parameters, such as the fermenter size, the composition of the medium, and the microorganisms under study, regulate the aeration rate. The influence of dissolved oxygen on the citric acid formation in the Czapek dox liquid medium by *A. niger* S11 is evident (Figure 13).Citric acid formation in the aeration experiment is the highest in comparison with the production under static pressure, at optimum fermentation conditions of 11%

(w/v) of sucrose, 5% (v/v) of inoculum size, and pH 4. Citric acid concentrations under static pressure and 1 vvm were 37.116 g/L (yield 33.742%, productivity 0.0859 g/L·h) and 47.248 g/L (yield 42.953%, productivity 0.1094 g/L·h), respectively. The sugar concentrations and the dry cell weight were 2 g/L and 26 g/L, respectively, for both aeration techniques at fermentation time 432 h. Vandenberghe et al. (2016) reported that the oxygen-air mixture employment in submerged fermentation increases citric acid production but is inapplicable for cost considerations. Relative low aeration rates initiate from 0.1 vvm and rise to 0.5–1 vvm are usually used in industrial applications (Vandenberghe et al., 2016). In the industry, the cost of the process should be taken into account.
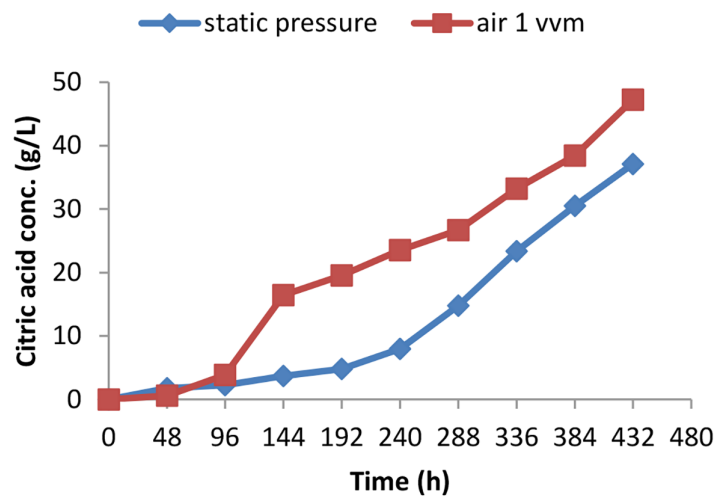

Figure 13. Comparison between citric acid production under static pressure and under oxygen supply of 1 vvm, by submerged fermentation using *A. niger* S11 at 30 ˚C
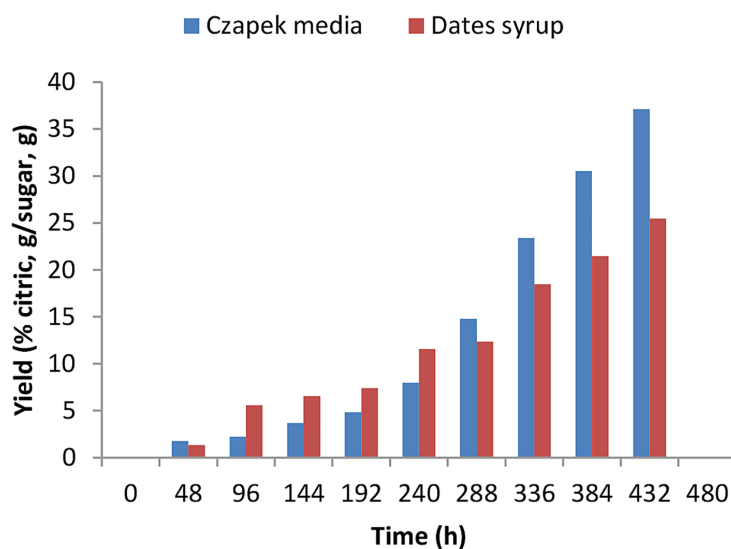

Figure 14. Comparison between dates syrup and Czapek medias

Citric acid from date syrup

Figure 14 compares citric acid production using the Czapek and dates syrup mediums with a sugar concentration of 11% (110 g/L). Without adding additional nutrients, date syrup produced citric acid with acceptable results because it already contained all the necessary ingredients. Maximum citric acid yields were 33.742% (37.116 g/L) and 23.139% (25.453 g/L) on Czapek dox liquid medium and dates syrup, respectively, at 30 °C and 150 rpm for 432 h. In this natural medium, the *A. niger* strain can produce a lot of citric acid, which can be enhanced by mutation, adding trace elements, or alcohol.

CONCLUSIONS

A. niger fungi are widespread. A variety of sources can be used to isolate these fungi. Purifying the isolated strains from Zahdi dates was unnecessary because they were already pure. In addition, it provided the highest yield compared to other strains isolated from other sources. The best citric acid formation (37.116 g/l, yield 33.742%, productivity 0.0859 g/L·h) was shown to be possible at 5% (v/v), 11% (w/v), and pH 4 in the presence of static air. A significant increase could be obtained using aeration of 1 vvm of air (47.248 g/L, yield 42.953%, productivity 0.1094 g/L·h). It was appropriate to make citric acid using dates syrup (25.453 g/L, yield of 23.139%).

Acknowledgements

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