Palm dates as a source for isolation of Aspergillus niger to produce citric acid by submerged fermentation; kinetics study

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Abstract. The study discussed here deals with the isolation of Aspergillus niger from palm dates, the formal and the most famous fruit in Iraq, to test and qualify this fungus isolate for its ability to produce citric acid. Submerged fermentation technique was used in the fermentation process. A. niger isolated from “Zahdi” Palme dates was used in the study of the fermentation kinetics to get the production efficiency of citric acid. Kinetics of CA production via fermentation by A. niger S11 was evaluated within 432 h fermentation time and under submerged conditions of 11% (w/v) sucrose, 5% (v/v) inoculum size, pH 4, 30 °C and 150 rpm. The maximum citric acid produced was (37.116 g/l). Kinetic criterions (product, exhaustion and growth rates “Cp/t, Cs/t, Cx/t”, yield coefficients “Yp/s, Yp/x, Yx/s”, and specific fermentation rate constants (“q_p, q_s”)) were studied and discussed to reach to a logical explanation of fermentation process.

Keywords: Citric acid, Aspergillus Niger, dates, submerged fermentation, fermentation kinetics.

1. Introduction

Citric acid (CA) is a weak acid with various uses in industries. Citric acid is a “tricarboxylic acid” (C₆H₈O₇.H₂O) and it was first isolated by Carl Scheele in 1784 in England. The premier trade origin of CA was from citrus like lime and lemon imported from Italy. For nearly a century, Italian industrialists had a monopoly on CA production [1]. Currently, the production is depending completely on microbial sources. The method of fermentation is considered the main technology in the field of citric acid production [2, 3, 4].

Citric acid is multifunctional and has a vital role in many industrial applications depending upon its positive characteristics of low-toxicity and high solubility. It is widely employed in pharmaceuticals, food and beverages, cosmetics, detergents and many chemical and metallurgical manufactures [3, 5, 6, 7, 8, 9, 10].

Biosynthesis of citric acid backs to 19th century where it was observed within formation of oxalic acid by some kind of mold Penicillium [11]. However, commercial citric acid production was begun according to J Currie (1917) who optimized the conditions to attain high CA concentrations in sugar based medium. Currie observed that significant quantities of CA could be accumulated by producing strains of Aspergillus niger in a surface fermentation process [1]. Since then, many developments have been applied on this process for the considerable industrial production of CA via using various carbohydrate, fundamentally cane sugar and molasses, and specified A. niger strains. The first group using sugar fermentation was Pfizer (the American pharmaceutical company) in 1923 for industrial manufacturing of
CA by *A. niger* [12, 13], and this project was applied by a Belgian collection, called “Citrique Belge” in 1929 [1, 11, 14, 15]. In 1948, molasses was used as a low-cost feedstock and an alternative for sucrose to citric acid production.

The commercial production methods of CA recorded are surface fermentation, solid-state fermentation, and submerged fermentation [16, 17]. The submerged technique is the favorable one for CA production especially by *A. niger* using starch or sucrose [18, 19].

Nowadays, the best commercial benefit to produce citric acid utilizes carbohydrates by submerged fermentation using fungi, yeast and bacteria. Obviously, *A. niger* is a saprophytic fungus growing on dead leaves, compost heaps (compost is organic matter and rich in nutrients), organic residues, and other decaying vegetation, and also its widely exists in soil. Its growth on the surfaces of certain fruits and vegetables, such as grapes, beets, banana, and onions, causes a disease called black mold [20, 21].

However, Among the citric acid producing microorganisms, today *A. niger* is the most commonly well-Known producer of CA from a waste materials because of its simple and safe of treating; its capability to consume various low-cost raw materials; and its elevated yields [1, 16, 22, 23, 24, 25, 26, 27).

In the present work, *Aspergillus niger* isolated locally from palm dates were conducted to find the ability of this strain in producing citric acid, especially with the concerns of the simplicity of getting this isolate from palm dates without serious contamination with other microorganisms, the availability of this fruit in Iraq, and relatively good citric acid yield. The study was examined the strain activity to produce citric acid from the point of view of kinetics study, which gives a clear observations about the yield and the course of fermentation process, at fermentation conditions adjusted well previously, which produce the best yield of citric acid. The selected carbon source used in the fermentation was sucrose.

2. Materials and Methods

2.1. Isolation of *Aspergillus niger*

Isolates of *Aspergillus niger* were isolated from dates “Zahdi” taken from the local markets. Isolation was done on Potato Dextrose agar medium (PDA, HIMEDIA, India). The sterilized medium was poured into Petri-dishes at aseptic conditions inside a biological cabinet and left to solidify. Petri-dishes were incubated overturned at “30°C for 2 days”. Then, clean uncontaminated plates were used for cultivation and isolation of *Aspergillus niger* from the sources mentioned above.

Dates samples were submerged in distilled water. Samples (0.1 mL) were taken from the resulting suspensions, and poured onto the center of the surface of PDA plates. The samples were spread evenly over PDA by a sterile L-shaped glass spreader (hockey stick) and incubated to isolate the fungi.

The inoculated plates were incubated at 30°C for 4-7 days in the incubator (memmert, Germany) and observed periodically. The isolated colonies obtained were picked out visually and observed under microscope using lacto phenol cotton blue as a coloring reagent, where the fungi were identified as *Aspergillus niger* depending upon predominance and distinct morphological properties of fungal isolates. The fungi were then purified “if necessary when it contaminant with other microorganisms” by repeated sub-culturing. *A. niger* isolates were then kept as slant cultures in universal bottles at 4°C [28].

2.2 Characterization “Morphology of the colonies”

The visually identified cultures were examined under a microscope with magnifications of X40 to study the cultural characteristics. The characterization was assisted by preparing slides using lactophenol cotton blue. The identification of *A. niger* was depended on shape and color of colonies (Morphological features) as well as shape, color and size of conidial heads and spores (Microscopic features) [29].
2.3 Selection of wild CA producer “qualitative screening”

“Aspergillus niger cultures” were screened qualitatively for CA formation by plate method using “Czapek-Dox agar medium and bromocresol green dye, HIMEDIA, India”. About ten milliliters of the sterilized Czapek Dox agar-dye media was aseptically putted into each aseptic Petri dishes and left it to solidify at ambient temperature. From the 7 days-old fungal culture, 1 cm$^2$ of fungal culture was transferred to the culture plates and labeled. After 5 days in the incubator at 30 °C, the development of yellow zones around colonies was observed and that indicates CA formation [27, 30]. The $A. \text{niger}$ isolates of the maximum yellow zone were cultivated on PDA plates for 7 days.

2.4 Inoculum

Conidial inoculum was made by pouring 10 mL of “sterilized” distilled water to a 7 days old culture on PDA. An aseptic inoculation loop was utilized to scrape from the plate culture and remove the spore clusters under sterilized conditions. The resulting spore suspension was poured into aseptic beaker. A Serial dilution technique was utilized to obtain the desired spores concentration by adding 1mL spore suspension to 9 mL sterilized distilled water. Spores estimation was carried out by direct microscopic counting by using Haemocytometer “Neubauer chamber” to adjust the spores’ account to about $(5-25 \times 10^6 \text{ spores/mL})$.

2.5 Submerged fermentation method

Isolates with the widest yellow zone were selected to determine the citric acid concentration by submerged fermentation. Prepared media of 100 mL of “Czapek-dox liquid medium, OXOID, UK” was charged into 500 mL Erlenmeyer flask. The sugar concentration of the media was adjusted to 11 wt.%, by dissolving pure sucrose “HIMEDIA, India”. pH of the fermentation was adjusted to 4, using (1 N) HCL solution and then sterilized. The flask was incubated with 5 mL of the specified isolates inoculum in the shaking incubator at 30 °C and 150 rpm. The shaking flasks were plugged with cotton and tested every 48 h. All the flasks, tools, and media used were sterilized at 121 °C for 15 minutes. Kinetic parameters was plotted versus time according to citric acid concentration, sucrose concentration and mycelia weight.

2.6 measurements

Concentration of citric acid was determined spectrophotometrically using Marier and Boulet (1958) method [31]. The concentration of CA was measured by using a calibration curve, of absorbency versus concentration, for CA (in the range 0 to 0.3 g/L) at wave length of 427 nm, using UV/VIS spectrophotometer (UV-1800, Shimadzu, Japan). Experimented samples must be diluted to the concentration within the standard curve range before measuring, by using distilled water, when the concentration exceeded the maximum (0.3 g/L).

Sugar concentration was measured spectrophotometrically by DNS method [32]. A UV/VIS scanning spectrophotometer (UV-1800, Shimadzu, Japan) was used for measuring color intensity at a required wavelength (523 nm). Calibration curve of glucose standard solutions must be performed between 0 to 1.2 g/L. In order to fit the range of calibration curve, samples must be diluted from its high initial concentration.

Determination of mycelia dry weight was achieved by filtering the fermentation products using “pre-weighed Whatman filter paper no. 44” according to Haq and Daud (1995) method [33] method. Mycelium was dried at 90 °C overnight in an oven (HYSC, Korea), then the dry mycelia was weighted using sensitive balance (ADAM, USA).[34].
3. Results and discussion

3.1 Isolation

Through the colonies characteristics observed and depending on their morphological characteristics, the identification process was accomplished [29], as shown in Figure 1 and 2. **Texture:** granular; **Colour:** starting with white / pale yellow mycelium, then gradually covering with dark-brown / black conidial heads; **Reverse:** colourless or creamy; **Topography:** flat, often with radial folds; **Conidia:** spherical to oval shape, biserite, rough-walled; **Predominant features:** large black sporing heads “black conidia”, black or very dark brown spores; **Conidiophore:** smooth and thick walled, colourless stalks; large round vesicle with phialides and metulae over entire surface. Isolation of the fungi, on PDA agar, from dates fruit was very easy without any contamination by other microorganisms.

![Figure 1. Appearance of A. niger.](image1)

![Figure 2. Microscopic conidia of A. niger.](image2)

3.2 Screening

3.2.1 Preliminary screening (qualitative). The qualitative detection test of citric acid was conducted by using “Czapek Dox agar” supplementing with “bromocresol green” as an indicator, where, yellow- zones indicated CA formation. Figure (3) shows bromocresol green indication method for detection of organic acids including citric acid. There is an agreement with studies by Almousa et al. (2018) [35], which also used Czapek dox agar with bromocresol green as indicator for best citric acid producer.
3.2.2 Quantitative screening. Strains S11 of *A. niger* (from Zahdi dates) produced maximum concentration of citric acid (37.116 g/l), recorded at 432 h fermentation time and under submerged conditions of 11% (w/v) sucrose, 5% (v/v) inoculum size, pH 4, 30 °C and 150 rpm. The dates source “Zahdi”, represents an excellent source of *A. niger* isolate, because of the relatively good yield of citric acid produced from these strains isolates and the simplicity of initial isolation of the fungi, on PDA agar, from dates fruit without contamination by other microorganisms. The relatively good yield of citric acid produced by this strains isolates, may be due to the prolonged exposure of the palm fruit “Zahdi” to the ultraviolet light of the sun shine, which may be enhance its ability to produce citric acid by mutagenesis. UV mutation was used by many researchers to obtain microbial mutants for enhanced yield of metabolites including CA [30, 36, 37].

3.3 Kinetics

Kinetic parameters were determined according to the procedure of Ishaq and his colleagues [38]. Rate of CA biosynthesis by *A. niger* S11 was evaluated under submerged conditions of 11% (w/v) sucrose, 5% (v/v) inoculum size, pH 4, at 30 °C and 150 rpm and for 432 h of fermentation time. Different kinetics such as “product (CA), consumption (sugar) and growth (mycelia) rates (C_p/t, C_s/t, C_x/t, respectively), product and growth yield coefficients (Y_p/s, Y_p/x, Y_x/s), and specific rate constants (q_p, q_s)” were studied as shown in Figures (4), (5), and (6).

In Figure (4), the mass rate of sucrose consumption was significantly increased after incubation to (0.204 g/L/h) at 2 days then to (0.3076 g/L/h) after 8 days. This is the early exponential phase so there is a
high demand for carbon source resulting to rapid uptake and breakdown of sucrose into readily utilizable glucose or fructose. The sucrose consumption was 59.06 g/L after 8 days. Mass rate consumption of sucrose was decreased to 0.249 g/L/h and relatively stable after 8 days, where the consumption of sucrose limited to citric acid production and only slightly to \textit{A. niger} growth.

The long fermentation time greatly depends on the microorganisms, and as know, fungi have a relatively slow growth rate. The product formation rate of \textit{A. niger} S11 oscillated slightly within the range of (0.023 - 0.037) g/L/h at incubation time (96 – 192) h, respectively.

After 240 h of incubation time, the production rate of CA increased gradually and reached 0.0859 g/L/h at 432 h. Also, the growth rate was increased to 0.06 g/L at 432 h. Further increase in the fermentation period caused a decrease in citric acid yield thus the mass rate (C$_x$/t, C$_p$/t, C$_s$/t) decreased.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Comparison of volumetric rates for citric acid fermentation C$_x$/t, C$_p$/t, C$_s$/t.}
\end{figure}

Figure (5) shows the “production and growth yield coefficients (Y$_{p/x}$, Y$_{p/s}$, Y$_{x/s}$)” with fermentation time. There is an approximate comparable between the resulting trend in the figure below and in the research by Anyanwu and Okerentugba (2013) [39] of CA production by \textit{A. niger} EE-12 on sucrose based medium containing 15%(w/v) of sucrose, pH 3.5, 30°C, 200 rpm and inoculated with 2% (v/v) spore suspension for 144 h.
Maximum yield of CA based on *A. niger* S11 was produced in the early stage of fermentation (48 h), in harmony with the high yield of CA based on sugar consumed (Figure (5)), where a rapid consumption rate of sugar was established within the whole incubation time (Figure (4.20)). Yield of *A. niger* based on sugar consumed (Yx/s), in early stage, was less than (Yp/s and Yp/x). This can be explained by that more amount of sugar bio-converted to CA rather than to *A. niger* growth in this stage of fermentation. This situation can be represented by specific rate constants (q_p and q_s) for CA and substrate, respectively, and plotted in Figure (6).
4. Conclusions

Maximum yield of CA based on A. niger formation was produced in the early stage of fermentation (48 h), in harmony with the high yield of CA based on sugar consumed, where a rapid consumption rate of sugar was established within the whole incubation time. Yield of A. niger based on sugar consumed (Y_{x/s}), in early stage, was less then (Y_{p/s} and Y_{p/x}) means that more amount of sugar transfers to citric acid than to A. niger growth, in this stage of fermentation, and this situation can be represented by identification of specific rate constants q_{p} and q_{s} (g/g_{cell}/h) for citric acid and substrate, respectively.

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Figure 6. Comparison of specific rate constants for CA fermentation by A. niger S11 under submerged conditions.
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