

Optimisation of Citric Acid Production from a Novel Strain of *Aspergillus niger* by Submerged Fermentation

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Fungi isolated from various rotten fruits and vegetables were screened for the capability of producing citric acid. *Aspergillus niger* (*A. niger*) was obtained in the decayed onion peels and denoted as KON13 and this fungi strain was used in the production of citric acid by submerged fermentation. Critical process parameters affecting the fermentative citric acid production were investigated, including the types of carbon source, initial pH and initial sugar concentration in the fermentation medium. It was demonstrated that sucrose is the best carbon source in the fermentation process for this new isolate with a produced citric acid concentration of 1.76 g/L as compared to glucose and lactose. The study of the effect of initial pH within the range of pH 2 to 7 in the fermentation medium revealed that a maximum concentration of 1.82 g/L of citric acid was produced at an initial pH of 2. The highest citric acid concentration of 2.02 g/L was achieved at an initial sugar concentration of 140 g/L in the fermentation medium. This present study has demonstrated that the novel isolate strain of *A. niger* from onion peels is capable of producing citric acid with optimum concentration when sucrose was selected as the carbon source in the fermentation process, and at the fermentation conditions where initial pH of 2 and initial sugar concentration of 140 g/L in the fermentation medium.

1. Introduction

Citric acid is a naturally occurring weak organic acid found in all citrus fruits. Its name is derived from the Latin word *citrus*, a tree with lemon-resembling fruit. In its pure form, citric acid is readily soluble in water, colourless and has a molecular weight of 210.14 g/mol (Angumeenal et al., 2013). At room temperature, it exists in solid form. The melting point of citric acid is 153 °C and it decomposes at high temperature. It possesses three functional groups of carboxylic acid and therefore contributed to three pKa values at pH of 3.1, 4.7 and 6.4 (Papagianni et al., 2007). Citric acid can be derived from either natural sources such as lemons or synthetic method using microorganisms.

The study conducted by James Currie in 1916 discovered the great capability of *A. niger* producing citric acid has given a breakthrough for a successful economical industrial citric acid production from *A. niger* (Currie et al., 1917). Over the years, diverse types of microorganisms have been studied ranging from fungi, bacteria and yeasts. Various species of fungi such as *Aspergillus wentii*, *Aspergillus foetidus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus fonsecaeus*, *Aspergillus phoenicis*, *Aspergillus carbonaries*, *Trichoderma viride* and *Mucor pyriformis* were reported to produce significant amount of citric acid (Currie et al., 1917). Besides, yeasts mainly species such as *Candida tropicalis* (Käppeli et al., 1978), *Candida oleophilis* (Anastassiadis et al., 2006), *Candida guillemondi* (Angumeenal et al., 2003), and *Yarrowia lipolytica* (Silva et al., 2012) are capable of producing citric acid. However, the drawback of using yeast in the citric acid production is because of yeast produces tremendously large quantities of isocitric acid during fermentation process, which is an unwanted bio-product, therefore mutant strains that have a low aconitase activity is required.

The production of citric acid using fungi is already renowned, but the continuously growing demand for citric acid urged more studies on the optimisation of citric acid production. Modeling and simulation of production of citric acid from *Aspergillus niger* has been carried out to investigate the effect of process parameters and optimise the process conditions (Kana et al., 2012). However, there is little research in the area of novel strain development, necessitates the need for improvements in the fermentative citric acid production. The present study was conducted to identify, isolate, screen and select of novel *A. niger* strain which capable of producing citric acid from the source of rotten fruits and vegetables by using submerged fermentation. Fermentation process parameters such as types of carbon source, initial pH and initial sugar concentration in the fermentation medium were studied and the corresponding effects on the citric acid production concentration, sugar consumption during fermentation process and biomass generated were discussed.

2. Materials and methods

All the sugars and chemicals utilized were of analytical grade. Sucrose, glucose and lactose were obtained from Sigma Aldrich.

2.1 Preparation of fungi culturing

The citric acid producing strain was isolated from rotten fruits and vegetables including onion peels, rotten dragon fruit, rotten cabbage and rotten chilli, which were obtained from a local shop in Semenyih, Malaysia. All the samples were prepared using serial dilution to about 10^5 times. Potato dextrose agar (PDA) medium was prepared by dissolving 39 g of the PDA powder in 1,000 mL of distilled water, and was adjusted to pH 5.5 using 0.1 M NaOH or HCl solution. 0.1 mL of the dilute solution from each of the sample was aseptically transferred to prepared PDA agar plate and was spread uniformly on the PDA. The petri plates were placed in incubator (model: Binder KB 240) at 30 ± 0.5 °C for 4 - 7 d for maximum sporulation and culture development.

2.2 Identification and selection of fungal strains

After incubation process, PDA plates were observed for the presence of *A. niger*. The identification of the *A. niger* was based on the cultural, macroscopic (Diba et al., 2007) and microscopic characteristics (Nwoba et al., 2012). After sampling, petri plates that suspected to have growths of *A. niger* were sub-cultured, by using a sterilized laboratory cork borer (5 mm) to cut out and placing one disc of the suspected fungal hyphae on a fresh PDA slant. After the sporulation period, a small portion of the fungal culture was scrapped using a sterilized needle, placed on a microscope slit and about two drops of iodine or methyl blue was added to ease visibility for observation using the microscope (model: Nikon eclipse 80i).

2.3 Screening of fungal culture using dye method

Czapek Dextrose-Agar (CDA) medium was used to measure the citric acid producing capability of the identified *A. niger*. The CDA medium was prepared by dissolving 24.5 g of the CDA powder in 1,000 mL of distilled water in a Schott bottle. Bromocresol green dye was prepared by dissolving 0.1 g of bromocresol green powder in 75 mL of 99.5 % ethyl alcohol. 2 - 3 drops of bromocresol green dye were added to the CDA solution as an indicator. The medium was adjusted to a pH of 7 using 0.1 M NaOH or HCl solution and was sterilised.

Next, the CDA medium was left to cool to about 50 °C. About 20 mL of the CDA medium was aseptically poured into sterilized petri plates in a laminar chamber and was allowed to solidify at room temperature for about 20 min. A sterilized cork borer was used to transfer the *A. niger* to the solidified CDA medium and then placed in the incubator at 30 °C for 3 - 5 d. The fungi strain that demonstrated the largest yellow zone on Czapek Dextrose Agar was used for further culturing and fermentation process. The strain was transferred aseptically to the PDA petri plates, and incubated for 4 - 6 d at a temperature of 30 °C to achieve maximum sporulation. The strain was kept in a refrigerator at 4 °C for further fermentation process use.

2.4 Preparation of conidial inoculum and conidial count

The conidial inoculum was prepared using the strain placed in the 4 °C refrigerator having ample amount of conidial growth. 0.01 % Tween-80 solution was used as a wetting agent, and a sterilized wire loop was then used to gently break the conidial clumps and recover the spores. The homogenous suspension obtained was transferred to a conical flask. The spores in the suspension were counted using the conidial neubauer haemocytometer slide bridge. The haemocytometer was used to calculate the amount of spores in a given volume of the homogenous mixture. The counting chamber consists of a ruled glass slide which was used to cover a specific amount of the medium volume in a given depth, in 0.1 mm, before it was

placed under a research microscope (Model: Nikon ellipse 80i). Dilution with the Tween-80 solution continues till the suspension was found to contain approximately 1×10^8 spores/mL.

2.5 Fermentation conditions

The synthetic fermentation medium was prepared comprising of 100 g/L carbon source (sucrose, glucose or lactose), 2.50 g/L NH_4NO_3 , 1 g/L KH_2PO_4 and 0.25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Jernejc et al., 1982), to make up 500 mL of the basal medium. 0.04 g/L of CuSO_4 was added to provide for the heavy metal requirement (Hossain et al., 1984). The initial pH of the medium was adjusted to 3, by using 0.1 M NaOH or 0.1 M HCl. The flask was covered with aluminium foil and autoclaved at 121 °C for 15 min, after which it is left to cool. The flask was inoculated with 1 mL each of the conidial inoculum containing approximately 1×10^8 spores, and placed on rotary shaker (model: KNM TS-520 orbital shaker) at a speed of 120 rpm at room temperature for 168 h.

2.6 Effect of types of carbon source

Three different sugars (glucose, sucrose, and fructose) were used as carbon source to find out which is the most suitable carbon source for citric acid conversion.

2.7 Effect of initial pH

The effect of initial pH of fermentation medium was evaluated by manipulating the initial pH from the range of 2-7, while keeping the other process parameters at constant.

2.8 Effect of initial sugar concentration

The initial sugar concentration of fermentation medium was varied from 10 %, 12 %, 14 %, 16 % and 18 %, in order to investigate the optimum amount of initial concentration sugar for the production of citric acid by submerged fermentation.

2.9 Citric acid assay

After fermentation process, the fermentation medium was filtered using a pre-weighed Whatman filter paper no. 4. Titration was used to determine the amount of citric acid in the filtrate. 0.1 M NaOH was titrated against 15 mL of the filtrate and 2 - 3 drops of phenolphthalein was used as an indicator. The titration was performed in triplicates and the average titre was determined and used for the calculation using eq(1).

$$C = \frac{V_{\text{NaOH}} \times N_{\text{NaOH}} \times W_{\text{acid}}}{V_{\text{sample}}} \quad (1)$$

where C=concentration of produced citric acid in g/L, V_{NaOH} =volume of NaOH used for turning sample solution into pink colour in mL, V_{sample} =volume of filtered sample in mL, N_{NaOH} =normality of NaOH solution in moles/L, and W_{acid} =equivalent weight of citric acid in g/moles.

2.10 Biomass dry weight measurement

The mycelial biomass that was remained at filter paper after filtration of fermentation medium was dried in oven (model: Memmehrt, Germany) at a temperature of 105 °C until a constant weight was achieved. The bone dry weight of the mycelia biomass was measured using a mass balance.

2.11 Measurement of sugar consumption by fungi

Sugar consumed by *A. niger* for the production of citric acid was estimated using refractometer.

3. Results and discussion

3.1 Identification of fungi

After incubation period, the colonies formed in all the samples were selected based on their macroscopic and cultural properties such as conidial colours, mycelial colours, and colony reverse. It was observed that only the cultures that obtained from onion peels demonstrated a microorganism with a resemblance to *A. niger*, which was denoted as KON13 isolate. Further examination using a microscope and the microorganisms was observed to possess yellowish brown and round conidia, brown and round vesicles, as shown in Figure 1. It has been studied that the microscopic parameters matched the description of *A. niger* (Diba et al., 2007). By using a haemocytometer under a research microscope, it was determined that the homogenous mixture contains 1×10^8 spores/mL.

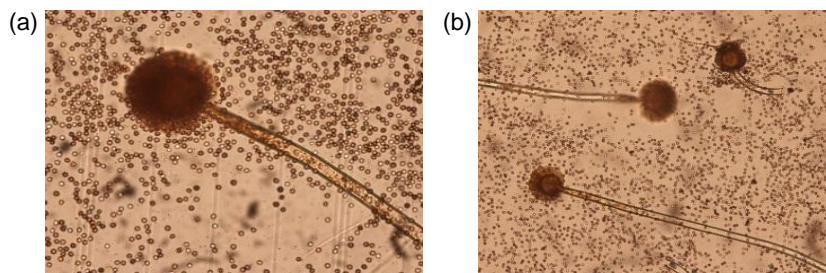


Figure 1: View of the onion peel culture under electron microscope (a) with magnification of 40X; (b) with magnification of 20X.

3.2 Effect of types of carbon source

Carbon source was manipulated among glucose, lactose and sucrose to be utilized in the citric acid production, at constant pH of 3 and at a temperature of 22 ± 2 °C. The maximum citric acid was produced by sucrose rich medium (1.76 g/L), compared to a production of 1.57 g/L and 1.63 g/L for lactose and glucose containing medium. The high citric acid concentration in the sucrose medium can be attributed to the fact that sucrose gives rise to the intracellular concentration of fructose-2, 6-diphosphate, which is the strongest activator of phosphofructokinase gene (PFK 1). Gutierrez-Rojas and co-workers reported that sucrose is readily transported into the cells of the microbes by *A. niger*'s strong extracellular mycelium (Gutierrez et al., 1995).

3.3 Effect of initial pH in fermentation medium

A maximum concentration of 1.82 g/L of citric acid was acquired at pH 2 of the initial fermentation medium, whereas the minimum concentration (0.83 g/L) was obtained at pH 7, as shown in Figure 2. A significant increase of citric acid concentration was observed as the initial of fermentation medium was increasing from pH 2 - 3. However, initial pH of 4 and 5 of fermentation medium has been observed to produce similar amount of citric acid and further increase in the initial pH decreases the citric acid concentration significantly. This trend can be attributed to the fact that at high pH values, the enzymes necessary for citric acid production are deactivated and at the same time glucose oxidase are activated and thereby reduce the citric acid produced.

The results of biomass weight over the diverse range of initial pH of fermentation medium demonstrated that the conidial fungi are able to flourish over a wide range of pH. The fungi can sporulate and grow maximally at a pH near to neutral. However, fungi are tolerant at a pH in the range of 4 - 9. The results showed that the mycelial growth have increased as the initial pH of fermentation medium increased from pH 2 - 5, and then drops dramatically at a pH of 5 and 6. The maximum mycelial weight (5.10 g/L) was observed at an initial pH of 5, which is in the range of the tolerated pH. Besides, it can be observed that there was an overall slight increase of 0.60 g/L of sugar consumed over the pH range of 2 - 7. This increment can be associated to the higher availability of sugar for the fungi's use. However, the increase in sugar consumption did not result in increase in citric acid formation.

3.4 Effect of initial sugar concentration in fermentation medium

The effect of initial sugar concentration in fermentation medium on the citric acid produced was shown in Figure 3. The highest citric acid concentration (2.02 g/L) was obtained at initial sucrose concentration of 140 g/L. The mycelial growth in the 140 g/L sucrose rich medium was in the form of small pellets and thereby resulting in a better agitation and, in turn, better aeration in the fermentation broth. It was reported that the citric acid production will be inhibited at concentrations less than 140 g/L as a result formation of polyalcohol (Aurora et al., 1997).

A further increase of sucrose concentration above 140 g/L resulted in a reduction in citric acid formation. This trend can be attributed to the overgrowth of the mycelial cells which increases the viscosity of the medium, thereby limiting mass transfer in the medium (Honecker et al., 1989). Besides, Tsay and co-workers reported that the reduction of citric acid concentration at high sugar concentration is due to the formation of oxalic acid (Tsay et al., 1987). In addition, Demirel et al. (Demirel et al., 2005) and Suzuki et al. (Suzuki et al., 1996) observed the same trend and reported the maximum citric acid production at an initial sugar concentration of 140 g/L.

The highest dry mycelial weight (0.42 g/L) was observed at an initial sugar concentration of 160 g/L. There was a continuous increase in the dry mycelial weight as the sugar concentration increased until a maximum value at 160 g/L, where at the same concentration there was a reduction in citric acid produced.

An increase in dry weight of mycelia with the increase in the sugar concentration was found in agreement with the work reported earlier by Ali et al (Ali et al., 2002) and Anwar et al. (Anwar et al., 2009), who made similar findings that the citric acid concentration will be reduced as sugar concentration was increased above 150 g/L, as a result of overgrowth of the mycelial which increases the viscosity of fermentation medium, resulting poor mass transfer.

The amount of sugar consumed was observed to increase as the initial sugar concentration was increased from 100 g/L through 180 g/L. This might due to the increasing of the initial sugar concentration enhances the availability of sugar for both citric acid formation and mycelial growth. This result was found in agreement with the observation done by Anwar's group (Anwar et al., 2009) in the study of fermentation of hydrolysed raw starch by *A. niger*.

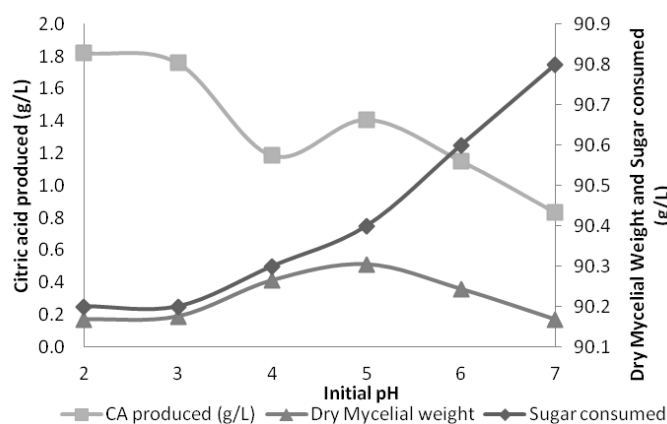


Figure 2: Effect of initial pH on produced citric acid concentration, dry mycelial weight and sugar consumed

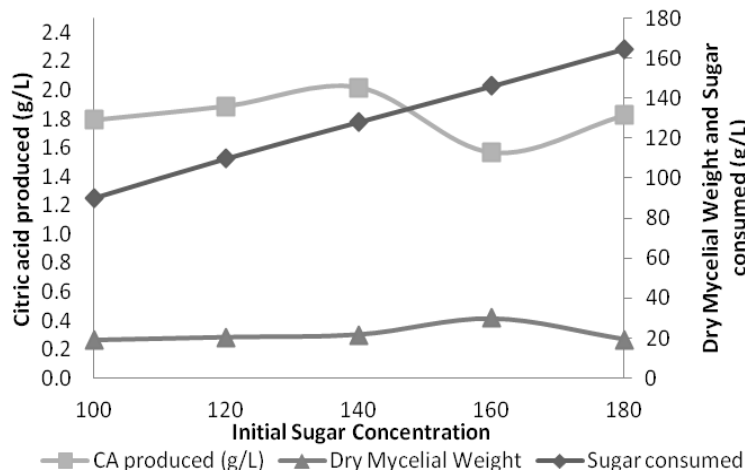


Figure 3: Effect of initial sugar concentration on produced citric acid concentration, dry mycelial weight and sugar consumed

4. Conclusions

This study has identified, isolated and screened a novel citric acid producing strain of *A. niger* from onion (*Allium cepa*) and successfully use the strain for citric acid production using submerged fermentation. Also, this study has demonstrated the effect of the essential process parameters of submerged fermentation process for optimum citric acid production concentration. This study is informative for the economic feasibility as it relates to the performance of the system, under different conditions. Further research is however necessary, before this novel isolate can be recommended for commercial production of citric acid.

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