Malaysian Journal of Microbiology, Vol 12(3) 2016, pp. 199-204

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

# Citric acid production by *Aspergillus niger* using different substrates

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Received 15 April 2015; Received in revised form 7 November 2015; Accepted 16 December 2015

## ABSTRACT

**Aims:** Citric acid is a commercially important acid that has many applications in varying sectors of industries. It is produced by various substrates through solid state or submerged fermentation. The capabilities of potato and rice as substrates for citric acid production using *Aspergillus niger* were tested in this experiment under submerged fermentation.
**Methodology and results:** Potato and rice extract media were prepared and inoculated with *A. niger* and titrations were carried out to determine the amount of citric acid produced. It was shown that rice extract media proved more useful than potato extract media as it produced the highest citric acid production. Rice extract media was supplemented with varying concentrations of glucose and sucrose and 5% sucrose (w/v) proved to be the best as it produced the highest amount of citric acid. The rice extract media with 5% sucrose (w/v) were supplemented with varying concentrations of ammonium nitrate and ammonium sulphate and 0.25% ammonium nitrate proved more effective in citric acid production. A low pH (1.9-2.3) was found during the maximum production of citric acid.
**Conclusion, significance and impact of study:** The results depict that potato and rice extract media can produce citric acid, hence providing an alternate substrate for citric acid production.

*Keywords: Aspergillus niger*, carbon source, citric acid (CA), nitrogen source

## INTRODUCTION

Citric acid is a ubiquitous intermediate product of metabolism and is found in practically all plants and animals (Papagianni, 2007). The widespread presence of citric acid in animal and plant kingdom is proof of its non-toxic nature and it has high water solubility (Padvi and Pawar, 2011; Ghosh, 2013), biodegradability, palatability and is a product adjudged to be Generally Recognized As Safe (GRAS) (Nwoba *et al.*, 2012; Bezalwar *et al.*, 2013). It is a biotechnological and biochemical product which is most used and produced through fermentation in tones with an annual production of 1.6 million tonnes (Nadeem *et al*., 2010; Nwoba *et al.*, 2012). About 70% of total citric acid produced is consumed by food industry, 12% by pharmaceutical industries and the remaining 18% consumed by other industries (Da Silva *et al.*, 2012). Its applications include acidulation, preservation, anti oxidation, flavour enhancement, plasticizer and synergistic agent (Nadeem *et al.*, 2010; Femi-Ola and Atere, 2013; Bezalwar *et al.*, 2013; Ghosh, 2013).

Citric acid can be produced by many microorganisms and related yeast species (Pawar and Pawar, 2014). At the present day most citric acid is produced using fungi *A. niger* (Ali *et al.*, 2002). The reasons for choosing *A. niger* over other potential citric acid producing microorganisms are; its high citric acid productivity at low pH without secretion of toxic metabolites (Nwoba *et al.*, 2012; Haider, 2014), ease of handling (Nadeem *et al.*, 2010), and ability to ferment a variety of cheap raw materials such as brewers spent grain (Femi-Ola and Atere, 2013), orange peel (Torrado *et al.*, 2011), cotton waste, cane molasses, bagasse, wheat bran, coffee husk and pumpkin (Majumder *et al.*, 2010; Kareem and Rahman., 2011; Pawar and Pawar., 2014).

Commercial production of citric acid is generally performed by submerged fermentation using *A. niger* (Kareem *et al.*, 2010; Prasad *et al.*, 2013; Yadegary *et al.*, 2013). Nitrogen and phosphorus limitation are crucial factors in citric acid production by *A. niger* and the interaction between both nutrients makes the study of their combined effect necessary, with nitrogen sources having two effects. One effect being negative as excess, nitrogen promotes a bigger growth and consequently diverts the source of carbon toward energy and biomass production. The other effect being positive as a moderate input contributes to the maintenance of citric acid productive biomass (Pintado *et al.*, 1998). Nutritional composition of the media, environmental conditions, manganese deficiency, pH, dissolved oxygen tension, influence of different sugar types and concentrations (El-Holi and Al-Delaimy, 2003; Patil and Patil, 2014) are other factors which affect citric acid production. According to Haider (2014), citric acid production is mainly affected by

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

cultural conditions such as carbon sources and concentration, nitrogen sources and concentration, acidity of the medium and aeration.

This study was focussed on determining the ability of potato and rice extracts in producing citric acid in order to meet the high demand of citric acid, as these agricultural products are very abundant in India. Determination of the effect of sugar supplements and different nitrogen sources on citric acid production using the above mentioned substrates was also under consideration.

## MATERIALS AND METHODS

### Collection and cultivation of citric acid producing fungi

Citric acid producing fungi, *A. niger*, was obtained from the Microbiology Laboratory Department of Life Sciences at ITM University and then cultured on Sabouraud Dextrose agar media. These were incubated at 28 °C for 24 h and actively growing hyphal regions were obtained and cultured in 100 mL of inoculum media. The inoculated inoculum media was incubated in an incubator shaker at 120 rpm at 27 °C for 24 h.

### Fermentation using potato extract media

Potato extract preparation was carried out by peeling and boiling potatoes until cooked and 100 g of the cooked potato was weighed out. This was placed in a 250 mL conical flask to which 200 mL of distilled water was added and the mixture was mashed and the mash solution obtained was then filtered using filter paper and procedure was repeated until required amount of potato extract was obtained. The potato extract media (300 mL) was prepared by mixing: potato extract 100 mL, $KH_2PO_4$ 0.38 g, $MgSO_4$ 0.50 g, distilled water 200 mL and a pH 7.0 and separated equally into 3 conical flask of 250 mL capacity. All the flasks were autoclaved at 121 °C at 15 psi of pressure for 15 min. After autoclaving the contents of the flasks were cooled and inoculated with 20% v/v of inoculum media with *A. niger,* while one conical flask was not inoculated and used as a negative control. The flasks were incubated at 27 °C in an incubator shaker at 110 rpm for 144 h (6 days).

### Fermentation using rice extract media

Rice (100 g) was boiled in 250 mL of distilled water for 1 min and the rice extract solution was separated from the rice using a number 2 sieve or sieve cloth. The procedure was repeated until required amounts of rice extract were obtained. The rice extract media (300 mL) was prepared by mixing: rice extract 100 mL, $KH_2PO_4$ 0.38 g, $MgSO_4$ 0.50 g, distilled water 200 mL and a pH 7.0 and separated equally into 3 conical flasks of 250 mL capacity. All the flasks were autoclaved at 121 °C at 15 psi of pressure for 15 min. After autoclaving the contents of the flasks were cooled and inoculated with 20% v/v of inoculum media with *A. niger,* while one conical flask was not inoculated and used as a negative control. The flasks were incubated at 27 °C in an incubator shaker at 110 rpm for 144 h (6 days).

### Fermentation with sucrose, glucose and nitrogen supplements

The media (potato and rice extract) which showed the maximum citric acid production was selected and supplemented with glucose and sucrose (5% and 10% w/v concentrations) and the sucrose or glucose concentrations that produced the maximum citric acid production was selected and then supplemented with varying concentrations of ammonium nitrate and ammonium sulphate (0.25%, 0.5%, 0.75% and 1% w/v) to test for effect of nitrogen on citric acid production. All the flasks were autoclaved at 121 °C at 15 psi of pressure for 15 min. After autoclaving the contents of the flasks were cooled and inoculated with 20% v/v of inoculum media with *A. niger,* while one conical flask was not inoculated and used as a negative control. The flasks were incubated at 27 °C in an incubator shaker at 110 rpm for 144 h (6 days).

### Determination of citric acid concentrations

After 24 h, 10 mL of fermentation media from each of the inoculated flasks was removed aseptically and the pH was recorded using a digital pH meter. Citric acid concentration was determined titrimetrically. One millilitre of rice extract fermentation media solution from the 10 mL removed aseptically was mixed with 5 mL of distilled water. Phenolphthalein was used as the indicator and titrated against 0.05 N NaOH. Titrations were done in triplicate and average values obtained and recorded.
The citric acid concentration was calculated according to the following:

$$N_1V_1 = N_2V_2$$

Where $N_1$ is concentration of NaOH and $V_1$ is volume of NaOH titrated and $N_2$ is concentration of citric acid and $V_2$ is volume of citric acid.

Concentration of citric acid obtained (mg/mL) was then multiplied by the equivalent weight of citric acid as follows:

$N_2$ x 64.04 (mg/mL)

(This was then converted to g/L Citric acid)

## RESULTS AND DISCUSSION

Citric acid production has been shown to be viable with many cheap agricultural raw materials (Pawar and Pawar, 2014) and this was also obtained in this experiment as the potato and rice extract media managed to produce citric acid. With reference to Figures 1 and 2, the highest maximum citric acid production was 1.47 g/L (96 h) and 2.62 g/L (48 h) for potato and rice extract media respectively, with the rice extract media being the better of the two. According to Kareem *et al.* (2010), sucrose is the principal substrate for citric acid production though,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

glucose, fructose and maltose can be used though not as much as sucrose. As a result we can say, rice extract media contains and readily made more sugars available as compared to the potato extract media, hence the higher citric acid production levels. This need for a shorter production time is of importance as confirmed by Prasad *et al.* (2013) that a reduction in production time is essential for reducing cost of production and rice media obtained maximal acid production at 48 h. The results showed that rice extract media proved to be better than the potato and hence was selected for the following stages of the experiment.

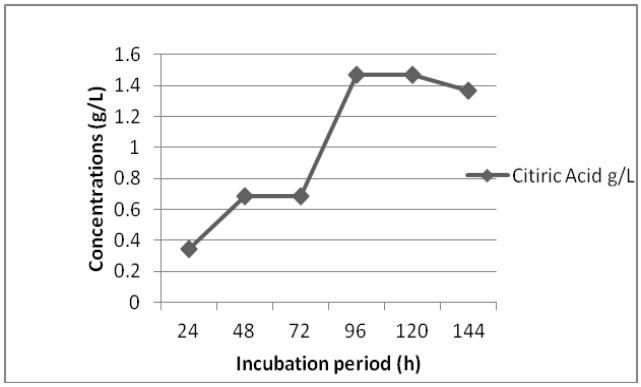


**Figure 1:** Illustration of citric acid production for potato extract media using *A. niger* at different incubation period.

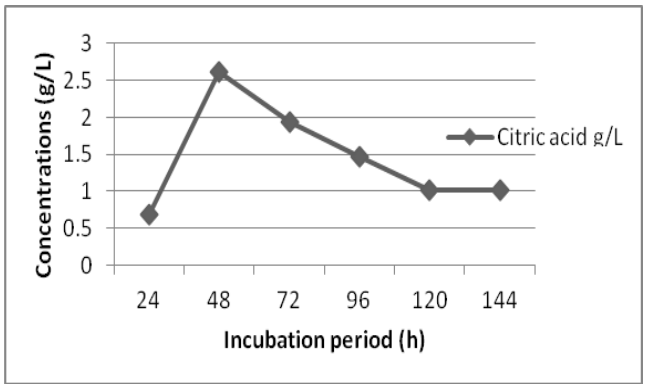


**Figure 2:** Illustration of citric acid production for rice extract media using *A. niger* at different incubation period.

**Effect of varying concentrations of sugars**

Citric acid production with rice media supplemented with different and varying sugar concentrations was presented in Figure 3. Media supplemented with 5% sucrose had the highest citric acid production at 14.44 g/L after 120 h while the control had 2.62 g/L after 48 h. Sucrose is the main substrate for citric acid production and is of low molecular weight and readily transported for hydrolysis by intracellular enzymes in microbial cells though other sugar substrates like glucose, fructose and maltose can also be used (Kareem *et al.*, 2010; Padvi and Pawar, 2011). According to Amenaghawon *et al.* (2013) sucrose is preferred over glucose due to the fact that *A. niger has* an effective mycelium-bound invertase that is active at low pH. In this study, media supplemented with sucrose produced more citric acid than the media with glucose, which agreed with the fact that sucrose is the principal and preferred substrate for citric acid production. Kareem *et al.* (2010) also showed that sucrose increases citric acid production more than glucose on their work with pineapple waste substrate. The type and concentration of sugar has an effect on the citric acid production (Papagianni, 2007; Kareem *et al.*, 2010) and the maximal production rates are usually between 14-22% of sugar and no citric acid was produced in media that contained less than 2.5% sugar (Papagianni, 2007). High levels of sugar supplements were accompanied with high levels of intracellular fructose 2,6 biphosphate which is not readily used for citric acid production like sucrose hence the low levels of acid produced (Kareem *et al.*, 2010). While, Prasad *et al*. (2013) who showed that when sugar concentration in medium was increased there was a reduction in the citric acid produced by *A. niger* and attributed this anomaly to the fact that high sugar concentrations cause an overgrowth of mycelium which causes increased viscosity in the medium and hence a reduction in citric acid production, while lower sugar levels lead to low citric acid production due to accumulation of oxalic acid in culture broth. In this study, highest citric acid production levels were obtained for 5% sucrose followed by 10% sucrose, with the later having a lower citric acid concentration than 5% sucrose. This was in agreement with Prasad *et al.* (2013) as an increase in sugar levels (for both different sugar sources) led to a decrease in citric acid production. This was however not in agreement with Papagianni (2007), who showed that maximal production rates were usually between 14-22%, and was also not in agreement with Kareem *et al.* (2010) who showed an increase in citric acid production as sucrose concentration was increased up to 15%. The varying sugar supplements all produced their maximum citric acid concentrations after 120 h of culture as compared to the control which produced its maximum at 48 h showing that the addition sugar supplements prolonged the time required to reach maximum citric acid production, whilst it also meant increased levels of citric acid production, though further incubation did not increase citric acid production. This was in agreement with Prasad *et al.* (2013), as the maximal culture time in this study (120 h) was near the 144 h maximal culture time obtained by Nwoba *et al.* (2012) and Prasad *et al.* (2013). Nwoba *et al.* (2012) and Prasad *et al.* (2013) also showed that further increases in incubation time beyond the 144 h did not lead to increase in citric acid production. In addition, reduction in production time as shown, results in a lower production cost for the citric acid, hence culture period reduction is of great importance. They attributed this reduction in citric acid production to inhibitory effects of high citric acid concentrations, age of fungi, decreased available nitrogen, depletion of sugar contents and decay of enzymes responsible for synthesis of citric acid.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

**Effect of varying nitrogen sources and concentrations on citric acid production**

Nitrogen has a profound effect on citric acid production as it is not only important for metabolic rates in the cells but is also a basic part of cell proteins and was shown to induce pellet formation in filamentous fungi (Ali *et al.*, 2002). Nitrogen has been reported to be an important factor in fermentation processes due to an increase in C/N ratio (Kareem and Rahman, 2011; Patil and Patil, 2014). The present study showed that nitrogen has an effect on citric acid production using *A. niger* as shown by Figures 4 and 5 where the maximum citric acid production with nitrogen supplements was 5.22 g/L (0.25% after 168 h) while the positive control had a maximum of 4.4 g/L (after 168 h). Kareem *et al.* (2010) showed that the effect of the nitrogen on citric acid production varied with the specific type of nitrogen source used (e.g. ammonium nitrate or phosphate or sulphate). In this study it was seen that ammonium nitrate (5.22 g/L at 168 h) had the highest citric acid production as compared to ammonium sulphate (5.02 g/L at 168 h) which was in agreement with Kareem *et al.* (2010) that different nitrogen source types have varying effects on citric acid production.

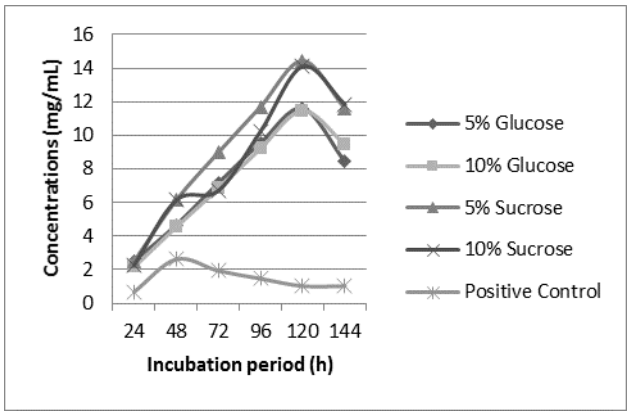


**Figure 3:** Illustration of citric acid production for rice extract media supplemented with varying glucose and sucrose concentrations using *A. niger* at different incubation period.

An increase in citric acid production was seen for media supplemented with 0.25% w/v ammonium nitrate and an increase in the basal nitrogen concentration above 0.25% w/v resulted in disturbance of fungal growth hence a reduction in citric acid production (Kareem *et al.*, 2010). Ali *et al.* (2002) showed that maximum citric acid concentration amount was obtained at 0.2% $NH_4NO_3$ with an increase or decrease affecting citric acid production.

This result of Kareem *et al.* (2010) was in agreement with results obtained for ammonium nitrate in this study, which had its maximum citric acid production at 0.25% w/v (5.22 g/L) and the higher concentrations saw a decrease in citric acid production (2.73 g/L at 120 h for 0.5%, 2.83 g/L at 144 h for 0.75% and 3.41 g/L at 168 h for 1%). However, findings of Ali *et al.* (2002) and Kareem *et al.* (2010) highlighted above, were not in agreement with results for ammonium sulphate, which had maximum citric acid production at 0.5% (5.02 g/L) while the 0.25% concentration had 3.65 g/L. Patil and Patil (2014) showed that ammonium sulphate produced more citric acid than casein, sodium nitrate, yeast extract and ammonium nitrate. Prasad *et al.* (2013) showed that citric acid production was increased with 2.4 mM concentration of ammonium sulphate. Results in this study showed that concentrations above 0.25% for ammonium sulphate had higher citric acid production as compared to the positive control, which agreed with Prasad *et al*. (2013) and Patil and Patil (2014), that ammonium sulphate increases citric acid production. Results for ammonium sulphate further contradicted with Da Silva *et al.* (2012) who showed that 0.7 g/L of ammonium sulphate reduced citric acid production, while 0.75% concentration of ammonium sulphate in this study had positive effect on citric acid production (4.88 g/L at 144 h) as it produced more citric acid than the positive control (4.44 g/L at 168 h).

Considering the incubation period, in this study the maximum citric acid was produced at 168 h for 0.25% ammonium nitrate and 0.5% ammonium sulphate as well as the control which was contrary to the findings of Nwoba *et al.* (2012) and Prasad *et al.* (2013) who showed that further increases in incubation time beyond the 144 h did not lead to increase in citric acid production. They explained that the reduction in citric acid production was due to inhibitory effects of high citric acid concentrations, age of fungi, decreased available nitrogen, depletion of sugar contents and decay of enzymes responsible for synthesis of citric acid. In addition, reduction in production time as shown, results in a lower production cost for the citric acid, hence culture period reduction is a of great importance which was not successfully obtained in this study as a longer incubation time was obtained though higher acid production levels were obtained.

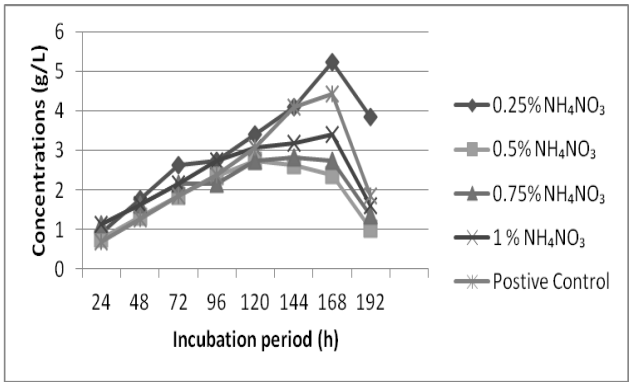


**Figure 4:** Illustration of citric acid production for rice extract media supplemented with 5 % sucrose at varying $NH_4NO_3$ concentrations using *A. niger* at different incubation period.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

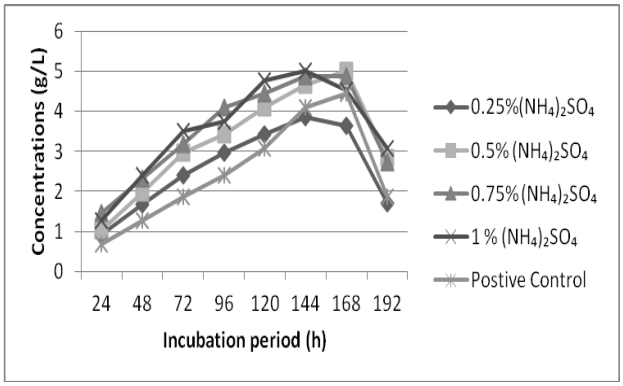


**Figure 5:** Illustration of citric acid production for rice extract media supplemented with 5% sucrose at varying $(NH_4)_2SO_4$ concentrations using *A. niger* at different incubation period.

**Observation of pH during citric acid production**

The pH is of great importance in citric acid production as during the fermentation process with *A. niger*, metabolism of nitrogen causes a release of protons which lower the pH of the medium (Papagianni, 2007; Amenaghawon *et al.*, 2013). The maintenance of a favourable pH is essential for successful citric acid production and a decrease in the initial pH causes a reduction in citric acid production due to poor mycelium growth due to a low initial pH (Papagianni *et al.*, 1999; Ali *et al.*, 2002; Prasad *et al.*, 2013). Results in this study, for the varying sugar concentrations, showed that the maximum citric acid productions were obtained at low pH ranging between 1.9 and 2.1. The highest maximum citric acid concentration was obtained for 5% sucrose supplement and at a pH of 1.9 which was the lowest of all observed and recorded pH for maximum production concentrations. This was in agreement with Papagianni (2007) who showed that a pH ≤ 2 was effective for maximal citric acid production using *A. niger* after maintenance of a higher initial pH.

In this study, the varying nitrogen supplements also showed the effect of a low pH on citric acid production as the maximum citric acid concentrations were obtained at pH levels between 2.2 and 2.4. This was in agreement with Vandenberghe *et al.* (1999) who proposed that a pH of 2.2 was optimal for citric acid production but was slightly above the findings of Papagianni (2007) of a pH ≤ 2 being essential for maximal citric acid production. A decrease in pH as the incubation time increased was noted by Kareem and Rahman (2011) and this was also evidenced in this study as pH decreased with increasing incubation time, which was attributed to the formation and accumulation of citric acid by *A. niger* (Kareem and Rahman, 2011; Nwoba *et al.*, 2012). This showed that a low pH was necessary for maximal production of citric acid which agreed with the work of Amenaghawon *et al.* (2013) who also showed that decrease in pH as fermentation proceeds is an indication of citric acid production. The low pH was seen to provide sterile conditions which reduce contamination and inhibits production of unwanted organic acids, like oxalic acid, which make recovery of citric acid difficult and also improves citric acid production (Nwoba *et al.*, 2012; Amenaghawon *et al.*, 2013).

## CONCLUSION

In conclusion, citric acid was produced from potato and rice extract media successfully using *A. niger*. This study also managed to show that sugar and nitrogen supplements can increase the citric acid production with *A. niger* with the particular concentrations being 5% sucrose and 0.25% ammonium nitrate respectively. The maximum citric acid concentration obtained for the sugar supplements was 14.44 g/L (5% sucrose) and for nitrogen supplements was 5.22 g/L (0.25% ammonium nitrate) with rice extract media. Study managed to show that rice extract can be considered as a potential substrate for citric acid production.

## ACKNOWLEDGEMENTS

Sincere gratitude to, ITM University Gwalior for providing facilities, Head of Dept Life Sciences for permission to work in the Laboratory, Head of Department Science and Technology for financial assistance, the Microbiology and Food technology Department staff for making this project viable.

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