Citric acid production by *Aspergillus niger* using different substrates

Chirova Travers Kudzai*, Kumar Ajay and Panwar Ambika

Department of Biotechnology, School of Engineering and Technology, ITM University, Gwalior (MP) India.

Email: traverschirova@gmail.com

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 it 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-Delaimey, 2003; Patil and Patil, 2014) are other factors which affect citric acid production. According to Haider (2014), citric acid production is mainly affected by...
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 hynphal 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, KH₂PO₄ 0.38 g, MgSO₄ 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, KH₂PO₄ 0.38 g, MgSO₄ 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).

RESULTS AND DISCUSSION

Cultural 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,
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.

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, resulted 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.

**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).
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% NH₄NO₃ 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 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 NH₄NO₃ concentrations using A. niger at different incubation period.
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 Vandenbergh 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.

REFERENCES

Ali, S., Haq, I., Qadeer, M. A. and Iqbal, J. (2002). Production of citric acid by Aspergillus niger using cane molasses in a stirred fermenter. Electronic Journal of Biotechnology 5, 258-271.

Amenaghawon, N. A., Arequamen, S. O., Agbroko, N. T., Ogbeide, S. E. and Okieimen, C. O. (2013). Modelling and statistical optimisation of acid production from solid state fermentation of sugar cane bagasse using Aspergillus niger. International Journal of Sciences 2, 56-62.

Bezawar, P. G., Gomashe, A. V., Sanap, H. M. and Guliha, P. A. (2013). Production and optimization of citric acid by Aspergillus niger using fruit pulp waste. International Journal of Current Microbiology and Applied Sciences 2, 347-352.

Da Silva, L. V., Taveres, C. B., Amaral, P. F. F. and Coelho, M. A. Z. (2012). Production of citric acid byconst Yarrowira lipolytica in different crude glycerol concentrations and different nitrogen sources. Chemical Engineering Transactions 27, 199-204.

El-Holi, M. A. and Al-Delaimy, K. S. (2003). Citric acid production from whey with sugars and additives by Aspergillus niger. African Journal of Biotechnology 2, 356-359.

Femi-Ola, T. O. and Atere, V. A. (2013). Citric acid production from brewers spent grain by Aspergillus niger and Saccharomyces cerevisiae. International Journal of Research in Biosciences 2, 30-36.
Ghosh, P. (2013). Comprehensive review on citric acid fermentation. Research Journal of Pharmaceutical, Biological and Chemical Sciences 4, 890-914.

Haider, M. M. (2014). Citric acid production from carob pod extract by Aspergillus niger. Journal of Pharmacy and Biological Sciences 9, 112-116.

Kareem, S. O. and Rahman, R. A. (2011). Utilization of banana peels for citric acid production by Aspergillus niger. Agriculture and Biology Journal of North America 4, 384-387.

Kareem, S. O., Akpan, I. and Alebiowu, O. O. (2010). Production of citric acid by Aspergillus niger using pineapple waste. Malaysian Journal of Microbiology 6, 161-165.

Vandenbergh, L. P. S., Soccol, C. R., Pandey, A. and Lebeault, J. (1999). Review: Microbial production of citric acid. Brazilian Archives of Biology and Technology 42, 263-276.

Majumder, L., Khalil, I., Mushni, M. K., Alam, K., Rashid, H., Begum, R. and Alam, N. (2010). Citric acid production by Aspergillus niger using molasses and pumpkin as substrates. European Journal of Biological Sciences 2, 1-6.

Nadeem, A., Syed, Q., Baig, S., Irfan, M. and Nadeem, M. (2010). Enhanced production of citric acid by Aspergillus niger M-101 using lower alcohols. Turkish Journal of Biochemistry 35, 7-13.

Nwoba, E. G., Ogbonna, J. C., Ominyi, M. C., Nwagu, K. E. and Gibson-Umeh, G. (2012). Isolation of citric acid-producing fungi and optimization of citric acid production by selected isolates. Global Journal of Bio-Science and Biotechnology 1, 261-270.

Padvi, M. A. and Pawar, S. V. (2011). Factors affecting production of citric acid. International Referred Research Journal 3, 37-38.

Papagianni, M. (2007). Advances in citric acid fermentation by Aspergillus niger: Biochemical aspects, membrane transport and modelling. Biotechnology Advances 25, 244-263.

Papagianni, M., Mattey, M., Berovic, M. and Kristiansen, B. (1999). Aspergillus niger morphology and citric acid production in submerged batch fermentation: Effects of culture pH, phosphate and manganese levels. Food Technology and Biotechnology 37, 165-171.

Patil, N. G. and Patil, V. R. (2014). Production and partial characterization of citric acid by local isolate of Aspergillus niger using sorghum. International Journal of Pharmacy 5, 229-231.

Pawar, V. A. and Pawar, P. R. (2014). Effect of various parameters on citric acid production using different substrates. Indian Journal of Research 3, 13-15.

Pintado, J., Torrado, A., Gonzalez, M. P. and Murado, M. A. (1998). Optimization of nutrient concentration for citric acid production by solid-state culture of Aspergillus niger on polyurethane foams. Enzyme Microbiology and Technology 23, 149-156.

Prasad, M. P. D., Sridevi, V., Surendra babu, N. V., Reddy, O. V. S. and Harsha, N. (2013). Studies on fermentative production of citric acid by Aspergillus niger isolate using sorghum malt and its optimization. International Journal of Innovative Research in Science, Engineering and Technology 2, 2961-2968.

Torrado, A. M., Cortes, S., Salgado, J. M., Max, B., Rodriguez, N., Bibbins, B. P., Converti, A. and Dominguez, J. M. (2011). Citric acid production from orange peel waste by solid-state fermentation. Brazilian Journal of Microbiology 42, 394-409.

Yadegary, M., Hamidi, A., Alavi, S. A., Khodaverdi, E., Yadaghi, H., Sattari, S., Bagherpour, G. and Yahaghi, E. (2013). Citric acid production from sugarcane bagasse through solid state fermentation method using Aspergillus niger mold and optimization of citric acid production by Taguchi method. Jundishapur Journal of Microbiology 6, 1-6.