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STIMULATION OF CITRIC ACID PRODUCTION BY HEAT SHOCK PROMOTING *ASPERGILLUS NIGER* USING NITROGEN ENRICHED *DIOSCOREA BULBIFERA*

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ABSTRACT

High cost of substrate and how to stimulate citric acid are among the factors that mitigate citric acid production. Exploiting a cheap substrate for citric acid production will be a viable alternative in order to reduce the cost of citric acid production. Therefore, the aim of this research was to evaluate the potential of wild Dioscorea bulbifera tubers for citric acid production. Experimental procedures were designed to determine the effect of different concentrations of wild Dioscorea bulbifera, different nitrogen sources and effect of different concentrations of nitrogen sources on citric acid production. The data obtained were analyzed using one- way analysis of variance (ANOVA). The results of the experiment showed that wild Dioscorea bulbifera tuber was able to produce citric acid. The concentration of acid produced increased from 5 % to 20 % citric acid up to 8.5 ±1.0 g/lafter 96 hours of fermentation. Among the different nitrogen sources used to supplement wild Dioscorea bulbifera, the medium with ammonium nitrate produced the highest citric acid concentration of 10.5 ±1.5 g/l after 96 hours of fermentation. Meanwhile, when the concentration of the amonium nitrate were varied, 0.4 % was the optimum for the maximum citric acid concentration of 14.8 ±2.0 g/l after 96 hours of fermentation (P< 0.05). In conclusion, Dioscorea bulbifera tubers is suitable for citric acid production and supplementation of the medium with ammonium nitrate as a nitrogen source had a positive effect on the yield

Keywords: citric acid, submerged culture, wild *Dioscorea bulbifera*, nitrogen source and heat shock

INTRODUCTION

A number of wild crops contain carbohydrate in high quantities which could be converted to useful metabolites, but are yet to be fully exploited. Gandhi *et al*. (2021) determined the carbohydrate content of three wild aerial tubers of *Dioscorea bulbifera*. The researchers reported 73.50, 78.20 and 72.50 % carbohydrate per 100 grams of wild *Dioscoreabulbifera* tuber A, B and C, respectively. However, wild *Dioscorea bulbifera*tuber may be a good substrate for citric acid production. The wild uncultivated varieties of *Dioscorea bulbifera* is commonly known as wild aerial yam or wild potato yam. They are found mostly in virgin lands and forests, unlike the normal edible cultivated varieties that are found in farmland.

Citric acid is an important intermediate of the tricarboxylic acid cycle (TCA). Citric acid is a

product of fermentation by *Aspergillus niger* and find its application in food, pharmaceutical, cosmetic and beverage industries (Ali *et al.,* 2002; El-Holi and Al-Delaimy, 2003; Lingappa *et al*., 2007, Ganne *et al*., 2008; Majumder *et al.,* 2010; Alam*et al*., 2011; Hamdy, 2013; Auta *et al*., 2014; Ezea*et al.,* 2015; Show *et al*., 2015; Almousa*et al*., 2018, Chergui*et al.,* 2021; Ezea*et al.,* 2021). Citric acid production requires substrate optimization parameters such as limited nitrogen source and minerals for maximum production. Physiologically, utilization of nitrogen compound such as ammonium salt, peptone, malt extract, urea and yeast extract by *Aspergillus niger* leads to decrease in pH which is essential for fungal growth and citric acid production (Grewal and Kalra, 1995; Papagianni, 2007; Ezea, 2012; Ezea*et al.*, 2015; Boufaris*et al.*, 2017). Furthermore, nitrogen is not only important for metabolism but it is also a basic component of cell proteins and DNA structure (Ezea*et al*., 2015; Shankar and Sivakumar, 2016). Aside substrate optimization with nitrogen sources, citric acid production requires microbial stimulation such as heat shock for efficient secretion and accumulation. Since microorganisms developed resistance to both heat shock and lethal shock as a result of metabolic rearrangement and the synthesis of nascent polypeptides, which can stimulate the production of metabolites, the response of fungal cells to heat shock (HS) has drawn interest (Tereshina *et al*., 2013).

However, the cost of substrate and microbial stimulation remains a challenge in the citric acid production industries as it represents a significant percentage of the total production cost. There is need to continue searching for cheap raw material and a way to stimulate the fungus for better citric acid production which could in turn or as a result reduce the cost of citric acid production and at the same time create job opportunities for the teaming youths in developing countries, especially in the Africa countries. Therefore, the aim of this research was to stimulate citric acid production by heat shock *Aspergillus niger* using wild *Dioscorea bulbifera* as a substrate.

METHODS

Sample collection and pretreatment

The wild *Dioscorea bulbifera* tubers were collected from virgin lands (bushes) and forests at Nsukka in Enugu State, Nigeria. The tubers were peeled, sundried, ground and sieved into a fine powder using muslim cloth. The fine powder flour of *Dioscorea bulbifera* tuber was suspended in 100 ml basal nutrients medium and thermally pretreated at 121^{o}C for 20 minutes using an autoclave as described by Ezea and Ezaka (2022).

Microorganism and ioculum preparations

Aspergillus niger strain was obtained from the Institute of Agricultural research and training moor plantation Ibadan and maintained on potato dextrose agar (PDA) slant at 4^{0}C. At intervals, the fungus was subcultured until the study was concluded. The inoculum was prepared according to the method of Ezea *et al.* (2015)**.** The spores of *Aspergillus niger* was harvested from potato dextrose agar slant using a sterile solution of 0.01% Tween 80 with inoculation wire loop. A 10 ml of 5 x10^{7} spores/ml was counted using haemocytometer and was used as the inoculum.

Submerged fermentation of wild *Dioscorea bulbifera* tuber (flour)

The modified Ezea*et al*. (2015) method of submerged fermentation was used. A 250 ml foam-plugged Erlenmeyer flask. Using a DENVER digital weighing balance (Model: MXX-123 USA), 5 g of wild *Dioscoreabulbifera* flour was weighed. It was then suspended in 100 ml of nutrient medium that contained peptone (2 g/l), KH_2PO_4 (0.2 g/l), $ZnSO_4.7H_2O$ (0.01 g/l), $Fe(SO_4)_2.7H_2O$ (0.01 g/l), and $MgSO_4.7H_2O$ (0.5 g/l). Prior to pretreatment, the pH was initially adjusted to 5.0 using 0.1MHCl and 0.1MNaOH. Ten millilitres (10ml) of *Aspergillus niger* spores were used to inoculate the sample, which was then incubated for 144 hours at 30^{o}C using a rotating incubator shaker (model: VWR

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International, made by B. Bran Scientific & Instrument Company, England) spinning at 225 revolutions per minute

Effects of wild *Dioscorea buldifera* concentration on citric acid production

The effect of different concentration of *Dioscorea bulbifera*tuber was investigated on citric acid production by suspending different percentage of wild *Dioscorea bulbifera*flour from 5 % to 25 % in 100 ml nutrient medium into 250 ml foam-plugged Erlenmeyer flask and incubated under rotary incubator shaker (model: VWR International by B. Bran Scientific & Instrument Company England) at 225 rotations per minutes (rpm) for 144 hours.

Effects of different nitrogen sources on citric acid production from wild *Diocoreabulbifera*

The production of citric acid from wild *Dioscorea bulbifera* tuber was optimized by supplementing the medium with different nitrogen sources (yeast extract, urea, sodium nitrate, ammonium sulphate and ammonium nitrate). The nitrogen source was added at 0.2 % (w/v) to the medium. The effects of different concentrations of nitrogen source (0.2 to 0.6 %) on citric acid production from wild *Dioscorea bulbifera* were also investigated for citric acid production.

Heat shock stimulation of*Aspergillusniger*spores for citric acid production

Heat shock stimulation of *A. niger* spores for citric acid production was done by pre-incubating of the harvested spores at different temperatures; 35, 40, 45, 50 and 55^{o}C for various lengths of time (5, 10, 15 and 20 minutes) in a water bath before cultivation in *Dioscorea bulbifera* medium enriched with nitrogen.

Citric acid determination

Citric acid was estimated using pyridine acetic anhydride method by Marrier and Boulet (1958**),** as reported in Ezea and Ezaka (2022).

Statistical analysis

Data obtained were subjected to one- way analysis of variance (ANOVA) and the means were separated using the least significant difference.

RESULTS

Production of citric acid from *Dioscorea bulbifera* tubers increased as the fermentation time increased up to 96 hours with the maximum concentration of 2.0 ± 0.09 g/l citric acid (Fig. 1). The effects of different concentrations of *Dioscorea bulbifera* tubers on citric acid production showed that as the percentage of *Dioscorea bulbifera* tubers increased up to 20 % concentration, the amount of citric acid produced significantly increased to 8.5 ± 1.0 g/l ($P< 0.05$) after 96 hours of fermentation when compared with the control (5 % wild *Dioscorea bulbifera* tubers) that had maximum citric acid concentration of 2.0 ± 0.09 g/l (Fig. 2).

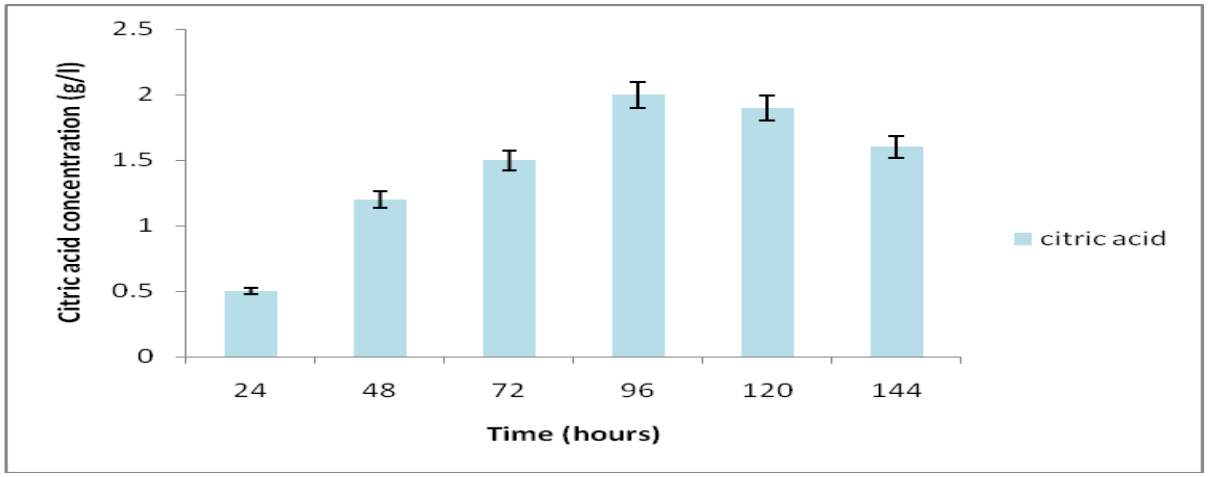

Fig 1: The concentration of citric acid produced from *Dioscorea bulbifera* flour (flour)

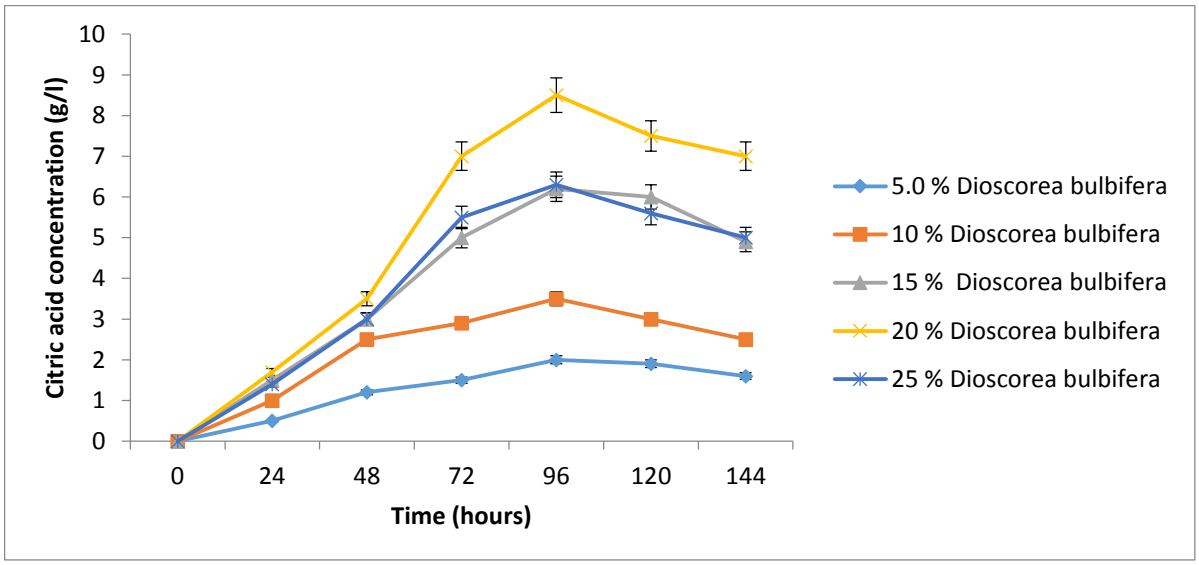

Fig 2: Effect of different concentration of *Dioscorea bulbifera* tubers (flour) on citric acid production

Evaluation of the effects of different nitrogen sources on citric acid production from wild *Dioscoreabulbifera*showed that amonium nitrate was the best nitrogen source for citric acid production yielded maximum citric acid concentration of 10.5 ± 1.5 g/l compared with the control and other nitrogen sources (Fig. 3). Citric acid concentration significantly increased when the concentration of ammonium nitrate increased to 0.4 % which yielded the highest citric acid concentrationof 14.8 ±2.0 g/l compared with the control, 0.2 % ammonium nitrate (Fig. 4). There was a decreasein the concentration of citric acid producedas the percentage concentration of amonium nitrate increased to 0.5 and 0.6 percent.

The effect of stimulating citric acid production by heat shock *Aspergillus niger* is shown in figure 5. Pre incubation of the *Aspergillus niger* spores for 15 minutes at 45°C was the optimum heat shock temperature for maximum citric acid production (23.0 ±2.5 g/l). As the temperature of heat shock *Aspergillusniger* increased to 45 ^{o}C, it favoured the stimulation of citric acid production using *Dioscorea bubifera*. Heat shock temperature of 50 and 55°C did not favour citric acid stimulation in *Aspergillusniger* during the production using *Dioscorea bulbifera* enriched with different nitrogen sources.

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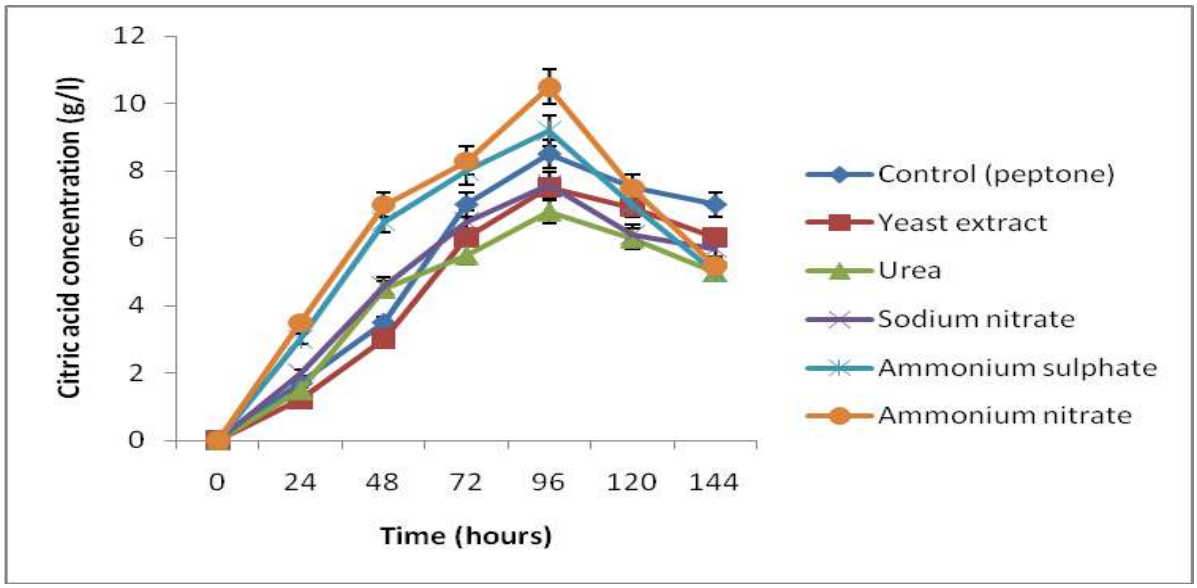

Fig 3: Effect of different nitrogen sources on citric acid production from *Dioscoreabulbifera* tubers (flour)

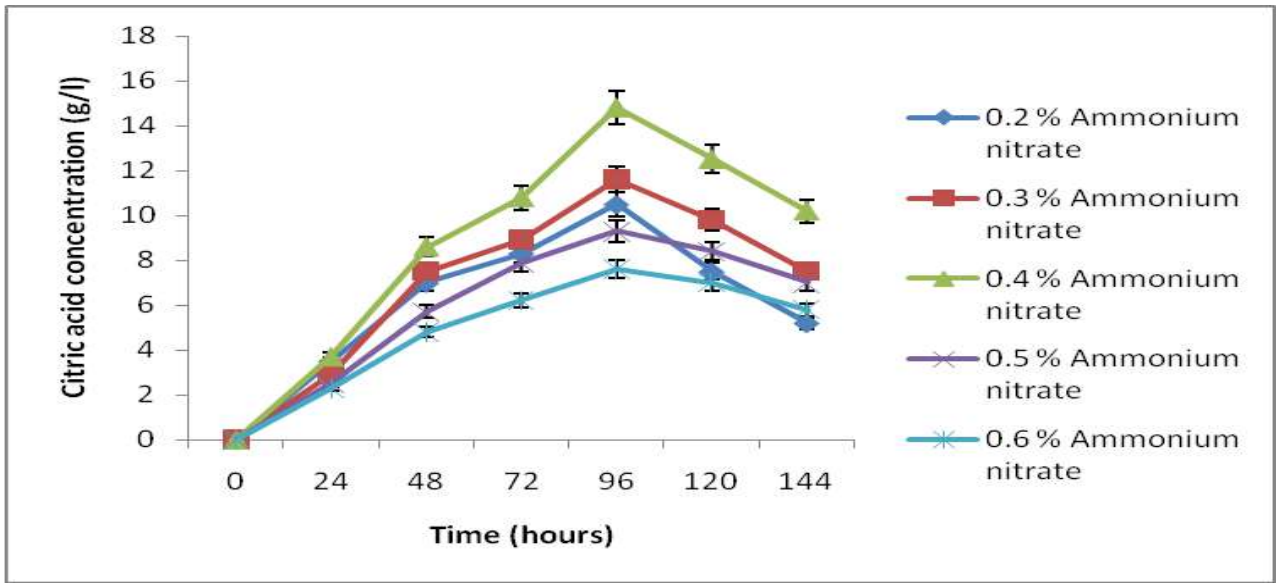

Fig 4: Effect of different of Ammonium nitrate on citric acid production from *Dioscorea bulbifera* tubers (flour)

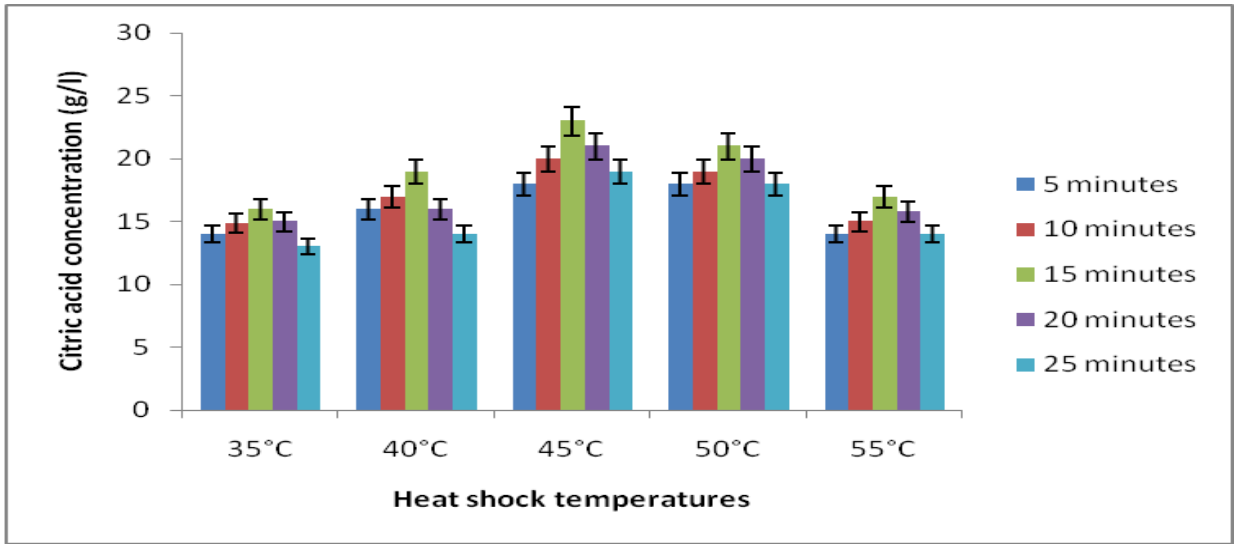

Fig. 5: Stimulation of citric acid production by heat shock *Aspergillus niger* at various length of time

DISCUSSION

Citric acid production from *Dioscorea bulbifera* tubers increased as the fermentation time increased to 96 hours. As the percentage of *Dioscorea bulbifera* tubers increased up to 20 % concentration, the amount of citric acid produced also increased from 2.0 ± 0.09 g/l to 8.5 ± 1.0 g/l after 96 hours of fermentation. This is in agreement with Ezea and Ezaka, (2022) and Ezea, (2022). whose works reported the same substrate concentration during citric acid production in submerged culture of *Aspergillus niger*. Auta*et al*. (2014) reported a similar result from their research. Ezea et al. (2021) reported 20 % substrate concentration as the optimum condition for the maximum citric acid production after 120 hours of fermentation. Okareh*et al*. (2016) reported the same trend during citric acid production from solid state fermentation of sugar cane waste. In a related study that involved a longer fermentation time, Sharma *et al*. (2021) reported citric acid production from different waste substrates such as banana peels, coconut husk and rice straw after 216 hours of fermentation. These results suggested that different substrates have different effects on citric acid production. Substrates with more cellulostic material take more fermentation time to accumulate citric acid by *Aspergillusniger*. Substrates with starchier material take less fermentation time for the production of citric acid (Sharma *et al*. (2021). The amount of citric acid production during fermentation may depend on the nature of the substrate, the strain used and the fermentation condition.

The effects of different nitrogen sources in enhancement of citric acid production from *Dioscorea bulbifera* flour evaluated in this study showed that ammonium nitrate is the most preferable. Shankar and Sivakumar (2016) reported a similar result during optimization of citric acid production. The nitrogen constituent of the medium has a profound effect on citric acid production because nitrogen is not only important for metabolic rate in the cell but it is also part of cell protein. The researchers reported that the maximum amount of citric acid was produced when ammonium chloride was used as nitrogen source followed by ammonium sulphate. *Ezea et al*. (2015) reported that the maximum citric acid was produced in cassava flour supplemented with ammonium nitrate as nitrogen source. Boufaris*et al*. (2017) reported that the following nitrogen sources; ammonium chloride, ammonium sulphate, ammonium dihydrogen phosphate, ammonium hydrogen phosphate, peptone, urea and sodium nitrate improved citric acid

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accumulation compared with the control without nitrogen source.

The concentration of citric acid increased when the concentration of ammonium nitrate increased to certain level with maximum concentration of 14.8 ±2.0 g/l citric acid. The results obtained from this study is in agreement with Ikram-ul-Haq*et al*. (2005) who reported that nutrients especially nitrogen sources had a marked influence on citrate production because it is an essential constituent of basal cell proteins. It has been established that nitrogen source is a limiting factor during citric acid productionFindings from this study showed that the growth rate of *Aspergillus niger* was decreased and the biosynthesis of citric acid was delayed at higher concentrations of ammonium nitrate. Odu*et al*. (2020) reported during optimization of citric acid production in solid state fermentation using banana peel substrate that different concentrations of nitrogen source had different effects on citric acid production. The mechanism of citric acid accumulation by *Aspergillus niger* is still not clear. Papagianni *et al*. (2005) reported that in the early stages of citric acid fermentation, *Aspergillus niger* produce more citric acid when ammonium ions combine with a carbon- containing metabolite inside the cell. This suggests that interaction between ammonium and carbon containing compound may trigger overproduction of citric acid by *Aspergillus niger*. Nitrogen limitation is necessary for citric acid production because it encourages pellets formation in filamentous fungi which is one of the factors that has been considered.

Pre-incubation of the *Aspergillus niger* spores at different temperature heat shock stimulated citric acid production in *Dioscorea bulbifera* enriched with different nitrogen sources. This is in agreement with Anastassiadis and Rehm (2006) who reported a continuous citric acid secretion at an elevated temperature shock during citric acid production. The exposure of *Aspergillus niger*, to heat stress conditions led to an upregulation of the relative expression levels of proteins with repair and protective functions. These proteins functioned in the citric acid cycle (TCA), pyruvate metabolism, porphyrin and chlorophyll metabolism, oxidative phosphorylation, and metabolic pathways (Deng *et al*., 2020). The upregulation and protective repair functions in *Aspergillus niger* may have led to the production of nasant polypeptides and induced citric acid secretion.

CONCLUSION

Wild *Dioscorea bulbifera* tuber has the potential of producing citric acid in reasonable quantity. Despite the fact that wild *Dioscorea bulbifera* tuber is found in virgin lands and forests uncultivated, its utilization as substrate has positive effect on citric acid production. Different concentrations of wild *Dioscorea bulbifera* tuber (flour) vary significantly on citric acid production. The use of ammonium nitrate as a nitrogen source had a significant positive effect on the citric acid production. The application of heat shock stimulation on *Aspergillus niger* spores promoted citric acid production using *Dioscoreabulbifera* flour as a substrate on *D. bulbifera*. Therefore, harnessing wild *Dioscorea bulbifera* tuber for citric acid production would promote its cultivation in the developing countries like Nigeria and other Africa countries, thereby reduce the cost of citric acid production and create more job opportunities.

Abbreviations
Not applicable

Declarations

Ethics approval and consent to participate
Not applicable

Availability of the data and materials
All data generated or analyzed during the study are included in this article

Consent for publication
Not applicable

Competing interests
The authors declare no competing interests relevant in this work

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Author's contributions

IBE drafted the proposal, participated in laboratory work and writing of the manuscript; CFA participated in proofreading the manuscript and statistical analysis of the work and EE participated in laboratory work and proofreading the manuscript.

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