



Investigating the Usefulness of Wild *Dioscorea esculenta* (Wild Yam) Flour as Substrate for Citric Acid Biosynthesis

Ifeanyi Boniface Ezea^{a*}, Anthonia Ngozi Ewoh^a
and Roseline Ifeyinwa Ezugwu^a

^a Department of Applied Microbiology and Brewing, Enugu State University of Science and Technology, Enugu, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background and Objective: The cost of substrate represents a significant percentage of the total biosynthesis cost of citric acid. There is need to continue searching for better and cheap underutilize starchy raw material for citric acid biosynthesis. Therefore, the objective of this research was to biologically synthesis citric acid in submerged fermentation of *Aspergillus niger* using wild *Dioscorea esculenta* (wild yam) tubers.

Methodology: Submerged fermentation was conducted and the effect of *Dioscorea esculenta* concentrations, combined pretreatment and membrane permeability induction on citric acid biosynthesis were carried out.

Results: Citric acid concentration increased as the concentration of the wild yam increased with maximum citric acid production of 10.5 ± 0.9 g/ l after 96 hours of fermentation. The overnight combined pretreatment with 0.3 % NaOH before pretreatment at 121°C for 20 minutes as one time

*Corresponding author: E-mail: ifeanyi.ezea@esut.edu.ng;

combined pretreatment yielded 15.0 ± 1.2 g/l citric acid. Calcium chloride membrane induction produced 24.5 ± 1.3 g/l citric acid concentration respectively after 96 hours of fermentation.

Conclusion: The result showed that wild *Dioscorea esculenta* tubers can be used as a substrate for the biosynthesis of citric acid. The biosynthesis of citric acid from *Dioscorea esculenta* tubers was enhanced by increased in concentration, combined pretreatment and membrane permeability induction.

Keywords: Biosynthesis; citric acid; wild *Dioscorea esculenta*; pretreatment; permeability induction.

1. INTRODUCTION

“Citric acid ($C_6H_8O_7$, 2-hydroxy-123-propane tricarboxylic acid) is a very important organic acid in the field of food and pharmaceutical” [1,2,3,4]. Aside food and pharmaceutical, citric acid is found useful in the field of cosmetics and other related Industries such as detergent and toiletries [5].

Owing to the numerous applications of citric acid, the cost of citric acid is high due to its demand. Citric acid is the most produced organic acid measured in tonnage. To meet the demand for citric acid, more tonnes are expected to be produced.

However, one of the challenge hindering citric acid production is the cost of substrate or raw material to meet the current demand. Commercially citric acid is produced by submerged fermentation using malasses based medium by *Aspergillus niger* [6]. Some carbohydrate rich agro resources have been found as promising substrate for citric acid production [7,8,9]. Many wild carbohydrate crops are unexplored in the area of citric acid production. Some wild carbohydrate crops, if well harnessed can be converted to useful metabolites. Limitation of mineral salts and some metallic ions in the medium during citric acid production is necessary.

Wild *Dioscorea esculenta* commonly known as the lesser yam or wild yam is a yam species but with a small corm than most other yams. Wild *Dioscorea esculenta* is not edible though some cultivated varieties are edible. Wild *Dioscorea esculenta* is one of underutilized tubers that can be found during dry season mostly in the bushes in Nigeria and some other African countries. The carbohydrate and amylose contents of wild *Dioscorea esculenta* flour are 86.69 % and 29.92 % respectively indicating its suitability or potential for energy source [10]. Utilization of wild *Dioscorea esculenta* for citric acid production will

be a better alternative and will help to meet the production demand for citric acid. This research was designed to produce citric acid in submerged culture of *Aspergillus niger* using African wild *Dioscorea esculenta* flour and also to know the effect of different pretreatment and calcium ion induction on citric acid production.

2. MATERIALS AND METHODS

2.1 Microorganism and Culture Maintenance

Aspergillus niger strain previously obtained from Department of Microbiology University of Nigeria, Nsukka and maintained on potato dextrose agar (PDA) slants at $4^\circ C$ and sub-cultured at intervals was used.

2.2 Collection of Samples and Pretreatment

The wild *Dioscorea esculenta* (wild yam) tubers were collected from a virgin lands (bushes) and forests at Nsukka in Enugu State, Nigeria. The tubers were peeled, sundried, ground and sieved into a fine powder using Muslin cloth. A 10 g of wild *Dioscorea esculenta* flour was suspended in 100 ml basal nutrients medium and thermally pretreated at $121^\circ C$ for 20 minutes using an autoclave.

2.3 Inoculum Preparations

The inoculum was prepared according the method of Ezea et al. [11], the spores of *Aspergillus niger* was harvested from potato dextrose agar slant using a sterile solution of 0.01% Tween 80. The inoculation wire loop was used to dislodge the spores and to ensure proper mixing of the culture with the Tween 80 thereafter, 10 ml of 5×10^7 spores/ml was counted using haemocytometer and was used as inoculum.

2.4 Submerged Fermentation of Wild *Dioscorea esculenta* Flour

Submerged fermentation was carried out as described by Ezea et al. [11], 10 g wild *Dioscorea esculenta* flour was weighed using DENVER digital weighing balance, model: MXX-123 USA and suspended in 100 ml nutrient medium in a conical flasks containing NH_4NO_3 , 2 g/l; KH_2PO_4 , 0.2 g/l; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l; $\text{Fe}(\text{SO}_4)_2 \cdot 7\text{H}_2\text{O}$, 0.01 g/l and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l at pH 5.0 before pretreatment. The sample was inoculated with 10 ml of *Aspergillus niger* spores and incubation at 30 °C for 144 hours under rotary incubator shaker (model: VWR International by B. Bran Scientific & Instrument Company England) at 225 rotations per minutes.

2.5 Effects of Different Percentage of Wild *Dioscorea esculenta* Flour on Citric Acid Biosynthesis

Different percentage of *Dioscorea esculenta* flour (5, 10, 15, 20 and 25 %) were investigated on citric acid production by suspending 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.

2.6 Effect of Different Pretreatment of Wild *Dioscorea esculenta* Flour on Citric Acid Biosynthesis

The effects of African wild *Dioscorea esculenta* flour pretreatment was carried out by soaking 20 g of wild *Dioscorea esculenta* flour in 100 ml of hot water, 0.1 % NaOH and 0.1 % HCl containing the basal nutrients and allowed to stay at room temperature overnight in 250 ml conical flasks. Thereafter, medium was adjusted to pH 5.0 before autoclaving at 121 °C for 20 minutes.

2.7 Effect of Permeability Induction Using Calcium Chloride on Citric Acid Biosynthesis

Membrane permeability induction of *Aspergillus niger* on citric acid production using Calcium chloride was investigated by varying the percentage of Calcium chloride from 0.1 - 0.4 % (w/v). The percentage of Calcium chloride was introduced in 100 ml of *Dioscorea esculenta* flour nutrients medium before the pretreatment.

2.8 Citric Acid Determination

Citric acid was estimated using pyridine acetic anhydride method as reported by marrier and Boulet [12]. "A 1 ml of diluted culture filtrate along with 1.30 ml of pyridine was added in the test tube and swirled briskly. Then 5.70 ml of acetic anhydride was added in the test tube. The test tube was placed in a water bath at 32 °C for 30 minutes. The absorbance was measured on a Spectrophotometer 722S B. Bran Scientific and Instrument Company, England at 420 nm against the blank and the citric acids of the samples were estimated with reference standard" [12].

2.9 Statistical Analysis

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

3. RESULTS

Fig. 1 shows the evaluation of citric acid production from wild yam. Citric acid concentration increased from 0.3 ± 0.01 g/l to 2.5 ± 0.06 g/l after 96 hours of fermentation of wild yam flour with the *Aspergillus niger*. Citric acid concentration increased as the concentration of the wild yam increased from 5 % to 20 % with maximum citric acid production of 10.5 ± 0.9 g/l after 96 hours of fermentation with 20 % wild yam concentration, however, 5 %, 10 % and 25 % wild yam had lower citric acid accumulation when compared with 15 % and 20 % (Fig. 2).

Fig. 3 shows the effect of combined pretreatment with 0.1 % NaOH, 0.1 % HCl and hot water, followed by general pretreatment at 121 °C for 20 minutes. The result shows that the overnight combined pretreatment with 0.1 % NaOH, 0.1 % HCl and hot water before pretreatment at 121 °C for 20 minutes produced 15.0 ± 1.2 , 11.8 ± 0.8 and 9.0 ± 0.7 g/l of citric acid respectively after 96 hours of wild yam flour fermentation. Different percentages of NaOH ranging from 0.1 to 0.5 % were investigated on wild yam for citric acid production. 0.3 % NaOH was the best on citric acid production when compared with other percentage of NaOH with 22.5 ± 2.0 g/l citric acid concentration after 96 hours (Fig. 4).

Fig. 5 presents the effect of permeability induction on *Aspergillus niger* for citric acid production from wild yam using different concentrations of Calcium chloride. A 0.1%,

0.2 %, 0.3 % and 0.4 % induction with Calcium chloride produced 14.6 ± 1.0 , 24.5 ± 1.3 , 16.8 ± 0.9 and 12.2 ± 0.5 g/l citric acid concentration respectively after 96 hours of fermentation.

4. DISCUSSION

“Biosynthesis of citric acid from *Dioscorea esculenta* flour (wild yam) by *Aspergillus niger* in submerged fermentation was in agreement with the direct production of citric acid from corn and potato starch, cassava waste, starch hydrolysate, whole maize flour, pap processing waste and wild cocoyam as reported” by [13,14]. “Citric acid concentration increased as the concentration of

the *Dioscorea esculenta* flour (wild yam) increased from 5 % to 20 % with maximum citric acid production. Citric acid production with potato extract media supplemented with different and varying concentration increased as the potato extract and its supplement increased” [15]. Kudzai et al. [16] reported “potato and rice starch extract during citric acid production by *Aspergillus niger* and found that different substrates have different effects on biosynthesis of citric acid depending on their concentrations. These suggested that carbon source and its concentration had a great influence on citric acid production depending on the nature and type carbon used for fermentation”.

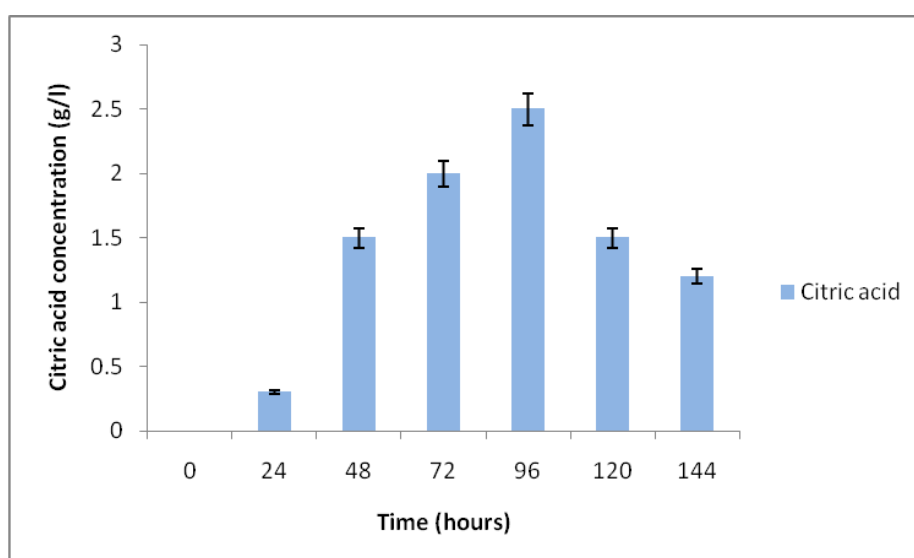


Fig. 1. Biosynthesis of citric acid from *Dioscorea esculenta* flour (wild yam)

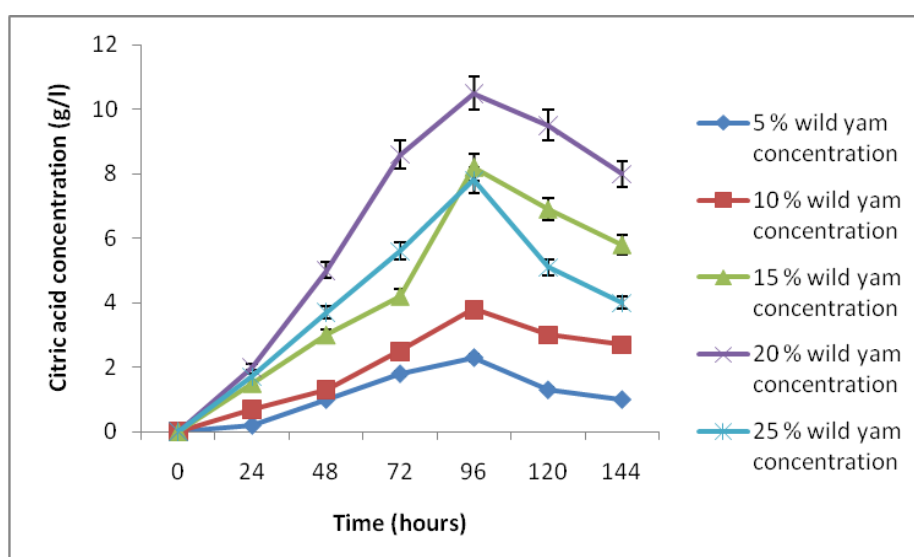


Fig. 2. Effect of different concentrations of *Dioscorea esculenta* flour (wild yam) on citric acid biosynthesis

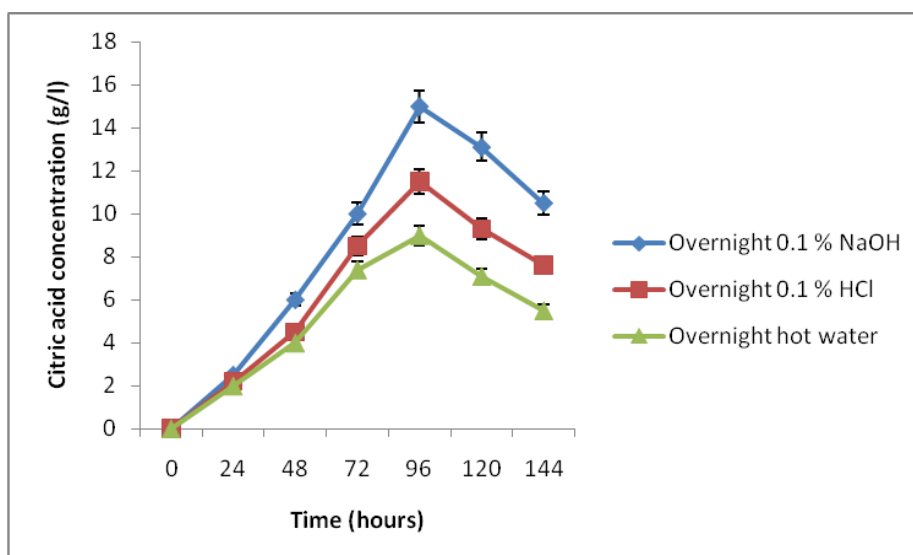


Fig. 3. Effect of combined pretreatment of *Dioscorea esculenta* flour (wild yam) on citric acid biosynthesis

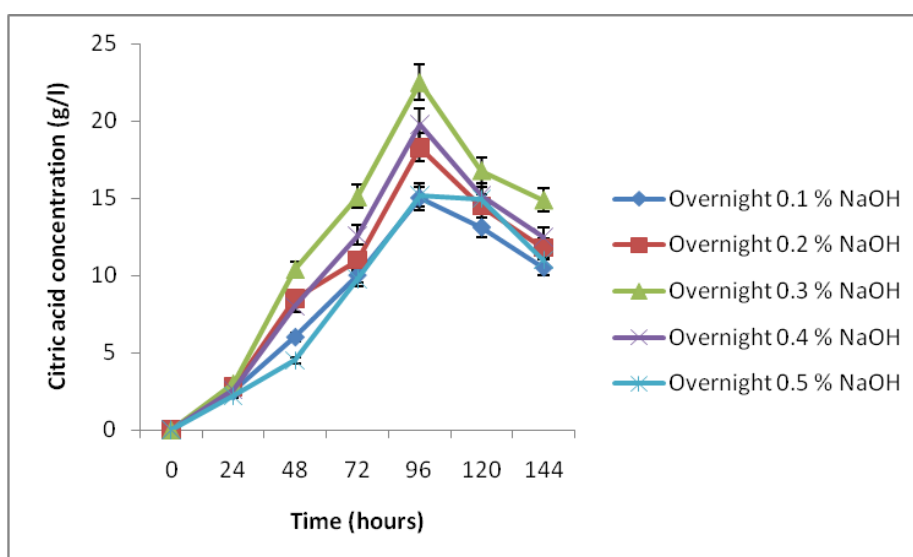


Fig. 4. Effect of different percentage NaOH pretreatment of *Dioscorea esculenta* flour (wild yam) on citric acid biosynthesis

Overnight combined pretreatment with 0.1 % NaOH, 0.1 % HCl and hot water before pretreatment at 121 ° C for 20 minutes produced 15.0 ± 1.2, 11.8 ± 0.8 and 9.0 ± 0.7 g/l of citric acid respectively after 96 hours of wild yam flour fermentation. A 0.3 % NaOH was the best on citric acid production when compared with other percentage of NaOH with 22.5 ± 2.0 g/l citric acid concentration after 96 hours. Soaking overnight and heating at 121 ° C for 20 minutes exploded the substrate which might have lead to more sugar production that favored overproduction of citric acid wild yam. Xue et al. [17] reported that

“*Aspergillus niger* exhibited a reduction of the total sugar content and reducing sugar from starch while the final citric acid production was significantly increased”. Adudu et al. [18] pretreated at 60 ° C for 2 hours which favored biosynthesis of citric acid. Torrado et al. [19] pretreated at 100 ° C for 1 hour with positive effect on citric acid biosynthesis. Ali et al. [20] used “combined pretreatment of 1 % HCl and temperature at 30 ° C for 1 hour which led to efficient citric acid synthesis. Chemically induced heat combined with high temperature released more starch from the water yam tubers which

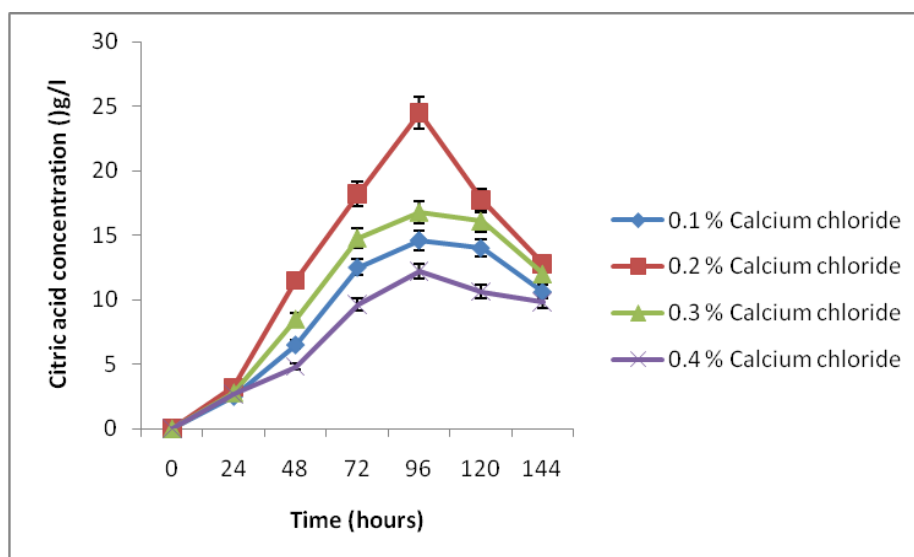


Fig. 5. Effect of permeability induction by Calcium chloride on *A.niger* for citric acid biosynthesis from *Dioscorea esculenta* flour (wild yam)

may trigger overproduction of citric acid. Also at an elevated temperature P, Mn, Fe and Zn content of the tubers are decreased which might have triggered overproduction of citric acid. Though, the mechanism of formation of citric acid is not yet clear, but hydrolysis and fermentation by *Aspergillus niger* favored citric acid biosynthesis which is the intermediate of the krebs cycle”.

Membrane permeability induction of *Aspergillus niger* by Calcium chloride enhanced citric acid production with maximum concentration of 24.5 ± 1.3 g/l citric acid using 0.2 % Calcium chloride. This is in agreement with Pera and Callieri [21] who reported that an addition of Calcium in fermentation medium induced a pellet form of growth which led to overproduction of citric acid in the medium. Pellet formation may have increased the permeability and increased the uptake of nutrients and the accumulation of citric acid by *Aspergillus niger*. This is also in agreement with Pera and Callieri [22] who reported that “incorporation of Calcium chloride to the fermentation medium at certain concentration highly stimulated citric acid production from sugarcane molasses - based medium by a strain of *Aspergillus niger*”. In contrary to the above report, Yasser and Saad [23] reported “lower citric acid production in the medium containing Calcium ion alone”. Yasser and Saad [23] also reported greater citric acid concentration on the control medium that had Tricalcium phosphate. This result suggested that the greater citric acid

concentration might have arisen from the presence of the Calcium ion in the Tricalcium phosphate compound.

5. CONCLUSION

Current results showed that *Dioscorea esculenta* tubers (wild yam) tuber has the potential to be exploited as substrate for citric acid biosynthesis. Utilization of wild yam as substrate had positive effect on citric acid biosynthesis. Different concentrations of wild *Dioscorea bulbifera* tuber (flour) vary significantly on citric acid biosynthesis. Combined pretreatment with NaOH had positive significant effect on the citric acid biosynthesis. A different percentage of Calcium chloride on membrane permeability induction varies significantly with positive effect on citric acid biosynthesis.

This work shows that *Dioscorea esculenta* tubers (wild yam) if well harnessed for citric acid biosynthesis will encourage cultivation of the crop, which in turn creates job opportunity for the teeming youth in Africa and other developing countries. Therefore, utilization of the wild crop for citric acid biosynthesis is cost effective with profit maximization as citric acid is applied in food, pharmaceutical, cosmetics and detergent industries.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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