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ISSN (PRINT) : 2320 -1967
ISSN (ONLINE) : 2320 -1975



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CHEMXPRESS 8(3), 201-207, (2015)

Biological production of citric acid in solid state cultures of *Aspergillus niger*

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Abstract : The effects of nutrient supplementation, initial pH and alcohol addition on citric acid production by *Aspergillus niger* under solid-state culture was studied, using cassava flour, corn flour or rice grain as the carbon source. Citric acid concentrations (g/kg) of 31.5, 17, and 20.5 were obtained from cassava flour, rice grain and corn flour, respectively. The highest concentration of citric acid (58.5g/kg) was accumulated when 2 mL of methanol and 0.02 g of NH_4NO_3 were added to 100 g of cassava flour. Simultaneous alcohol fermentation and

citric acid production from cassava flour by co-inoculation of *Aspergillus niger* and *Saccharomyces cerevisiae* gave citric acid concentration of (53.5 g/kg) which was comparable to the value (54 g/kg) obtained when ethanol was added to a monoculture of *Aspergillus niger* under the same culture conditions. © Global Scientific Inc.

Keywords : Solid state fermentation; Citric acid; Nutrient supplements; Cassava flour; Corn flour; Rice grains.

INTRODUCTION

Citric acid is one of the most versatile industrial organic acids that are used in food preparations, cosmetics and pharmaceuticals. As at 2003, the global demand for citric acid was about 6.0×10^5 tones per year^[1], and about 70% of the citric acid is utilized in food industry, confectionery and beverages as an acidulant, flavor enhancer, preservative, chelator, buffer, emulsifier, stabilizer and antioxidant, while about 10% is used in cosmetics and pharmaceutical industries^[2-5]. Thus, availability of cheap citric acid can facilitate developing of many indus-

tries, especially in poor developing countries in tropical African and Asian countries. Commercial production of citric acid is generally by submerged fermentation of sucrose or molasses using the filamentous fungus *Aspergillus niger*^[6-9]. Several other raw materials such as hydrocarbon and starchy materials, have also been investigated as substrates for submerged citric acid production^[10].

Solid-state fermentation (SSF) refers to the cultivation of microorganisms in a low-water activity environment (no free running water) on a non-soluble material acting as both nutrient source and physical support^[11]. Solid state fermentation has been inves-

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tigated as an alternative to submerged fermentation in the production of microbial metabolites^[12]. The main advantages of solid state cultures include simplicity in terms of both the type of bioreactors and production process, low water requirement and high product yields. It can even be operated without constant power supply by occasional manual mixing. Solid state cultures are therefore more suitable than sub-merged cultures for developing countries. The cost of citric acid is high, mainly due to the high cost of the substrates and this has necessitated the search for cheap and easily available substrates for citric acid production^[13,14]. Many developing countries within the tropics have a lot of underutilized, cheap starchy materials that can be converted to citric acid and other useful metabolites. Development of industries for value addition and processing of agricultural produce of these countries is a sure way of creating jobs and alleviating poverty in these countries.

This work was therefore designed to investigate citric acid production from different starchy materials that are abundant in developing countries within the tropics, using simple solid state cultures. The feasibility of enhancing citric acid productivity by addition of nutrient supplementation cocktail and alcohols was also investigated.

MATERIALS AND METHODS

Collection and preparation of the substrates

Cassava tuber, corn and rice grains were obtained from the Faculty of Agriculture, University of Nigeria, Nsukka, Enugu State, Nigeria. The cassava tubers were peeled, washed, sliced, dried and milled into powder. The corn was also milled, using a hand grinding machine.

Microorganisms and culture conditions

Aspergillus niger and *Saccharomyces cerevisiae* were obtained from the Department of Microbiology, University of Nigeria, Nsukka. The cultures were maintained on Potato Dextrose Agar (PDA) slants at 4°C and sub-cultured at two weeks intervals. The spores of *A. niger* were 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. The concentration of the spores was counted using haemocytometer.

A 100 g dry weight of rice grain, cassava flour or corn flour was soaked in 70ml of distilled water for 2 hours and steamed. After cooling, the rice, cassava paste and corn paste were inoculated with 10ml of spore suspension (5×10^7 spores/mL) and mixed properly, wrapped in a clean muslin cloth and incubated at $30 \pm 2^\circ\text{C}$ for 5 days. During the incubation, 10 g fermented samples were taken every day, suspended in 100 ml of distilled water, mashed, mixed well, allowed to settle and filtered using Whatman no. 1 filter paper. Ten (10) ml of the filtrate was used to determine the citric acid concentration and the pH.

The effects of the initial pH on citric acid production in solid state cultures were determined by varying the pH from 3.5 to 7.5. The effects of nutrient supplementation on solid state citric acid production by *A. niger* were also investigated. A 5 ml, 10 ml, 15 ml or 20 ml of a nutrient cocktail 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 or each of the individual components was added to 100 g of the substrate (rice grain, corn or cassava paste) and solid state citric acid fermentation was carried out as described before. The effects of methanol and ethanol addition on citric acid production by *Aspergillus niger* were determined by adding 2 ml of absolute methanol or ethanol to the substrate under aseptic condition. Simultaneous alcohol fermentation and citric acid production was also investigated by simultaneously inoculating 5 ml each of *Saccharomyces cerevisiae* (5×10^7 cells/mL) broth and *A. niger* spores suspension (5×10^7 spores/mL) into the substrate under aseptic condition and incubating as described before. Samples were taken periodically for citric acid analysis.

Citric acid determination

The concentration of citric acid in the culture was estimated titrimetrically as described by Kareem et al.^[12] using 0.1M NaOH and phenolphtha-

lein as indicator. The percentage citric acid in the sample was calculated from the following equation

% Citric acid =

$$\frac{\text{Normality} \times \text{Titre value} \times \text{equivalent weight of citric acid} \times \text{dilution factor}}{\text{Weight of sample (g)} \times 10}$$

Statistical analysis

Data obtained were subjected to one-way analysis of variance (ANOVA) and the means were separated using the Least Significant Difference method.

RESULTS

Effect of pH on solid-state citric acid production

Figure 1 shows that at all the initial pH values tested, there were significant differences in citric acid production from the substrates ($P < 0.05$), and cassava flour gave the highest citric acid concentration among the three substrates. For each substrate, pH of 5.5 was the optimum for solid-state citric acid production. At this pH, the citric acid concentrations were 17.5 g/kg, 20.5 g/kg and 31.5 g/kg for rice

grains, corn flour and cassava flour, respectively. In the present study, the maximum amount of citric acid (31.5 g/kg) was produced from cassava flour, which was 1.54 fold higher than the value obtained from corn flour and 1.8 fold higher than that of rice grain.

Effect of nutrient supplementation on citric acid production

The effect of different volumes of nutrient supplement on citric acid production is shown in Figure 2. With each substrate, the concentration of citric acid increased with increase in the volume of the nutrient supplement from 5 to 15 ml but decreased when the volume was increased to 20 ml. Addition of 10 ml of nutrient supplement to cassava flour and rice grains enhanced citric acid production from 31.5 g/kg to 53.0 g/kg and from 17.0 g/kg to 25.5 g/kg, respectively. In the case of corn flour, 15 ml of the nutrient supplement was the best, resulting in an increase in citric acid concentration from 20.0 g/kg to 38.5 g/kg. In all the substrates, supplementation with 5 ml and 20 ml of the nutrient supplement gave lower citric acid concentrations than the values obtained with

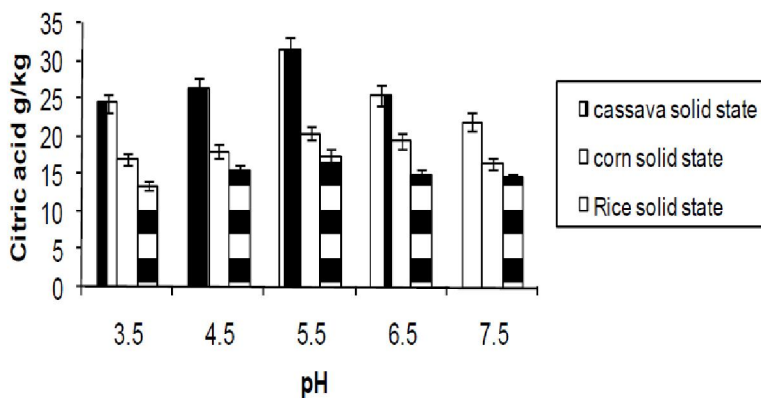


Figure 1 : Effect of substrate and pH on solid-state production of citric acid

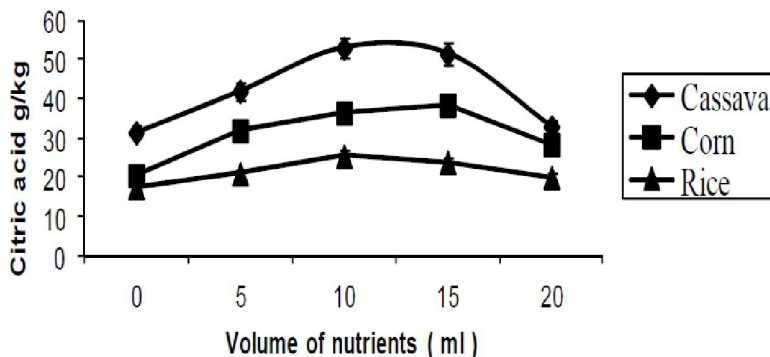


Figure 2 : Effect of nutrients supplementation on citric acid production; The nutrient supplement was composed of $(\text{NH}_4)\text{NO}_3$, 2 g/l; KH_2PO_4 , 0.2 g/l; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l; $\text{Fe}(\text{SO}_4)_2 \cdot 7\text{H}_2\text{O}$, 0.01 g/l and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l.

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TABLE 1 : Effect of supplementing substrates with individual nutrients on citric acid production in solid state culture. Citric acid obtained (g/kg)

Nutrient	Optimum concentration	Cassava (100 g)	Corn (100 g)	Rice (100 g)
Nutrient cocktail	10 mL	53.0±0.5	38.5±0.3	25.0±0.2
ZnSO ₄ .7H ₂ O	0.0001 g	14.5±0.2	12.5±0.3	11.0±0.2
MgSO ₄ .7H ₂ O	0.005 g	15.5±0.2	13.5±0.4	1.5±0.1
Fe(SO ₂).7H ₂ O	0.0001 g	24.5±0.5	21.5±0.2	17.5±0.2
KH ₂ PO ₄	0.002 g	21.5±0.2	18.5±0.5	15.5±0.3
NH ₄ NO ₃	0.02 g	37.5±0.3	24.5±0.5	24.0±0.5
Control	0.0	31.5±0.5	20.5±0.3	17.5±0.4

10 and 15 ml. Cassava flour produced a maximum of 53.0 g/kg citric acid on supplementation with nutrient cocktail. This value is 1.38 and 2.08 folds higher than the values obtained from corn flour and rice grains, respectively under the same conditions.

Effect of supplementing substrates with individual nutrients on citric acid production in solid state culture

Production of citric acid from 100g of cassava flour, corn flour or rice grain supplemented with either 0.0001 g of ZnSO₄.7H₂O, 0.005 g of MgSO₄.7H₂O, 0.0001 g of Fe(SO₄)₂.7H₂O, 0.002 g of KH₂PO₄ or 0.02 g of NH₄NO₃ is shown in TABLE 1. Although supplementation of the substrate with the nutrient cocktail (a mixture of all the nutrients) significantly enhanced citric acid production when compared with the control, supplementation with the individual minerals resulted in either lower or marginal increase in citric acid production. However, addition of NH₄NO₃ alone resulted in significant increase in citric acid production over the control but was significantly lower than the value obtained with the nutrient cocktail.

Effect of alcohol addition to cassava flour supplemented with NH₄N.O₃ as the nitrogen source on citric acid production

Figure 3 shows the effect of ethanol and methanol addition to cassava flour on citric acid production. Addition of either ethanol or methanol to cassava flour resulted in increase in citric acid production compared to the control. In each case, the citric acid concentration increased with increase in the cultivation time up to 120 h. A maximum citric acid concentration of 58.5 g/kg was obtained when 2 mL of methanol was added to 100 g of cassava flour. Simultaneous ethanol fermentation and citric acid production by co-culture of *Saccharomyces cerevisiae* and *Aspergillus niger* enhanced citric acid production. Under this culture condition, the citric acid concentration (53.5 g/kg) was almost the same as the value (54.0 g/kg) obtained by addition of ethanol to mono-culture of *Aspergillus niger*.

DISCUSSION

This study has shown that cassava flour is a bet-

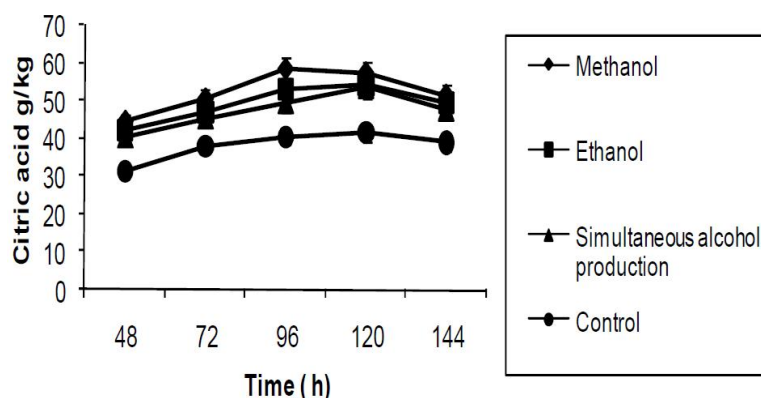


Figure 3 : Effect of alcohol addition on citric acid production from cassava flour by *A. niger* in solid-state cultures

ter substrate for citric acid production than maize and rice flours. Previous studies have indicated that the type of substrate has a significant influence on citric acid production^[15]. In their study, sweet potato starch produced the highest concentration of citric acid which was 1.52 fold higher than the value obtained with maize. In terms of economic and environmental considerations, cassava wastes would be a better substrate than cassava flour. However, the hydrolysable carbohydrate content may be too low for efficient production of citric acid. For example, Vandenberghe et al.^[16] reported lower citric acid production in dry cassava bagasse, probably due to lower starch content. The differences in the citric acid yields obtained from the various substrates may be due to the composition of the substrates (starch and other components) which may directly or indirectly affect citric acid synthesis or due to difference in the water activities. For example, when the same volume of water was added to the substrates, rice grain had the highest water activity, followed by corn paste while cassava paste had the lowest water activity.

Generally, many mineral elements are required either as major or minor elements in fermentation media. These elements affect both cell growth and secondary metabolites production and regulation of their concentration is a good method of inducing overproduction of secondary metabolites by the microorganisms^[17]. Starchy tubers and grains contain enough carbon but are usually deficient in nitrogen and other elements. Thus, there is a need to supplement with different sources of these essential elements. In the present work, citric acid was greatly enhanced by addition of a mixture of mineral salts. The result agrees with those of Mohamed et al.^[18] who reported enhanced citric acid production using a glucose basal nutrient medium containing salts of K, Mg, Fe, Zn, Cu and NH_4 . Many other previous studies have also shown that carbon, phosphorus, nitrogen and some trace elements have positive effects on citric acid production^[5, 12, 18, 19, 20]. Phosphorus, for example, affects enzyme activity, being the main component of ATP, a compound used to accumulate and transport energy within cells^[5]. The mechanisms of action of these elements vary since

synthesis of some metabolites is enhanced by addition while synthesis of others is enhanced by deficiency of some particular elements. Furthermore, their effects may be direct or indirect. Physiologically, consumption of nitrogen compound such as ammonium salt, peptone, malt extract, urea and yeast extract leads to decrease in the pH which is essential for fungal growth and citric acid production^[19]. Furthermore, nitrogen is not only important for cell metabolism but it is also a basic component of cell proteins. The present result that NH_4NO_3 was the best in terms of citric acid production is in agreement with the report by EL-Aasar^[4]. He reported that *Aspergillus niger* preferred 0.3% NH_4NO_3 as the nitrogen source in the optimum growth medium, and produced more protein and citric acid than other nitrogen sources. The lower yields obtained when some of the components were added individually may be due to other factors rather than toxicity since when they were added together with other minerals, citric acid production was enhanced. The results also imply that these substrates may contain enough mineral elements, except for nitrogen, for cell growth and citric acid synthesis. Thus, their addition is not necessary when impure substrates are used as the carbon source.

Aside from the nutrient composition, pH is another important factor that affects citric acid production. Although in the present study, pH 5.5 was the optimum for citric acid production, Moataza^[21] reported that citric acid concentration increased with increase in the initial pH from 3.5 to 6.5 and reached the maximal level at pH 6.5.

In the present study, the maximum amount of citric acid (58.5 g/kg) was produced when 2ml of methanol was added into the cassava flour supplemented with NH_4NO_3 as nitrogen source. Kareem et al.^[12] reported that 2% concentration of methanol induced citric acid production by *A.niger*. Sardar et al.^[15] reported that 1.5% (v/v) methanol added into the medium 24hours after inoculation induced citric acid production in potato starch by *A. niger*. In a pre-treated crude date syrup, maximum citric acid was obtained in culture grown in 3% (v/v) methanol^[21]. The mechanism by which alcohols induce citric acid production is not yet clear. Mohamed et al.^[18]

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observed that the addition of methanol into a medium containing NH_4NO_3 as a nitrogen source influenced the size of the pellets and the production of citric acid in submerged culture. The size of the pellets decreased with increasing amount of methanol added in submerged culture. Reduction in the size of pellets means increase in mass transfer into the pellets which could enhance citric acid production. However, the present work has shown that alcohols also enhance citric acid production in solid state culture where pellets are not formed. Hamissa and Radwan^[22] opined that the high stimulating effect of methanol can be attributed to the inhibition of spore formation and the increase of the microorganism tolerance to high levels of minerals contained in different sources of carbohydrate. Maddox et al.^[23,24] reported that the effect of methanol is at the cell permeability level, noting that it allows citrate to be excreted from the cell. The cells then respond by increasing its citrate production via repression of 2-oxoglutarate dehydrogenase in an attempt to maintain an adequate intracellular level of the metabolite. Furthermore, methanol markedly depressed cell proteins in the early stages of cultivation and also increased the enzyme metabolic activity^[25]

In the present study, the maximum concentration of citric acid (58.5 g/kg) produced from cassava flour compares with the maximum concentration of 60.6 g/kg, 52.0 g/kg and 45.0 g/l citric acid from pineapple waste, expanded clay solid substrate, and sweet potato starch hydrolysate respectively^[12, 15, 26]. Generally, people have expressed concern over processing of food crops into non food products, especially in Africa and other developing countries where starvation remains a serious problem. However, lack of value addition and technologies to process the agricultural produce is also partly responsible for the poverty and food insecurity in these poor nations. Lack of guaranteed market, price fluctuations and post harvest losses are some of the factors responsible for low agricultural productivity in Nigeria and many other African countries. Development of technologies for processing of some agricultural products into useful metabolites will guarantee market and prices, drastically reduce both in-farm and post harvest losses and thus stimulate agricultural

productivities^[27]. This study has demonstrated that cassava, which is efficiently cultivated in most parts of Nigeria and many tropical countries can be efficiently converted to citric acid using a simple solid state culture.

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