Citric Acid Production by Aspergillus Niger on a Corn Cob Solid Substrate using One-factor-at-a-time Optimisation Method

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Abstract: The potential of corn cobs as solid substrates for the production of citric acid was investigated using *Aspergillus niger* KA88, a local isolate as the fermenting organism. One-factor-at-a-time (OFAT) model was used to optimize the fermentation media to achieve the maximum yield of citric acid. The fermentation parameters studied and their resultant optimised levels were found to be 28 °C incubation temperature, 15% (w/v) sucrose for 6 days fermentation period, 50% each of (0.5-1mm and 3-5mm) particle sizes. Di-ammonium hydrogen phosphate was the preferred nitrogen source at a concentration of 4g/l. The final OFAT fermentation media gave 138.24 g anhydrous citric acid/kg dry corn cob. In general, corn cob proved to be a highly promising solid substrate for commercial citric acid production.

Keywords: Substrate, *Aspergillus niger*, fermentation, Temperature, One-factor-at-a-tme model.

I. INTRODUCTION

Citric acid is widely used to impart a pleasant, tart flavour to foods and beverages. It also finds applications as a function of additive detergents, pharmaceuticals, cosmetics and toiletries. It is therefore an important commodity in our everyday life with worldwide tonnage of 1.8 million in 2010 [1]. *Aspergillus niger*, a ubiquitous fungus, alone is credited with the production of over 1 million tonnes of citric acid annually. Among the microorganisms, the fungus *A. niger* has remained the organism best suited for commercial production, because, it produces more citric acid per unit time [2]. Although citric acid can be produced chemically from the juice of citrus fruits, fungal fermentation to produce citric acid has gained more prominence than chemical synthesis because the latter is a much more expensive endeavour [3]. Again, even though submerged fermentation is known to dominate worldwide citric acid production, recent works on solid state fermentation by [2-4] yielded considerable amount of citric acid. Over 60% of Ghanaians engage in agriculture in one way or the other, producing millions of tonnes of agro-wastes such as corn cobs.

These pose serious health and aesthetic environmental problems and efforts aimed at disposing these corn cobs have proven to be a mirage. Corn is an important cereal in Ghana and other parts of West and Central Africa. Citric acid production in Sub-Sahara Africa and for that matter Ghana has been on the quiet for one reason or the other despite increasing disposal problems and efforts at by-product utilisation. In Ghana, corn cobs are used as a fuel in direct combustion in cooking in maize growing areas, while in the United States, technology is well advanced in using cobs for cellulosic ethanol, co-firing and gasification projects [5-6]. Local industries have to spend the meagre profits earned to mob up the little foreign exchange in the system to import citric acid and other organic acids, which puts more pressure on an already struggling Ghanaian

currency. The average maize farmer earns GHC 296 per hectare as gross margin per season, with a meager GHC 0.88 as return on investment [7]. For not dumping or burning the corn cobs, but gathering and sending them to "Cobs Point of Sale" the farmer is able to earn extra income by transforming waste to wealth. In this regard, 'Cob Processing Units' could be established around major maize farming communities, where some microbial isolates from the naturally degrading cobs could be tested as starter cultures for the production of compounds of economic importance such as organic acids, sugars, bioethanol and nutritious animal feed supplements. This study therefore seeks to maximize citric acid production by *Aspergillus niger* using corn cob as the solid substrate by optimizing the fermentation media.

II. MATERIALS AND METHODS

A. Pre-treatment of corn cobs
The corn cobs were collected from local farmers after threshing in Antoa, Kwabre East District, Ashanti-Ghana, and brought to the lab for various pretreatments to be carried on them.

B. Isolation of *Aspergillus niger* KA88
This particular *Aspergillus niger* strain was isolated from a rotten cassava by serial dilution in the Microbiology Research Laboratory of the Kwame Nkrumah University of Science and Technology. One millimeter of each diluent was plated on the solidified agar plates by streaking. The plates were incubated at 37 °C for 3-5 days to ensure maximum fungal growth. The fungal strains were purified by re-streaking on the medium and each pure culture were maintained on Potato Dextrose Agar (PDA) slants and stored at 4 °C in a refrigerator. The isolate was therefore named as *Aspergillus niger* KA88. Sub-culturing was done monthly on slopes of potato dextrose agar at 30 °C for 7 days.

C. Solid state production of citric acid

To 10 g of substrate in each Erlenmeyer flask (250 ml), 35 ml stock mineral salt solution with a concentration of (g/l): (NH4)$_2$SO$_4$, 5; KH$_2$PO$_4$, 5; MgSO$_4$ .7H$_2$O, 0.25; NaCl, 0.25; and 15% (g/l) sucrose was added. The resultant corn cob in each flask gave the following salt concentration in terms of kg dry corn cob: 17.5 g (NH$_4$)$_2$SO$_4$, 17.5 g KH$_2$PO$_4$, 0.88 g NaCl, 0.88g MgSO$_4$·7H$_2$0 and 525g of sucrose. 0.1 M NaOH/HCl was used to adjust the pH of the medium to 5. The flask was cotton plugged and wrapped by two layers of aluminium foil and then sterilised at 121°C for 15 minutes.

After sterilisation by double autoclaving, the flasks were cooled and inoculated with 2 ml homogenised inoculum culture of 1.0 x 10^7 spores/ml under aseptic conditions and incubated at 30 °C for 6 days. Each fermentation parameter was investigated and the outcome introduced into the succeeding parameter – in a one-factor-at-a-time manner – as a means of gradually optimizing the fermentation media. At the end of the fermentation, 9 parts of distilled water was added to 1 part of the fermented substrate in the flask and vortexed for 10 mins to extract the impregnated citric acid to the mycelia. The broth was filtered with mash followed by Whatman no. 1 filter paper and the supernatant was analysed for citric acid content, a modification of [8].

D. Analytical Technique

Citric acid was determined titrimetrically (AOAC) [9] by using 0.1 NaOH and phenolphthalein as indicator.

III. RESULTS AND DISCUSSION

A. Effect of Incubation Temperature on Citric Acid Production

At 26 °C, low amount of citric acid was produced (Table I), which may be attributed to low enzyme activity of the fermenting organism. As temperature increases, rate of reaction also increased, until it reached its optimal threshold of 28°C.

At temperatures above 28 °C, deactivation effect of temperature on endogenous metabolism set in, leading to a reduction in the biosynthesis of citric acid. Selahzadeh and Roehr [10] asserted that, at lower temperatures, analysing the metabolic effects of the fermenting organism show a waste of large amounts of the carbon source, primarily due to an increase in the organism's respiratory activity. Roukas [11] also reports that, at higher temperatures, there is the inhibition of the enzyme citrate synthase; the terminal enzyme of the pathway responsible for the biosynthesis of citric acid. This rather aids in the accumulation of oxalic acid at such temperatures.

Furthermore, higher fermentation temperatures result in severe moisture losses, making the environment unfavourable for growth. Kareem and Rahman, [12] in their work on the production of citric acid using pineapple waste under solid state fermentation had the highest citric acid titre (43.5 g/kg) at 30 °C. The 28 °C optimum temperature falls within the range observed by Szewcyzk and Myszka [13] on the growth of *Aspergillus niger* to be between 26 °C – 34 °C.

TABLE I: Mean amount of citric acid produced at different temperature of incubation

Incubation Temp't (°C)	Citric Acid Produced (g/kg DCC)	Standard Deviation
26	32.64	1.985
28	46.72	2.161
30	34.00	2.014
32	16.00	1.880
34	12.80	1.564

(Substrate to moisture ratio: 80%, Incubation temperature: 30°C, pH: 5, Sugar source: 15% g/l sucrose, Fermentation period: 6 days, Particle size: 3-5mm, Initial inoculum size: 2ml of 1 x 10^7 spores/ml, Nitrogen source: 5g/l Ammonium sulphate)

B. Effect of Sugar Concentration at Different Fermentation Periods on Citric Acid Production

The low amount of citric acid produced (Table II) may be due to unavailability of readily metabolised carbon source in the control media. Sucrose and glucose are readily metabolised by *Aspergillus niger*, so it is added to the corn cob substrate for easy uptake, for the growth of the fungus. Karimi [14] reports that the fungi cultures after utilizing the readily metabolized sugar source are primed to hydrolyse the lignocellulosic components of corn cobs. The general rise in citric acid titres from day two and peaking on day six, and the ensuing fall on day seven may be due to the age of fungus, depletion of the readily metabolised carbon source or nitrogen. This exhaustion of fermentable sugars may activate a decay in the enzyme system. The organism then tends to feed on the citric acid, secreted into the fermentation media, or citric acid catalysis may have ensued due to the accumulation of waste products in the media. Largely, increasing the sugar concentration resulted in a considerable increase in the amounts of citric acid produced. Peksel and Kubicek [15], hold the view that, a high rate of acidogenesis in *A. niger* is observed only under conditions of high glycolytic metabolism and can be induced by the addition of an excess amount of sucrose or other carbohydrates. Tkacz and Lange, [16] report that, production of more citric acid by the sucrose induced culture to glucose may be attributed to the production of an extracellular mycelium bound invertase by *A. niger* that is active at low pH and rapidly hydrolyses sucrose but the presence of glucose represses the genes that encode invertase. Moreover, the two glucose transporters found in *A. niger* are strongly susceptible to citrate inhibition, thereby terminating citric acid accumulation at some point, even though much of the glucose substrate still exist in the medium. The 20% sucrose media showed a steady rise in citric acid titres and did not witness any drop on the day seven as evident in the other media. Roukas [11] reports that further increase of sugar concentration above 20% (w/v) results in a decrease in the rate of citric acid accumulation as a result of reduced water activity and plasmolysis. This reduced water activity and plasmolysis extend the lag phase of the fungus, reducing fungal numbers, and thereby the amount of citric acid secreted outside the hyphal walls. Ishaq *et al*.

[17] in their work reports that citric acid production starts after a lag phase of one day and peaks at the onset of stationary phase or even late. This observation is in line with the works of (Hossain *et al.*; Papagianni) [18-19] that

sucrose is the best sugar source for citric acid production. They further backed their conclusions by showing the direct relationship that aconitase has on the sugar source during citric acid accumulation.

TABLE II: Effect of Sugar Concentration at Different Fermentation Periods on Citric Acid Production

Fermentation media	Mean Citric acid (g/kg DCC) at different fermentation periods (days)/SD					
	2	3	4	5	6	7
Corn cob only (control)	**13.68**/2.352	**14.73**/2.332	**16.44**/2.340	**17.15**/2.411	**18.78**/2.377	**16.38**/2.329
Corn cob + 5% glucose	**18.84**/2.300	**19.78**/2.423	**20.24**/2.398	**21.96**/2.435	**22.03**/2.443	**21.34**/2.322
Corn cob + 10% glucose	**21.67**/2.401	**23.96**/2.421	**26.01**/2.427	**27.88**/2.403	**29.31**/2.433	**26.75**/2.392
Corn cob + 15% glucose	**24.50**/2.413	**26.44**/2.419	**30.98**/2.456	**32.17**/2.438	**33.00**/2.429	**31.33**/2.410
Corn cob + 20% glucose	**22.32**/2.406	**24.54**/2.422	**26.71**/2.430	**28.56**/2.428	**29.66**/2.387	**30.23**/2.392
Corn cob + 5% sucrose	**21.45**/2.408	**22.91**/2.405	**24.12**/2.414	**25.66**/2.426	**27.08**/2.425	**25.35**/2.404
Corn cob + 10% sucrose	**25.56**/2.427	**27.88**/2.439	**30.26**/2.420	**33.45**/2.423	**35.14**/2.436	**31.77**/2.408
Corn cob + 15% sucrose	**31.11**/2.416	**35.67**/2.453	**40.58**/2.466	**45.27**/2.472	**48.60**/2.512	**42.04**/2.500
Corn cob 20% sucrose	**28.50**/2.422	**32.82**/2.430	**35.63**/2.444	**39.25**/2.461	**41.94**/2.468	**42.68**/2.490

(Substrate to moisture ratio: 80%, Incubation temperature: 28°C, pH: 5, Sugar source: 15% g/l sucrose, Fermentation period: 6 days, Particle size: 3-5mm, Initial inoculum size: 2ml of 1×10^7 spores/ml, Nitrogen source: 5g/l Ammonium sulphate): SD = Standard deviation

C. Effect of Particle Size/Distribution on Citric Acid Production

The smaller the particle size, the larger the surface area of the corn cob particles. This increases the availability of substrate to the *Aspergillus niger*, hence citric acid production is favoured, as a consequence of a more pronounced contact catalysis. The results of this study contradicts this assertion, in that, the finest corn cob particles used in this study produced the lowest amount of citric acid (Table III).

As the particle size was increased to some extent, the citric acid titres also increased. Kumar and Jain, [20] in their findings concluded that the high packing densities evident in smaller sized particles restrict airflow and inhibit fungal growth. Further increases in the particle size beyond the (3.0 – 5mm = c), led to a decrease in the amount of citric acid produced. This is contrary to earlier assertion that relatively larger particle sizes increase the rate of heat and mass transfer in the media, to counteract the highly exothermic respiratory metabolism of *A. niger*, hence, resulting in increases in citric acid titres.

The reason may be, for the 10 g corn cob supplied in each fermenter, the media containing the larger particle sizes were not much exposed to the fermenting organism as there was a considerable reduction in the surface area. Therefore, an effective balance of increasing substrate availability to the fermenting fungus and ensuring adequate heat and mass transfer within the media has to be the utmost priority.

In this instance, localised build-up of temperature and non-homogenous fermentation conditions are eradicated. This is evident in Table III, where the finest particle size which favours substrate availability was mixed with a relatively larger particle size in a 1:1 ratio and ended up as the media with the maximum citric acid produced. This compares favourably with the results from Gopinath [21] on the influence of particle size on citric acid production by *Aspergillus niger* using rice chaff and sesamum oil cake as substrates; and also Bari *et al.* [22] on the effect of particle size on the production of citric acid from oil palm empty fruit branches as new substrate by wild *Aspergillus niger*.

TABLE III: Amount of citric acid produced at different particle size and/or distribution

Particle size and/or distribution	Citric Acid Produced (g/kg DCC)	Standard Deviation
(0.5 – 1mm = a)	17.92	2.316
(1.0 – 3mm = b)	26.88	2.421
(3.0 – 5mm = c)	37.12	2.465
(5.0 – 7mm = d)	35.20	2.445
(7.0- 10mm= e)	29.44	2.399
a+b (1:1)	42.88	2.560
a+c (1:1)	51.84	2.608
a+d (1:1)	48.08	2.597
a+e (1:1)	41.60	2.553

(Substrate to moisture ratio: 80%, Incubation temperature: 28°C, pH: 5, Sugar source: 15% g/l sucrose,

Fermentation period: 6 days, Particle size: varried, Initial inoculum size: 2ml of 1 x 10^7 spores/ml, Nitrogen source: 5g/l Ammonium sulphate)

D. Effect of Different Nitrogen Sources on Citric Acid Production

For the nitrogen compounds analysed, Di-ammonium hydrogen phosphate ; $(NH_4)_2HPO_4$, Di-ammonium hydrogen sulphate; $(NH_4)_2SO_4$, Urea ; $CO(NH_2)_2$ and Ammonium chloride; NH_4Cl reflected the amount of citric acid produced in decreasing order (Table IV). This same order also reflected the ability to excrete stoichiometric amounts of protons into the media, thereby reducing the pH. Brainard and Tsao [23] observed that, within 24 h after the inoculation of spores, ammonium ions are rapidly consumed with the excretion of stoichiometric amounts of protons.

As a result the vegetative growth of germinated spores slows down due to the decrease in pH and citric acid accumulation ensues. In contrast, the hydrolysis of urea liberates ammonia into the fermentation media, leading to a rapid alkalinisation of the media. Hence, biomass accumulation is favoured, to the detriment of citric acid accumulation. With regards to this assertion, a consciously chosen concentration of the nitrogen source, should take cognizance of the initial inocula levels of *A. niger* needed to utilise the ammonia present for the early onset of citric acid accumulation. In fact, literature shows that ammonium sulphate is the preferred source of nitrogen in microbial citric acid production [4, 19-24]. In this study, the di-ammonium hydrogen sulphate gave the optimum citric acid yield of 138.24 g citric acid/kg dry corn cob, with the highest for ammonium sulphate being 115.20 g/kg DCC. Nitrogen is a constituent of many vital compounds including amino acids, basic proteins, enzymes, coenzymes and nucleic acids. An optimum nitrogen concentration must be used to maintain proper C:N ratio in the optimum range because citric acid synthesis will decrease at both lower and higher nitrogen levels [4].

TABLE IV: Effects of Different Nitrogen Sources and their Limitations on citric acid production

Nitrogen Source	Conc.(g/l)	Citric Acid Produced(g/kg)	SD
[$CO(NH_2)_2$]	3	66.56	3.1
	4	44.16	2.6
	5	12.80	1.9
	6	10.24	1.8
[$(NH_4)_2SO_4$]	3	97.92	4.5
	4	115.20	5.2
	5	51.12	2.9
	6	19.84	2.0
[NH_4Cl]	3	38.40	3.2
	4	30.08	3.3
	5	21.76	2.1
	6	19.84	2.2
[$(NH_4)_2HPO_4$]	3	120.96	5.6
	4	138.24	5.9
	5	112.00	5.1
	6	51.20	2.8

(Substrate to moisture ratio: 80%, Incubation temperature: 28°C, pH: 5, Sugar source: 15% g/l sucrose, Fermentation period: 6 days, Particle size: 5 g each of (0.5 − 1mm and 3-5mm), Initial inoculum size: 2ml of 1 x 10^7 spores/ml)

IV. CONCLUSION

In conclusion, the results obtained in the study proved the effectiveness of the OFAT studies in maximizing citric acid production. The first parameter trial conducted on the effect of incubation temperature produced 46.72 g citric acid per kg dry corn cob. After three other parameters were investigated, with the outcome of each trial been incorporated into the succeeding one,138.24 g citric acid per kg dry corn cob was produced.

As a result, 91.52 g citric acid per kg dry corn cob was the amount of citric acid realized from the OFAT studies, thus the difference between the first and last trial. The study thus presents corn cob as a potential solid substrate for the commercial production of citric acid in Ghana.

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