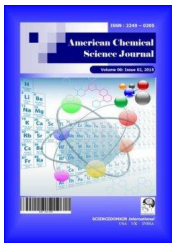

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Studies on the Production of Citric Acid by *Rhizopus stolonifer*

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

A fermentation process was developed for citric acid production from orange peel waste by *Rhizopus stolonifer*. In a screening programme to select the best citric acid producing fungus, it was showed that after 3 days of incubation, the amount of citric acid in the screening medium varied depending on the isolate used. *Rhizopus stolonifer* produced the highest citric acid titre (5.8 g/L) and was therefore selected for fermentation studies. The addition of 3% and 4% (v/v) methanol into the isolation medium resulted in a citric acid yield of 7.8 g/L and 7.1 g/L respectively. This level corresponded to 135 and 122% respective increases in citric acid yield in comparison to the control medium that produced only 5.8 g/L. The basal medium was supplemented with different concentrations of lactose, maltose and sucrose. It was found that sucrose at 15% (w/v) concentration caused the best citric acid yield (33.7 g/L). Yields of citric acid generally increased with sugar concentrations and maximum production rates were achieved at 10–15% of sugar.

Keywords: Citric acid; orange peel; sucrose; lactose; maltose.

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1. INTRODUCTION

Citric acid (CA) is the leading constituent of citrus fruit and is currently one of the most important organic acids produced by microbial fermentation. Chemically, CA is 2 hydroxyl propane – 1, 2, 3, - tri carboxylic acid. Because of its characteristics, it is widely used in the food industry as acidulants, flavourings and antioxidants and in the pharm aceutical industry as buffering, scavenging and chelating agent [1]. CA is produced by fermentation using inexpensive raw materials including crude natural products such as hydrolysate of starch, sugar cane bagasse, beet molasses, cassava bagasse, coffee husk, wheat brain, apple pomace, pine apple waste, grape pomace, citrus waste [2].

CA is a versatile and innocuous alimentary additive and it is accepted worldwide as generally regarded as safe (GRAS) by the joint FAO/WHO Expert Committee on Food Additives [3]. The food and pharmaceutical industries utilize citric acid extensively because of its general recognition of safety, pleasant acid taste, high water solubility and chelating and buffering properties [2]. CA has a wide range of applications in the food, pharmaceuticals and cosmetic industries. CA, present in citrus fruits was first crystallized from lemon juice in the form of calcium citrate [4]. CA fermentation is one of the largest biotechnological industries [5].

At present, a variety of agro-industrial residues and by – products are used as substrates for citric acid production such as cassava bagasse, coffee husk, wheat brain, apple pomace, pineapple waste, kiwi fruit peel, corn waste, banana peel, apple pomace, orange peel, grape pomace [6]. Several processes have been developed that utilize these as raw materials for the production of bulk chemicals and value – added fine products such as CA, ethanol, single cell protein (SCP), mushrooms, enzymes, other organic acids, amino acid, biologically active secondary metabolites etc. [7]. Application of agro-industrial residues in bio processes on the one hand provides alternative substrates, and on the other hand helps in solving pollution problems, which their disposal may otherwise cause. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new avenues have opened for their utilization. Considerable attention has been shown in using agricultural wastes for citric acid production [8,9,10]. Different agro-industrial residues including apple pomace, wheat straw, coffee husk, pineapple waste, cassava, bagasse, banana, orange lemon and kiwi fruit peel have been investigated as substrates for CA production. Karthikeyan and Sivakumar [11] used banana peel, while Khosravi and Zoghi [9] used bagasse. Dhillon et al. [12] used different kinds of agro-industrial wastes like apple pomace, brewery spent grain, citrus waste and sphagnum peat moss. Imandi et al. [13] used pineapple waste as a substrate for the production of citric acid.

Many microorganisms have been evaluated for the production of CA including fungi such as *Aspergillus niger* [14,15,16]; *Aspergillus awamori, A foetidus, Rhizopus* spp*, Penicillium restricum*; bacteria such as *Bacillus licheniformis*, *B. subtilis, Corynebacterium* spp.; yeasts such as *Candida lipolytica, C. intermedia* and *Sacharomyces cerevisiae* [17].

The citrus processing industry yearly generate, tonnes of residues, including peel and sequent membrane from the extraction of citrus juice in industrial plants. The management of these wastes, which produce odour and soil pollution represents a major problem for the food industry [18]. Orange peel contains soluble sugars and pectin as the main components [19]. According to Rivas et al. [20] the orange peel is constituted by soluble sugars 16.9% wt; starch, 3.75% wt, fiber (cellulose, 9.21% wt, hemicellulose, 10.5% wt, lignin, 0.84% wt; and pectin, 42.5% wt); ashes, 3.50% wt; fats, 1.95% wt; and proteins 6.5% wt. Their low cost and high carbohydrate content and susceptibility to fermentation make citrus by-products attractive raw materials for CA production. The objective of this study was to utilize orange peel waste in a fermentation medium for CA production by naturally – occurring *Rhizopus stolonifer* isolate as well as to study the effects of added sugars on the yields CA.

2. MATERIALS AND METHODS

2.1 Isolation of Fungi

Orange fruit undergoing spoilage was obtained and taken immediately into the laboratory for microbiological analysis. Spoiled sections (ca. 20 g) were removed with a sterile kitchen knife and homogenized in a mortar and pestle with 15 ml of sterile distilled water. The pH of the homogenate was measured and recorded as 3.9. The homogenate was serially diluted with normal saline and plated out onto Potato Dextrose agar

(PDA) plates containing 0.1% chloramphenicol solution to inhibit bacterial contaminants. The plates were incubated for 48 h at room temperature (28±2ºC). Pure cultures of the isolates were obtained by streaking slant cultures on fresh PDA plates and were given arbitrary numbers. The culture with code number AO19 which produced the highest concentration of CA (5.8 g/L) after the screening was selected and identified as *Rhizopus stolonifer* based on the taxonomic descriptions of Pitt and Hocking [21].

2.2 Preparation of Orange Peel

Orange fruits used in this study were purchased from Nsukka market and were washed with clean tap water. Fresh, ripe fruit peels were used and when not used immediately, they were stored at 4ºC and used within 24 h of collection. About 200 g of peel was minced into pieces and oven dried at 55ºC until a constant weight was achieved. They were ground into powder using a Corona mill (Medellin, Colombia).

2.3 Screening of the Fungal Isolates for Citric Acid Production

The screening medium for CA production was designated as medium A and had the following composition (glL): Dried orange peel, 5; NH_4NO_3, 2.5; KH_2PO_4, 1.0; $MgSO_4$, 0.25; $CuSO_4$, 0.048; $ZnSO_4$, 0.038; $FeSO_4$, 0.022 and $MnSO_4$, 0.01. Inocula for fermentation were prepared by growing the test organisms on PDA at 30ºC for 6 d. Spore suspension were harvested by adding 5 ml of sterile 0.1% Tween 80 solution to the slants and shaking gently for 1min. Medium was dispensed into 1L Erlenmeyer flasks and autoclaved at 121ºC for 15 min before use. Each flask was inoculated with 1.0 mL inoculum (2 x 10^7 spores/mL) and incubated in a Gallenkamp orbital shaker at 30ºC for 3 d. Aliquots (10 ml) were withdrawn daily and mycelium – free filtrates were obtained by centrifugation and analyzed for citric acid content.

2.4 Effect of Methanol on Citric Acid Production

Into the screening medium contained in flasks was each added various concentrations of methanol, namely, 1, 2, 3, 4, and 5%. A control flask containing no methanol was separately prepared. The flasks were incubated in a Gallenkamp orbital shaker at 30ºC for 3 d.

2.5 Effect of Additional Carbon Sources on Citric Acid Production

The carbon sources namely, sucrose, maltose and lactose were each added at 5, 10 and 15% (w/v) concentration into Medium A. The medium pH was adjusted to 3.9 and the flasks incubated in a Gallenkamp orbital shaker at 30ºC for 8 d.

2.6 Analysis

Aliquots samples (10 mL) were withdrawn daily and mycelium – free filtrates were obtained by centrifugation at 2515 x g for 15 min. The supernatant sample was analyzed for citric acid content by the pyridine acetic anhydride method of Marrier and Boulet [22].

3. RESULTS AND DISCUSSION

The experiment showed that after 3 days of incubation, the amount of CA in the screening medium varied depending on the isolate used. The culture, with code number AO19, which produced the highest citric acid titre (5.8 g/L) after screening, was therefore selected for further work (Table 1). The isolate was identified as *Rhizopus stolonifer* based on the taxonomic descriptions of Pitt and Hocking [21]. *Rhizopus stolonifer* is among the most commonly occurring fungal species in foods Pitt and Hocking [21].

The addition of 3% and 4% (v/v) methanol into the isolation medium resulted in a citric acid yield of 7.8 g/L and 7.1g/L respectively. This level corresponded to 135 and 122% increases respectively in CA yield in comparison to the control medium that produced only 5.8 g/L. When the methanol concentration was increased to 5%, lower CA yield of 4.9 g/L was obtained (Table 2). There are similar findings on the stimulation of citric acid production with the addition of methanol. Rivas et al., [20] adding 4% methanol to an orange peel aqueous extract as culture medium increased 20 fold maximum CA production with *Aspergillus niger* in submerged fermentation. Dhillon et al. [23] showed the effect of ethanol and methanol addition on the rate of production of CA where the addition of 3% (v/v) ethanol and 4% (v/v) methanol increased the citric acid production from apple pomace solid waste by 2-fold. Bari et al. [24] used sucrose as co-substrate for citric acid production and methanol as a stimulator. Kang et al. [25] found the optimal conditions in terms of maximum yield of citric acid (80.4%) from skins of mandarin by *Aspergillus niger* with the addition of 2.5%

methanol. Tran et al. [26] obtained the highest citric acid yield in fermentations using pineapple waste and *Aspergillus niger* ACM 4992. De Lima et al. [27] added 4% methanol using *Aspergillus niger* ATCC 1015 and pineapple waste in solid – state fermentation to achieve the highest citric acid production. Conversely, some authors have reported a decreased synthesis of citric acid after methanol addition. For instance, Hang et al. [28] observed that the supplementation of 0.74mmol methanol/L diminished citric acid production during fermentation of kiwi fruit peel by *Aspergillus niger* ATCC 9142 and Tsay and To [29] reported that methanol inhibited mycelial growth of *Aspergillus niger* TMB 2022 as well as citric acid production.

Table 1. Selection of fungal isolates based on their citric acid productivity

S/No.	Code	Citric acid yield (g/L)
1	AO 1	0.4
2	AO 2	1.8
3	AO 3	1.7
4	AO 4	2.2
5	AO 5	2.5
6	AO 6	4.2
7	AO 7	3.1
8	AO 8	1.8
9	AO 9	1.0
10	AO 10	1.1
11	AO 11	5.2
12	AO 12	3.5
13	AO 13	2.4
14	AO 14	2.6
15	AO 15	3.3
16	AO 16	4.1
17	AO 17	5.0
18	AO 18	2.2
19	AO 19	5.8
20	AO 20	3.4
21	AO 21	3.4
22	AO 22	2.8
23	AO 23	3.5
24	AO 24	1.7
25	AO 25	1.2

Citric acid production in a medium containing orange peel plus various concentrations of added sucrose is shown in Fig. 1. The lowest CA yield (0.1g/L) was observed with a sucrose concentration of 0.5%. This yield increased progressively with the days of incubation and peaked at 19.8 g/L on the 6th day and there after decreased on the 7th and 8th days. The addition of 10% sucrose into the cultivation medium resulted in a much higher CA yield of 27.9 g/L on the 5th day of incubation. However, lower yields were recorded thereafter. The overall best yield of CA was observed in a medium containing 15% sucrose with the production of 33.7 g/L on the 6th day. This level reduced to 21.8 g/L and 18.5 g/L on the 7th and 8th days respectively. Sucrose consumption was followed by a rapid rise in CA titre and the highest concentration of CA reached after 6 days. After this phase, CA level was depleted rather rapidly from the medium. This phenomenon may be attributed to microbial utilization of citric acid for growth and energy generation. Although sucrose stimulated citric acid production it had a little or no effect on the depletion of CA in the medium after the 6th day. Sucrose is the traditional commercial substrate for CA production [30]. Superiority of sucrose over other carbon sources for CA production was demonstrated by Xu et al. [31] and Hossain et al. [32]. Sucrose is of relatively low molecular weight and readily transported into microbial cells for hydrolysis by intracellular enzymes [33]. It was proved [34] that the increase in sucrose concentration had a positive effect on CA production. The enhancement of CA production in the presence of sucrose is similar to that reported by Soccol et al. [2] to occur in the fermentation of crude carbohydrate sources. El – Holi and Al – Delaimy [35] reported that whey with 15% (w/v) sucrose with or without 1% methanol was the most favourable medium producing the highest amount of CA.

Table 2. Influence of added methanol on citric acid production by *Rhizopus stolonifer*

Methanol (%)	Citric acid yield (g/L)
0	5.8
1	6.2
2	6.9
3	7.8
4	7.1
5	4.9

Fig. 2 shows the production of CA in the basal medium with added lactose. The best yield of CA (22.6 g/L) was observed on the 5th day of incubation in a medium containing 15% lactose. The lowest yield in the lactose containing culture medium (0.2 g/L) was observed in the medium containing 5% lactose (Fig. 2). After peak levels of CA were reached, the culture depleted citrate from the medium and this was taken as evidence that the galactose moiety of lactose was co-metabolized during the fermentation.

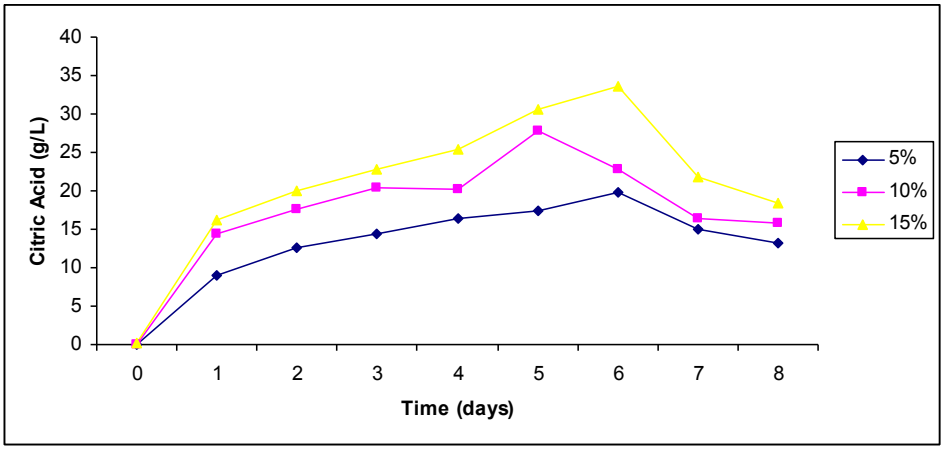

Fig. 1. Influence of added sucrose on citric acid production by *Rhizopus stolonifer*

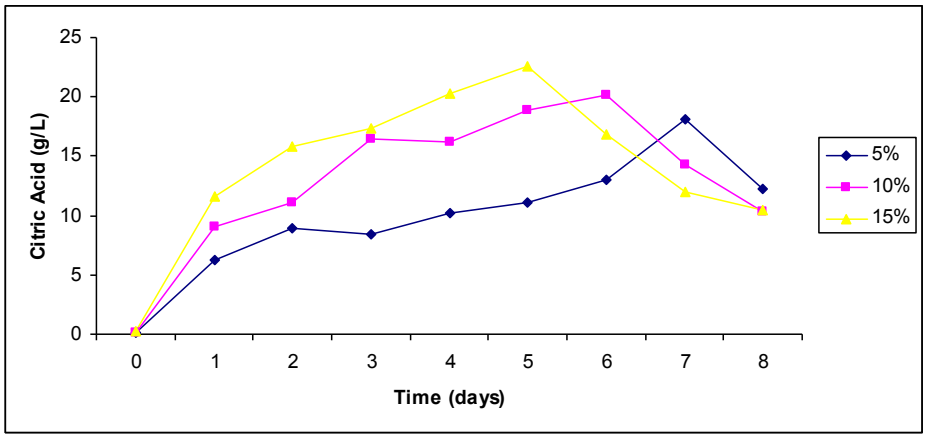

Fig. 2. Influence of added lactose on citric acid production by *Rhizopus stolonifer*

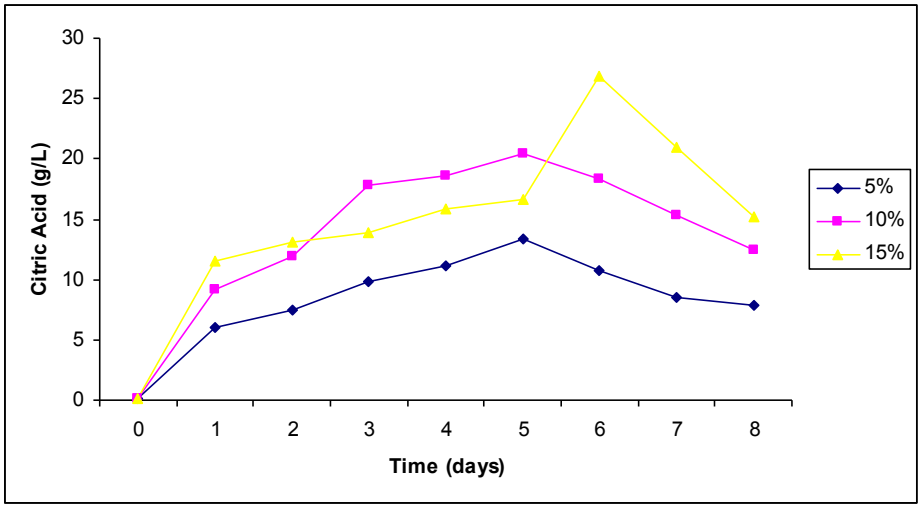

Fig. 3. Influence of added maltose on citric acid production by *Rhizopus stolonifer*

When maltose was added into the basal medium for CA production, it was observed that the best yield (26.8 g/L) was obtained on the 6th day with 15% maltose. This level decreased to 20.9 and 15.2 g/L on the 7th and 8th days respectively. Comparatively, lower concentrations of maltose in the basal medium resulted in lower CA yields. For instance, at a maltose concentration of 5%, the best CA yield was 13.4 g/L, at 10% concentration the best CA yield was 20.5 g/L while at a concentration of 15%, best CA yield was 26.8 g/L (Fig. 3)

The carbon source for CA production has been the focus of much research studies [36]. In general, only sugars that are rapidly taken up by the microorganism allow a high final yield of citric acid [37]. CA production is strongly affected by the nature of the carbon source and the presence of easily metabolizable carbohydrates has been found essential for good production of citric acid [38]. Hossain et al. [32] showed that sucrose was the most favourable carbon source followed by glucose, fructose and galactose. Galactose contributed to a very low growth of fungi and did not favour citric acid accumulation. Xu et al. [31] reported that *Aspergillus niger* needed an initial sugar concentration of 10 – 14% as optimal; no CA was produced at sugar concentration of less than 2.5%. Maddox et al. [39] reported the influence of different sources of carbon on CA production by *Aspergillus niger* and *Saccharornycopsis lipolytica*, Glucose, maltose, galactose, xylose and arabinose were tested. The best results were found for *Aspergillus niger* with 0.45 g of CA per g of glucose corresponding to 27 g/L. *S. lipolytica* produced 0.41 g/g of glucose or 9 g/L. Starch, pentoses (xylose and arabinose) sorbitol and pyruvic acid slowed down fungal growth with a resultant minimal CA production [40]. Socool et al. [2] reported positive effects on CA production with 14 – 22% carbon sources such as sucrose, glucose, fructose and galactose, but negative effects were reported for starch, xylose, arabinose, sorbitol and pyruvic acid.

4. CONCLUSION

Rhizopus stolonifer used for this study was selected among other isolates because it produced a maximum concentration of 5.8 g/L CA in the isolation medium. The addition of 3% and 4% (v/v) methanol into the isolation medium resulted in a citric acid yield of 7.8 g/L and 7.1 g/L respectively. This level corresponded to 135 and 122% increases respectively in CA yield in comparison to the control medium that produced only 5.8 g/L. When the methanol concentration was increased to 5%, lower CA yield of 4.9 g/L was observed. This study has shown that *Rhizopus stolonifer* is capable of producing CA using orange peel and disaccharides as carbon sources. The optimal medium consisted of 15% sucrose and this caused the production of 33.7 g/L CA after 6th day cultivation. Sucrose proved superior to lactose and maltose for CA production. Both the type of carbon source and its concentration were critical to CA fermentation. The final yields of CA increased with sugar concentration and maximum production rates were achieved at 10 – 15% of sugar.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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