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Effect of Incubation Period on Citric Acid Production by *Aspergillus niger* Using Fruit and Vegetable Wastes

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Received: September 2020 **Accepted: November 2020**

Abstract
The present study was conducted to study the effect of different incubation periods on the production of citric acid from fruit and vegetable wastes obtained from the local market in Khartoum State. Ten samples of fruit and vegetable wastes were screened i.e. mangoes, oranges, bananas, lemon, grapefruits, beets, carrots, pumpkin, potatoes, and sweet potatoes. The solid state fermentation method was adopted in this study to produce citric acid using *Aspergillus niger* which isolated from rotten onion and cultured on Potato Dextrose Agar (PDA) and incubated at 25ºC. Three different incubation periods were used in this study (seven, ten and thirteen days) at 25ºC for acid production. The produced citric acid concentration was determined and in parallel, the sugar concentration in the substrates was also determined before and after fermentation. The results showed that the highest production of citric acid was obtained through incubation period of seven days especially, by fermentation of mango wastes (130.50 mg/ml) followed by the grapefruit wastes (99.80 mg/ml)while, the lowest acid production was obtained by banana wastes fermentation (40.42mg/ml). It was also shown that citric acid concentration decreased by increasing incubation period in all the tested wastes substrates. This study also showed that the production of citric acid was accompanied with the reduction in the concentration of sugar content in manner depends on the type of the substrate used.

Keywords: *Aspergillus niger*, citric acid, solid-state fermentation.

Introduction
Citric acid, or 2-hydroxy-propane-1, 2, 3-tricarboxylic acid ($C_6H_8O7.H2O$) is a naturally occurring weak organic acid found in citrus fruits. The name of this organic acid is derived from Latin word *citrus*, which refers to trees of the genus *Citrus* (Papagianni, 2007). Citric acid can be derived from natural sources such as lemon, lime and orange or synthetic sources e.g. chemical reaction and microbial fermentation. A German botanist, Wehmer, in 1893 was the first observed the feasibility of obtaining citric acid through the fermentation of a sugar medium containing inorganic salts with *Penicillium glaucum* (Soccol *et al*., 2006). In 1916, a study conducted by James Currie made a breakthrough for successful economic industrial production of citric acid from *Aspergillus niger*. He discovered that significant amounts of citric acid could be obtained from various strains of *A. niger*. *Aspergillus niger* is superior to other microorganisms for the commercial synthesis of citric acid because of its better production yield**.** Microbial production of citric acid *via* fermentation by fungi

Aspergillus such as *A. wenti*, *A. foetidus*,*A. aculeatus* and *A. awamori* have been found to produce significant amounts of citric acid (Berovic and Legisa, 2007).Also some yeasts species such as *Candida guilermondi* (Angumeenal *et al.,* 2003), *Yarrowia lipolytica* (Angumeenal and Venkappayya, 2013) can produce citric acid. Also can produce considerable amount of citric acid by bacteria such as *Arthrobacter paraffinens* and *Bacillus licheniformis*, and the mould *Penicillium janthinellum* (Ikram-ul *et al*., 2004) are also used for citric acid production. Surface fermentation, also known as liquid surface culture, was the original citric acid industrial production technique. Surface fermentation offers advantages such as lower installation and energy cost, and is also foam free. This method consists of two phases; both of them are characterized by a rapid uptake of carbohydrates. The first phase is the development of the fungus as mycelia mat on the surface of the medium and the second phase was the utilization of carbohydrates by converting them to citric acid (Kiel *et al*., 1981).The process is conventionally performed in fermentation chambers, using trays made from materials such as special-grade steel. Process also controls the humidity and temperature (*via* evaporative cooling). The air also serves to remove carbondioxide, which is an inhibitor of citric acid production at concentrations higher than 10% (Soccol *et al*., 2006). Submerged fermentation is the most widely used fermentation technique in the world today. Eighty per cent of the world's production is estimated to be from the submerged method (Roukas, 1991).

About 60 % of citric acid product is mainly used in the food and beverage industry, because of its general recognition as safe, having pleasant taste, high water solubility and chelating and buffering properties. Addition of 0.02 % citric acid to liquid dosage forms complexes trace iron and copper ions (Marin and Matic, 2006) and retards degradation of active ingredients and other uses of citric acid. Sudan as an agricultural country and at the same time has some agro. industries, these make available large quantities of agricultural and agro-industrial by products wastes to be reformulated to new valuable products.

Solid-state fermentation or 'Koji' fermentation, originates from Japan, which has an abundance of agro-industrial residues/wastes (Kareem *et al*., 2010). The solid materials act as a physical support and source of nutrients for the microorganisms. Under optimal conditions, the process should be completed in 4 days (Drysdale and McKay, 1995).

The main advantage of solid-state fermentation is its superior yield and the ability to utilize inexpensive and widely available agro-industrial residues as substrates for bio-production, making it more environmentally friendly than submerged fermentation (Falony *et al*., 2006). It requires less water and has lower operating costs, and does not require complex equipment. There is no need for pretreatment as the system (solid state) is less sensitive to the presence of trace elements compared to submerged fermentation (Berovic and Legisa, 2007).There is also a limited pool of viable microorganisms, and strains with large nitrogen and phosphorus requirements cannot be used. Agro-industrial wastes that have been utilized include banana peel (Max *et al*., 2010), orange peel waste (Torrado *et al*., 2011), potato (*Solanum tuberosum*) and taro (*Colacasia esculenta*) (Arshad *et al*., 2014).

This work aimed to make use of the fruits and vegetables wastes from the local markets to produce citric acid using isolated fungal strain of *Aspergillus niger*, to optimize favorable conditions for citric acid production from these local produced fruits and vegetables wastes in order to reduce the agro-industrial wastes in the local environment.

Materials and Methods

Aspergillus ngier was isolated from rotten onion wastes, maintained on Potato Dextrose slants agar, stored at 4ºC and sub- cultured at 2 weeks interval. Solid State Fermentation was followed in this study (Drysdale and Mekay, 1995). Samples of vegetable and fruit wastes (orange, banana, grapes, pumpkin, carrot, beet, lemon, mango, potato and sweet potato). Were collected from Khartoum State (Alsouq Almarkazy) during July 2018.

Citric acid production: Inoculation of microorganism was done by adding *Aspergillus niger* spores in the fermentation medium. Spores were inoculated by mixing them aerobically with the substrates in the form of a spore suspension in conical flasks on the solid substrates (the wastes) at the optimum temperature (25ºC). Different incubation periods were applied for the optimization production of citric acid (7, 10, 13 days at 25 ºC). Pre-treatment of fruits and vegetables wastes bought from the local market in Khartoum State, were used in the present study. The different fruit and vegetable wastes were oven-dried at 60 °C for 2days and hand grounded (particles between 1.2 and 1.6 cm). Twenty gram grounded fruits and vegetables wastes were taken in to 250 ml conical flasks, 100 ml distilled water was added. The flasks containing substrates were sterilized at 121 º C for1 hour to provide proper cooking of the substrates and to increase its amenability for microbes. After sterilization, flasks containing substrates were allowed to cool to room temperature 25ºC and then inoculated with 1 ml of *A. niger* spores suspension (2×10^7 spores/ml) in sterilized distilled water and twin 80 solution by scratching *A. niger* spores on slants, well mixed and incubated in a humidity controlled incubator. Methanol (4%v\w) was added to the substrates after sterilization for its stimulatory effect on citric acid production.

Determination of citric acid, sugar and pH:

The fermented substrates were dried in an oven at 50°C and extracted by the addition of 100 ml distilled water. The mixtures were agitated on a rotary shaker for 2 hours then filtered with Whiteman filter papers. The supernatant were used for the estimation of total residual sugar and pH values and citric acid percentages. The sugar content was determined using a refractometer according to AOAC (2008). The pH was measured using Analog pH meter. Citric acid was estimated titrimetrically (AOAC, 1995) by using 0.1 M NaOH and phenolphthalein as indicator and calculated as percentage(%) according to the formula below .The detection of citric acid was also done chemically by the addition of three drops of bromocrysol green indicator to the 10 ml of the distillation yield (Soccol1 *et al*., 2006).

- Normality of citric acid = normality of NaOH× NaOH volume ÷ volume of citric acid
- Concentration of citric acid = Citric acid normality ×equivalent ×100 ÷ volume of distillation
- (Equivalent = 96, volume of distillation = 10).

The Results were expressed as a Mean ± SD. The data were analyzed by one way analysis of variance (ANOVA) using the statistical package of SPSS program, version 20 ($p \leq 0.05$).

Results and Discussion

Fruit and vegetable wastes usually, have higher sugar content that can be utilized by microorganisms. The results showed that the incubation period of seven days gave the highest production of citric acid in all the substrate. Mango wastes yielded the highest production of citric acid (130.50 mg/ml) followed by grapefruit wastes which yielded 99.80 mg/ml while the lowest acid production was shown in banana wastes yielding 40.42mg/ml (Figure 1).

It was observed that any increase in the incubation period after seven days did not

enhance the acid production and the amount of the acid decreased which it might be due to the depletion of sugar contents in the fermentation medium, the increase in age of fungi, the presence of inhibitors produced by fungi itself, and the utilization of the acid by the fungus itself (Figures 2-5). This finding was in agreement with Iqbal *et al*. (2015) and Rao and Reddy (2013) demonstrated that incubation period of 72h was the optimum for the production of the acid by the same fungus. The maximum production in mango media might be due to its relatively high sugar content (5.6%). This Result is disagreed with those of Ma *et al. (*1993) and Rivas *et al.* (2008) who used orange peel as a cheap source for the acid production. This result is quite comparable to the yields obtained by fermentation of other agricultural wastes such as kiwi fruit peel, soy-residue, and cane molasses which agreement with Hang and Wood (1986) in the present study, a parallel relationship between citric acid production and the incubation period was also observed.

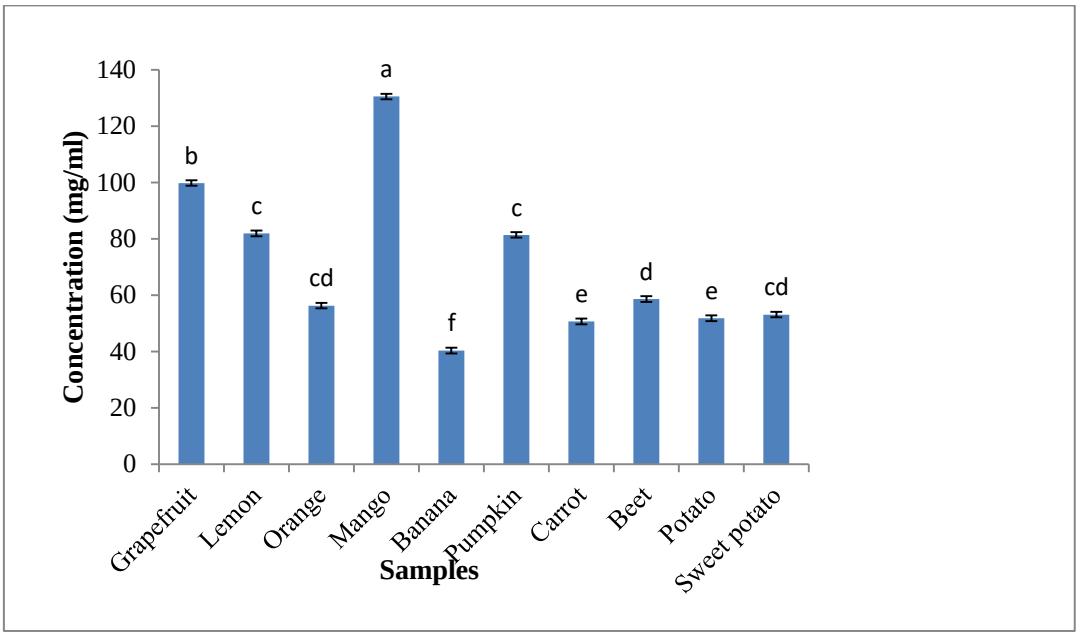

Figure (1) Citric acid production by *A. niger* after seven days of fermentation

The values with the same letters in the same column are not significantly different at P ≤ 0, 05

Figure 2 shows citric acid production after ten days of fermentation. Mango was shown the highest acid production (111.3mg/ml) followed by grapefruit (72.9mg/ml), lemon (71.6mg/ml) and pumpkin (65.2mg/ml).While beet, orange, potato, sweet potato and banana show middle production of citric acid (49.2-27.3mg/ml). Fermentation of carrot vegetable wastes showed the lowest level of the acid production (23.1mg/ml).Further increase in fermentation period decreased the citric acid production (Figure 2). One possible reason of this further reduction in the product value could be attributed to the age of fungus, depletion of sugar contents, other inhibitory metabolites and decreased available nitrogen in the fermentation medium. This result suggests that the optimum fermentation, incubation time period for enhancing citric acid yield varies with growth supported substrates as reported previously by (Rolfe *et al*. (2012), this also in agreement with the Steel *et al.* (1955) and Asad-ur-Rehman *et al.* (2002).who found that increase in the incubation time has been found decrease citric acid yield due to denaturation of the enzyme citrate syntheses and activation of oxalic acid synthesis pathway.

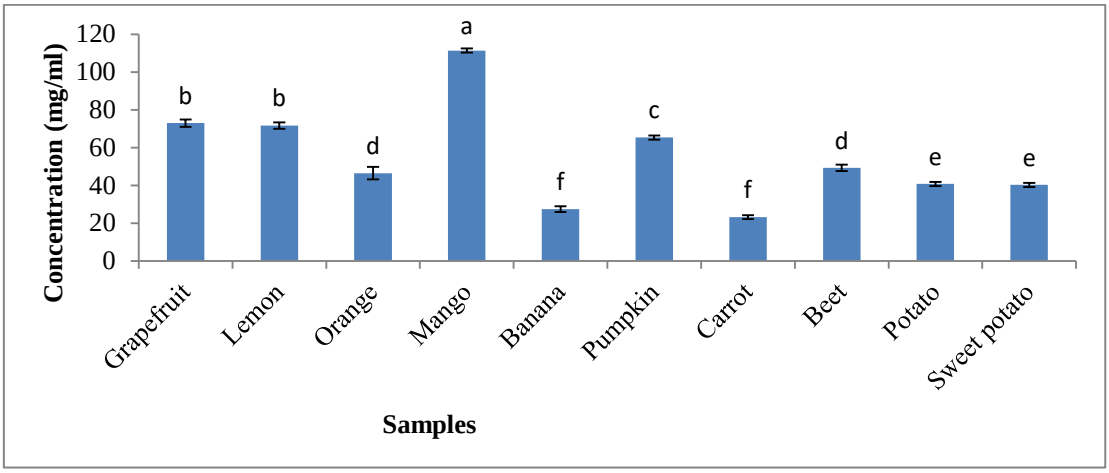

Figure (2) Citric acid productions by *A. niger* after ten days of fermentation

The values with the same letters in the same column are not significantly different at P ≤ 0, 05.

Figures 3 shows that citric acid production after thirteen days of fermentation.Also fermentation of mango wastes showed the highest acid production (97.9mg/ml) followed by grapefruit (59.5mg/ml), lemon (58.2mg/ml), pumpkin (48.0mg/ml) and orange(44.1mg/ml).while sweet potato, beet, potato and banana show middle production of citric acid (35.2-23.6mg/ml).Fermentation of carrot vegetable wastes showed the lowest level of the acid production (16.0mg/ml). These results disagreed with Crolla and Kennedy (2001) but agreed with Arzumanov *et al*. (2000). Finding these wastes are rich in moisture, carbohydrates and other compounds depending upon their origin. Apart from moisture and carbohydrates, they also contain considerable quantities of proteins, fats, natural colorants and in some cases, antioxidants and other bioactive compounds so they can be used as substrates for fermentation to produce value added products Hilde *et al*. (2009).

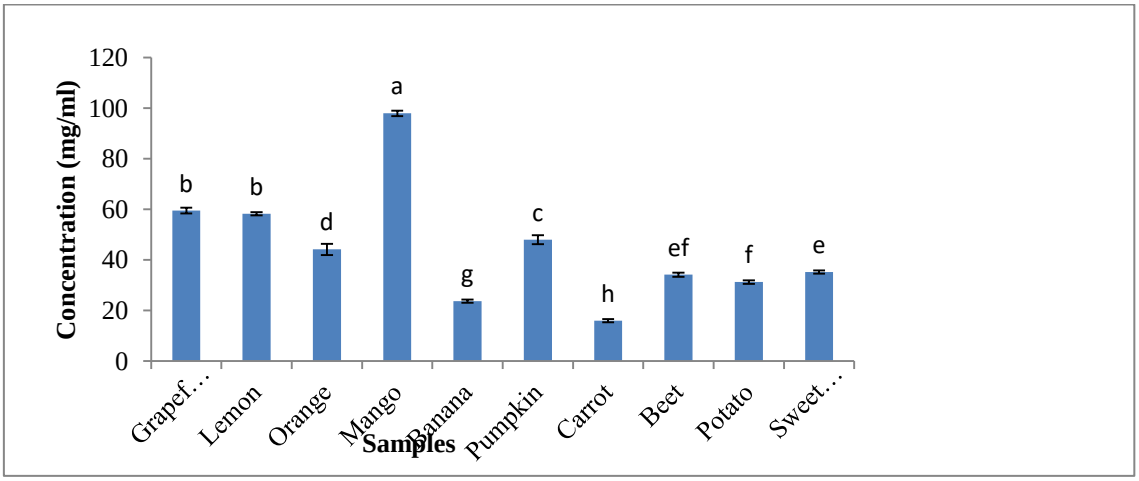

Figure (3) Citric acid production by *A. niger* after thirteen days of fermentation.

The values with the same letters in the same column are not significantly different at P ≤ 0, 05

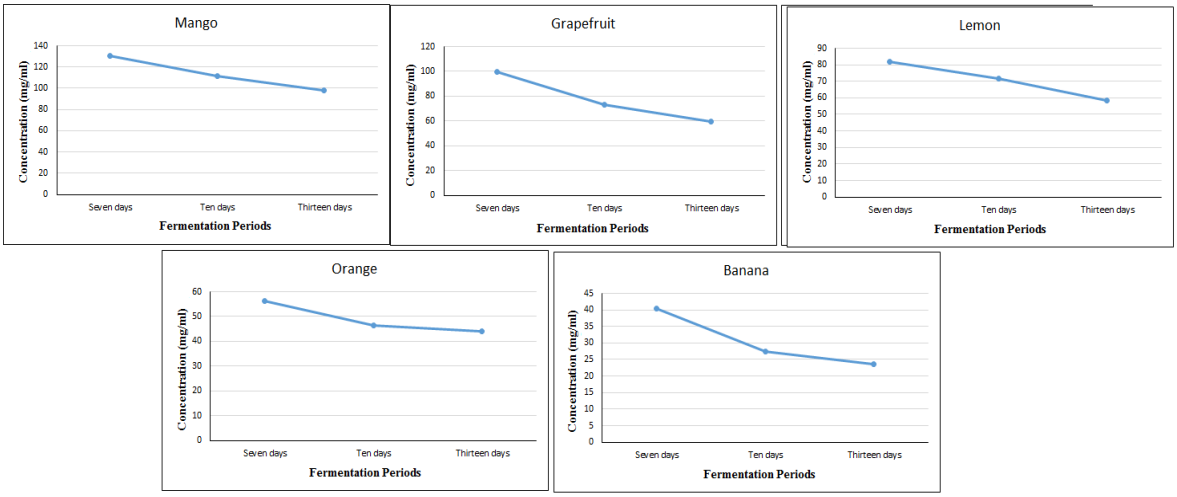

Figure (4) The effect of different Fermentation periods on the production of citric acid in fruit wastes

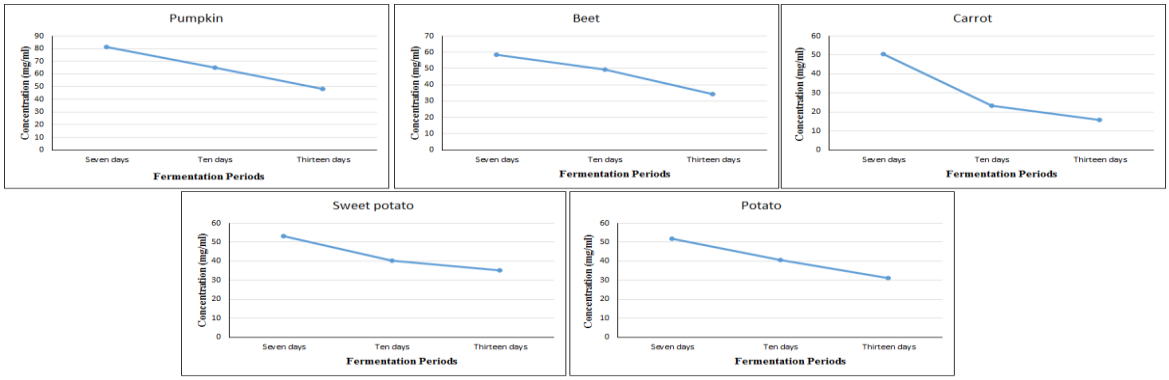

Figure (5): The effect of different Fermentation periods on the production of citric acid in vegetable wastes

The Results show that the production of citric acid by fermentation was followed by the reduction in the concentration of sugar. The highest reduction in sugar concentration after the first seven days incubation period was shown to be from 5.6% to 3.6% in mango which gave the highest acid production, while the lowest reduction in sugar concentration was recorded in orange (from 4.6% to 4.0%) as shown in Figure 6. In this study a parallel relationship between citric acid production and the consumption of sugar was observed which agreed with El-Holi and Al-Delamy (2003). Also it was shown that, the production of citric acid by fermentation was followed by continuous sugar concentration reduction with incubation time. On the tenth day of incubation, the highest reduction of sugar concentration was also shown in mango fruit (from 5.6% to 2.0%), where as in some vegetables like potato and sweet potato, the sugar nearly or completely depleted by fermentation process on the tenth day of incubation from 1.6% to 0.1% and from 2.0% to 0.0%, respectively (Figure 7).The production of citric acid approximately paralleled the consumption of sugar. This result agreed with Hang and Woodams (1986) who reported that the yields of citric acid from apple and grape

pomace based on the amount of sugar 60%, respectively. In this work, based on the amount of fermentable sugar consumed, it can be seen that the yield of citric acid was more than 90% under optimum solid-state fermentation conditions. As seen in Figure 8 the production of citric acid by fermentation continuously, accompanied with sugar concentration reduction. After thirteen days of fermentation, the reduction was low in orange (from 4.6% in orange to 3.3%), but it was highly reduced in other used substrates and completely depleted in grapefruit, potato and sweet potato (0.0%). These results agreed with the report of Honecker *et al.* (1989) and Haq*et al.* (2003).

consumed which were about 88% and

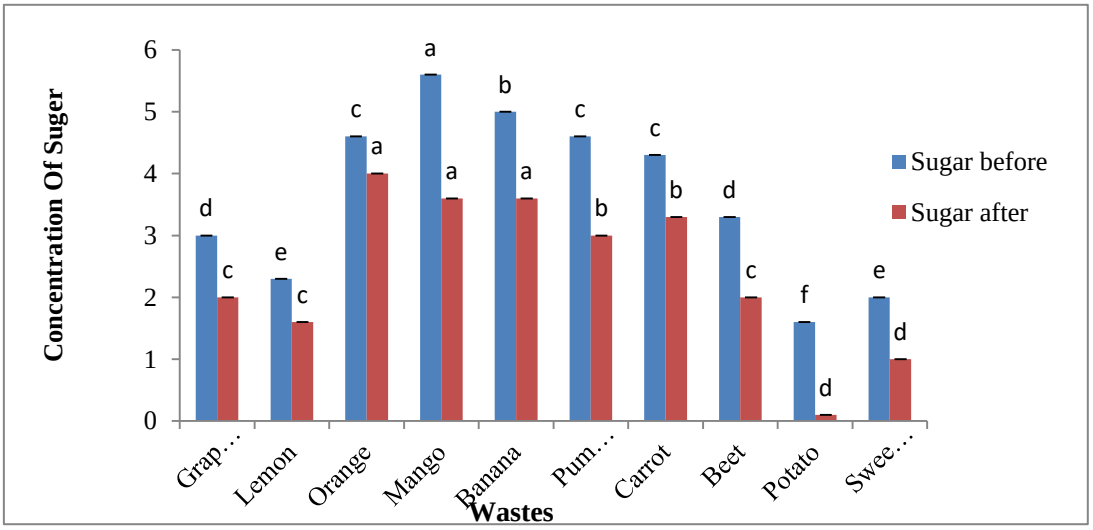

Figure (6) Sugar content before and after seven days of fermentation
The values with the same letters in the same column are not significantly different at P ≤ 0, 05.

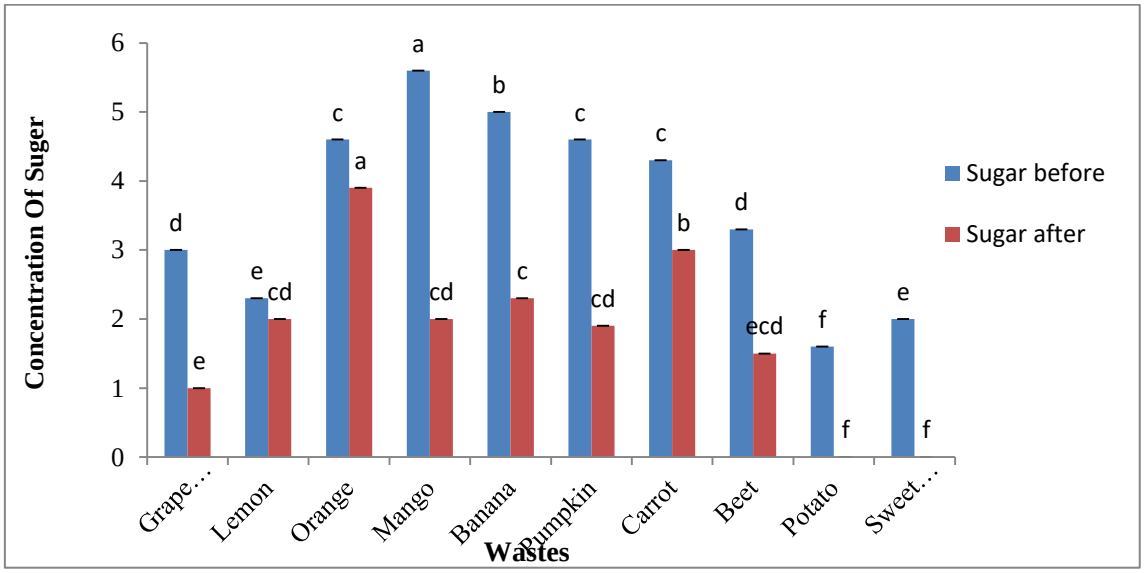

Figure (7) Sugar content before and after ten days of fermentation
The values with the same letters in the same column are not significantly different at P ≤ 0, 05.

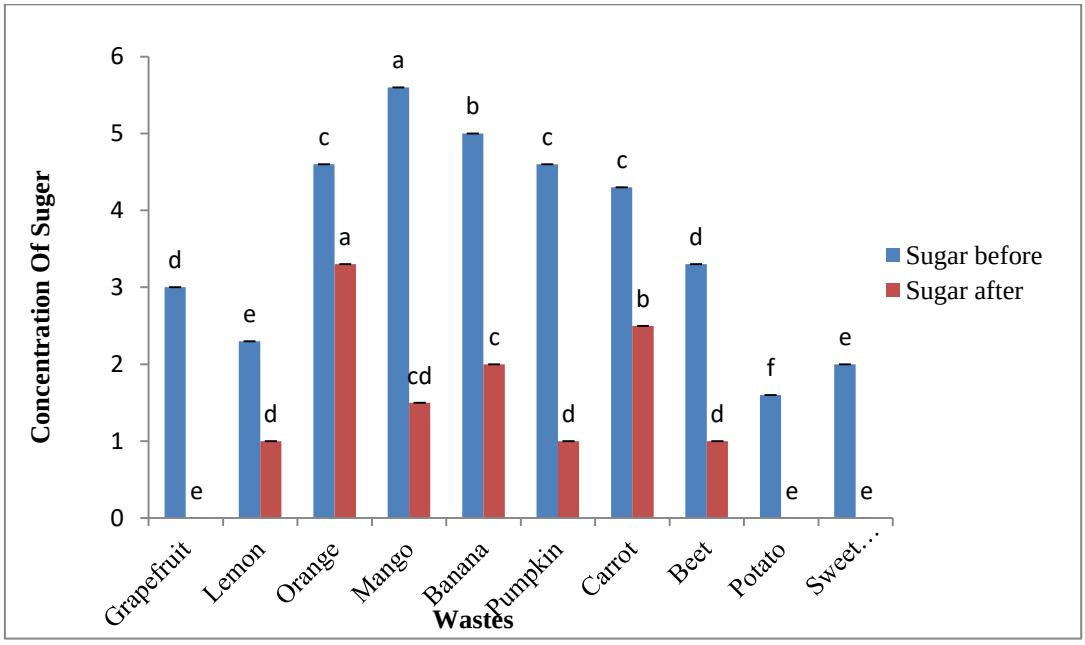

Figure (8) Sugar content before and after thirteen days of fermentation

The values with the same letters in the same column are not significantly different at P ≤ 0, 05.

Conclusions

From the present obtained Results it can be concluded that *Aspergillus niger* can produce considerable quantity of citric acid by utilizing various substrates of fruit and vegetable wastes as a cost-effective growth extensive potential to support media for citric acid production. Seven days incubation period at 25°C was found to be the best incubation periods for citric acid production showing a maximum citric acid yield from mango wastes. The use of various fruit and vegetable wastes for production of citric acid signify an efficient outlook of minimizing waste disposal problems, indirectly reducing the health hazards faced due to dumping of the waste and concurrently produce organic acids of valuable importance for food and pharmaceutical industries. Efficacy of large scale citric acid production using fruit and vegetable wastes deserve further studies.

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أثر فترة التحضين في إنتاج حمض الستريك من مخلفات الفواكهه والخضروات بإستخدام فطر الاسبيروجلس نيجر(*Aspergillus niger*)

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3. جامعة افريقيا العالمية– كلية العلوم البحثية – قسم الاحياء الدقيقة

المستخلص

اجريت هذة الدراسة بغرض انتاج حمض الستريك من مخلفات الفواكهة والخضروات الماخوذة من السوق المحلي بالخرطوم حيث تم جمع عشرة عينات من مخلفات الفواكهة والخضروات وهي المانجو والبرتقال والموز والليمون والقريب فروت والبنجر والجزر والقرع و البطاطس والبطاطاالحلوة.وتم استخدام طريقة تخمرات الحالة الصلبة لانتاج حامض الستريك بإستخدام فطر الإسبيروجلس نيجر. تم عزل فطر الأسبيروجلس نيجر من البصل المعفن وتم تزريعة في وسط البطاطا دكستروز اجار وتم تحضينه في حضانة الفطريات عند درجة حرارة 25 درجة مئوية لمدة خمسة أيام ومن ثم تم التعرف علي الفطر تحت الميكروسكوب بصفاته المظهريه ومن ثم تم استخدامه لتلقيح اوساط التخمير. إستخدمت في هذه الدراسة ثلاثة فترات تحضين مختلفه لمدة سبعة ايام وعشرة ايام وثلاثة عشر يوما عند درجة حرارة 25 درجة مئوية لإنتاج حامض الستريك. وتم تحضين أوساط التخمير لفترة تحضين لمدة سبعة أيام عند درجة حرارة 25 درجة مئوية و أظهرت النتائج ان فترة التحضين سبعة ايام عند درجة الحرارة 25 درجة مئوية أعطت أعلي إنتاج من حامض الستريك في وسط تخمير مخلفات المانجو وكان 130,50 ملجم/مل ثم بعدها القريب فروت 99,80ملجم/مل وأقل إنتاج من حامض الستريك من الموزوكان 40,42 ملجم/مل.وعند فترة تحضين عشرة ايام عند درجة حرارة 25 درجة مئوية أعطيت أعلي انتاج من الحامض 111.36 مليجرام/ مل من فاكهة المانجو ثم بعدها في فاكهة القريب فروت 72.96 مليجرام/ مل. أقل إنتاج من الحامض لفترة تحضين عشرة ايام عند درجة حرارة 25 درجة مئوية من مخلفات الجزر وكان 23.16 مليجرام/ مل من حامض الستريك . وعند فترة تحضين ثلاثة عشر يوما عند درجة حرارة 25 درجة مئوية أيضا أعطيت أعلي إنتاج من فاكهة المانجو وكان 97.92 مليجرام / مل ثم بعدها من القريب فروت وكان 59.52 مليجرام/ مل ثم أقل انتاج من حامض الستريك اعطي من مخلفات الجزر وكان 16.00 مليجرام/مل. تبين أن كمية السكر الموجودة في مخلفات الفواكهة والخضروات قبل التخمير تؤثر علي ذيادة إنتاج الحامض والسبب في ذالك لأن فطر الأسبيروجلس نيجر يتغذي علي السكر لإنتاج حمض الستريك. كما لوحظ انه كلما طالت فترة التخمير يقل إنتاج الحامض.