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Citric Acid Production by *Aspergillus flavus* Using Sugarcane Bagasse Via Solid-State Fermentation

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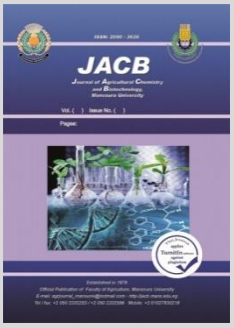

ABSTRACT

Citric acid is in high demand due to its low toxicity when compared to other acidulants commonly used in the food and pharmaceutical industries. The current study aims to identify effective variables for producing citric acid via solid-state fermentation (SSF) from sugarcane bagasse medium using *Aspergillus flavus.* A local fungus strain of *A. flavus* was isolated from spoiled fruits from Mansoura markets, was tested for its ability to produce citric acid, and then was used for maximizing citric acid production using sugarcane bagasse which was collected as a residue of juice shops as a low-cost fermentable material with high sugar content using solid state fermentation process. The highest yield of citric acid (14.41 g citric / 100 g bagasse) was obtained after 7 days of incubation at 25ºC with using bagasse medium with moisture ratio 1.0:3.0 (bagasse/solution), supplemented with 1% fructose and ammonium nitrate as carbon and nitrogen sources, respectively. Medium pH was adjusted at 6.0.

Keywords: citric acid production, *Aspergillus flavus*, solid-state fermentation, sugarcane bagasse.

INTRODUCTION

Citric acid is an organic acid which found primarily in citrus fruits. It has great importance, where it is produced in huge quantities and widely used in the pharmaceutical and food industries. Submerged fermentation is the primary method of producing citric acid by fungi utilizing a variety of carbohydrate sources, such as starch-based media and molasses. However, more research should be done on other fermentation methods, like solid-state fermentation, which has great potential for its production (Soccol *et al.*, 2006). Citric acid is a tricarboxylic acid, it has the formula $C_6H_8O_7$ and is 2-hydroxypropane-1,2,3-tricarboxylic acid (Vandenberghe *et al.*, 1999). It has three carboxylic functional groups with different pKa values (3.1, 4.7, and 6.4), and a molecular weight of 210.14 g/mol (Kumar and Jain, 2008). Citric acid is a primary metabolic product which produced during the Krebs cycle. There are many uses for citric acid, but it is usually utilized as an acidulate in the food industry worldwide (Anastassiadis and Finogenova, 2008). Citrus fruit is the primary source of the acid, but it can be synthesized industrially (Kareem *et al.*, 2010 and Max *et al.*, 2010). The sugar content of the fruit serves as the substrate for fermentation-based citric acid production. Though sucrose is preferred for its production, polysaccharide can also be used if the fermenting organism has the ability to secrete hydrolytic enzymes at low pH for relatively long periods of time.

One of the main advantages of solid-state fermentation over submerged fermentation is the reduction production cost, lower volume of wastes and industrial sewage (Yadegary *et al.*, 2013). Solid state fermentation is a process that carried out in a solid medium that contains little or no free water, with a percentage of moisture to support the metabolic activity and growth of microorganisms. (Thomas *et al.*, 2013). In recent years, the use of solid-state fermentation process for microbial citric acid production has a great interest as it can be used for many purposes. The resistance of microbial cells to catabolic repression with abundant substrates presence *i.e.*, glucose, glycerol and other carbon sources was considered as the most important phenomenon attributed to SSF (Chetan, 2018).

Sugarcane bagasse is considered as the most suitable substrate used via SSF processes in the production of citric acid by microorganisms, which considered as a low-cost method and beneficial citric acid production (Yadegary *et al.*, 2013). Regarding the cost and taking into account the volume of sugar plant production in Egypt, sugarcane bagasse is considered the most suitable substrate for citric acid production. Sugarcane bagasse is an environmental waste in Egypt, and it needs to be converted into a useful raw material.

Therefore, this study aims to use sugarcane bagasse as a low-cost fermentable substrate via solid-state fermentation process and obtain effective variables in producing citric acid from high citric acid-producing local fungus isolate by providing optimum circumstances for citric acid production and finally optimizing the production of citric acid.

MATERIALS AND METHODS

Isolation of fungal isolates: Samples of spoiled fruits: guava, pear, grape, banana, orange, and apple were collected from different Mansoura markets to some local fungi. The isolation process was carried out on potato dextrose agar plates' medium with addition of streptomycin as antibacterial agent. Plates were incubated at 30°C. Fungal colonies were observed after 5 days of incubation to isolate every single colony of different fungus on PDA slants.

Identification of the selected producing fungus: Morphological and molecular analysis was performed on a 5-days-old fungus culture grown on potato dextrose agar. According to Campbell and Johnson (2013), morphological examination includes colonial and microscopic appearance of fungi were carried out to determine fungal species**.** For the

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confirmation of the fungal strain, the molecular identification was carried out using a partial sequence of the fungal 5.8S rRNA genome by Sigma scientific services, Cairo, Egypt.

Preparation of Sugarcane bagasse medium: Sugarcane bagasse was collected from the sugarcane processing industry located in El-Dakahlya, Egypt. Bagasse was dried at 40°C for two days then cut into small pieces and ground, the chemical composition of the used sugarcane bagasse was (%): N 2.77, C 44.03, H 5.51, P 0.04, Ca 0.54, Mg 0.08, K 0.22 and Na 0.07. For the preparation of fermentation medium, 5 g of bagasse was placed in a 250 ml Erlenmeyer flask and moistened with 5 ml of a 1% NH_4NO_3 solution. To ensure that the substrate was properly cooked and to make it more vulnerable to microbial attack, the medium was autoclaved for 60 minutes at 121°C, then the medium pH was adjusted to 6.0**.**

Inoculum preparation: For the preparation of the inoculum, three slants of fungal growth were used to make a 50 ml of spore suspension. 5 ml of distilled water were added to 5 days old slant culture with addition of tween 80, the fungal spores were scratched gently and shaked vigorously for 1 min, and then spores were transferred to 50 ml of 0.8% NaCl solution. The suspension was adjusted to achieve spores' density up to 10^6 spores/ml (Selim *et al*., 2020).

Production of citric acid via SSF process: One ml of isolated fungi spore suspension (10^6 spore/ml) were transferred to Sugarcane bagasse medium, then the inoculated media flasks were incubated at 30°C for 7 days. After that, cultures were collected for citric acid extraction.

Extraction of the crude citric acid: the extraction of citric acid from fermented culture were done with modification according to (Yadegary *et al*., 2013). Fifty ml of distilled water was added to each flask of SSF cultures, mixed well, and shaken at 150 rpm for 30 minutes to ensure homogeneity. The mixture was filtered using double-layered gauze. Then, the filtrate was centrifuged at 6000 rpm for 20 minutes to separate the fungal spores and solid particles from the filtrate. The clear filtrate was collected for produced citric acid estimation.

Citric acid assay (Pyridine-acetic anhydride method): Citric acid estimation was carried out using the pyridine-acetic anhydride method according to the modification of, 1ml of culture filtrate (supernatant) was taken in clean test tubes and make up the volume with1ml distilled water. 8ml of acetic anhydride were added in each test tube and kept at 60°C for 10 min, after that 1ml of pyridine was added, and again kept at water bath for 40 minutes at 60°C. The mixture then cooled for 5 min in ice water. The yellow color that appears as a result of the reaction between acetic and pyridine was measured at 420nm using jenway 6305 spectrophotometer (Vaishnavi *et al.*, (2012). Results were signed on standard curve of citric acid and measured as amounts of citric produced from 100 grams of dry sugarcane bagasse**.**

Factors affecting citric acid production:

Effect of Incubation period: Sugarcane bagasse medium was inoculated with the most citric acid producing fungus and incubated for 5, 7 and 9 days to investigate the effect of incubation period on the production of citric acid by the selected fungal isolate.

Effect of Initial moisture: Sugarcane bagasse medium was moistened with different moisture ratios ranging from (1:1 - 1:7) using 1% Ammonium nitrate solution, with adjusting pH at 6.0. Flasks were inoculated with fungal spores and incubated at room temperature 25°C to determine the best moisture content.

Effect of carbon sources: To test the effect of carbon sources on citric acid production, the fermentation medium was enriched with 1% (w/w) of different sugars as additive carbon sources such as glucose, fructose, lactose, maltose and sucrose. Medium without addition of carbon source was used as control. Fermentation media were adjusted at 6.0 incubated at 25°C.

Effect of nitrogen sources: Nitrogen source in bagasse fermentation medium (ammonium nitrate) was replaced with different mineral nitrogen sources in ratio of 1% (w/w) to determine the best nitrogen source for citric acid production. Mineral nitrogen sources used were ammonium sulfate, ammonium chloride, and sodium nitrate as well as organic nitrogen sources such as beef extract, yeast extract and peptone. Medium with 1% ammonium nitrate was used as control. Medium were enriched with the best carbon source, then inoculated and incubated at 25°C for 7 days under pH value 6.0.

Effect of incubation temperature: Fermentation medium were prepared with the best moisture content, carbon source, and nitrogen source, then incubated at different temperatures from 20 to 35°C (interval 5), to determine the optimum temperature for citric acid production.

Effect of initial pH: To determine the best pH value for citric acid production, the initial pH of the bagasse fermentation medium was adjusted at different values from 4.0 to 8.0. All flasks were modified with the best moisture, best carbon source, best nitrogen source and incubated at the optimum temperature for 7 days.

RESULTS AND DISCUSSION

Isolation of citric acid producing fungi:

Eight local fungi isolates were isolated from the collected samples of spoiled fruits from Mansoura markets. Fungal isolates were tested for their efficiency of using sugar cane bagasse as low-cost substrate with high sugar content in the production of citric acid via SS fermentation. Data in Fig. (1) show the amounts of citric acid produced by the eight isolated fungi after 5 days of SSF process. Data showed that isolate No. 8 gave the largest amount of citric acid compared to the rest of other fungal isolates, where it yielded 0.084 *g* of citric / 100 g of bagasse after 5 days of incubation. As a results, fungal isolate No.8 was chosen to complete the subsequent experiments to maximize citric acid production through SSF process. There is great interest in the production of citric acid via SSF by fungi, numerous research has studied the microbial production of citric acid by fungi by using both solid-state and submerged state fermentation. Many fungi species could be involved in citric acid production, although most research showed that the species of *Aspergillus* are the most capable of producing citric acid such as *A. niger* (Hang & Woodams 1986); Roukas and Kotzckidou 2020; Pintado *et al.*, 1998 and Lu *et al*. 1997); *A. aculeatus* (El Dein & Emaish 1979); *A. awamori* (Grewal & Kalra, 1995); *A. wentii* (Karow & Waksman, 1947); and *A. foetidus* (Chen 1994 and Tran *et al.*, 1998). Also, Grewal & Kalra, 1995, reported that it could be use *Penicillium janthinelum* in citric acid production.

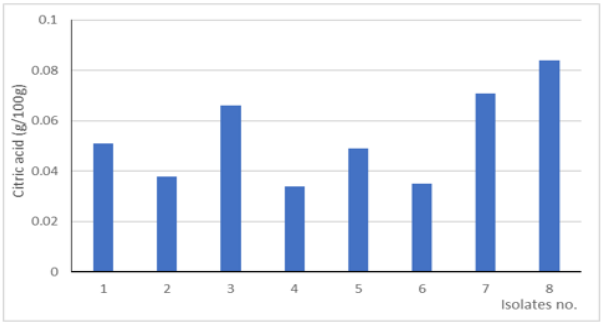

Fig. 1. Screening for citric acid production by fungal isolates via SSF on sugarcane bagasse medium after 5 days.

Identification of *Aspergillus flavus*

The colonial examination of the chosen isolate no. 8 after 10 days showed a yellow greenish color, colony diameters about 5 cm on czebeck medium and 7 cm on malt extract agar medium. Conidiophores are hyaline, about 0.6 mm long with rough walls. The conidial heads radiating. Conidia are globus, finely roughened and about 3-4µ diameter. Figs (2a) and (2b) shows the photograph of colony obverse and reverse, respectively, while Fig. (2c) shows the conidiophore and conidia of the isolate. The partial sequence of the 5.8S rRNA gene carried out by Sigma Scientific Services Co., Cairo, Egypt. The sequence was entered to national center of Biotechnology Information Database (NCBI), which resulted as our isolate was *Aspergillus flavus* as shown in the phylogenic tree shown in Fig. (3). Malik *et al.*, (2018) used *A. flavus* in citric acid production via SSF, while Daiwshala and Kamthane (2017) used *A. flavus* in citric acid production via SMF.

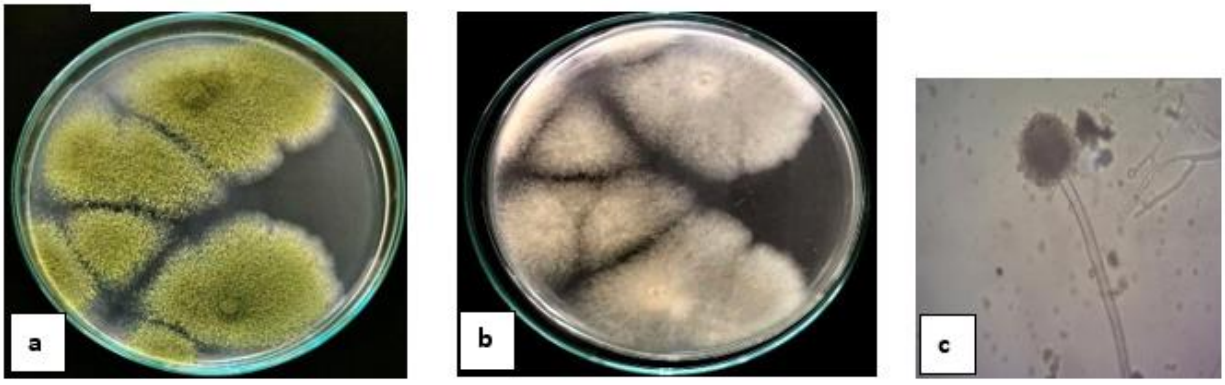

Fig. 2a. Colony obverse, b. Colony reverse photograph on peptone agar medium after 7 days and c. Microscopic photograph of conidia and conidiophore with 40X magnification of *A. flavus*.

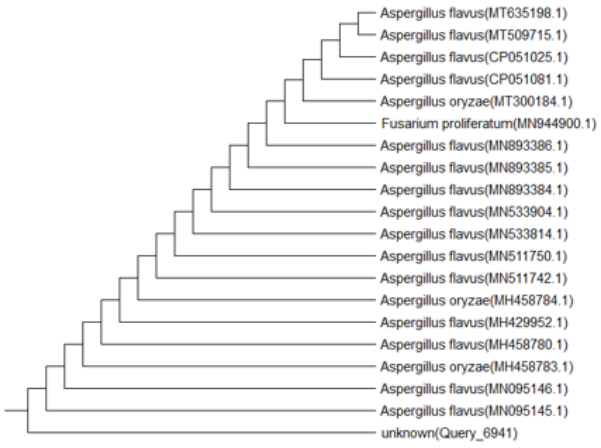

Fig. 3. Phylogenetic tree of *A. flavus*

Optimization of Citric acid production by the local isolate of *A. flavus*

Effect of Time course on citric acid production:

Data in Fig. (4) illustrates the effect of incubation time on citric acid production by *A. flavus.* Data indicate that citric acid production increased with time which reached the maximum production of citric acid at the seventh day which recorded 1.77 1.77 g / 100 grams of bagasse. After that, citric acid production decreased at 8th and 9th days.

The obtained results were within the range of those recorded by Kumar and Jain (2008), where after 8 days, they reached the maximum production of citric acid by *A. niger* via SSF using sugarcane bagasse. Kumar *et al.*, (2003), also reported that the maximum amounts of citric acid produced by *A. niger* using mixed fruit wastes as substrates through SSF process was obtained at the 9th day of fermentation.

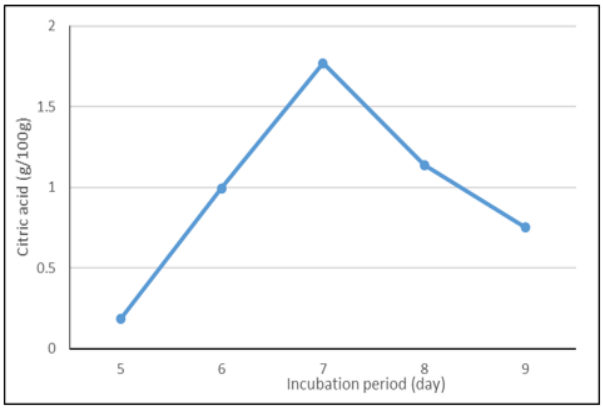

Fig. 4. Effect of Incubation period on citric acid production by *A. flavus* via SSF on sugarcane bagasse medium

Effect of moisture content

Dutt and Kumar, (2014) explained the important role of moisture ratio in SSF which considered as a key factor in the solid-state fermentation process, affecting microbial growth and citric acid production. The optimum moisture content for a solid-state fermentation process varies according to the type of fermentation substrate as reported by Chugh *et al.*, (2016). Moisture improves the substrate usage by the

microorganisms. Where the lower moisture ratio compared to the optimal leads to reduce the swelling of the substrate, thus resulting in a low transfer of different nutrients, the higher moisture ratio compared to the optimal might inhibit the fungi growth moreover encourage bacterial contamination because of both lowing oxygen diffusion rate and porosity in the substrate.

Fig. (5) shows the effect of different moisture ratios from 1:1 to 1:7 on the production of citric acid by *A. flavus* after 7 days of incubation. Citric acid production gradually increased with increasing moisture content, which achieved maximum production of citric acid 9.49 g/ 100g with moisture ratio 1:3. With increasing moisture ration over 1:3, the citric production gradually decreased.

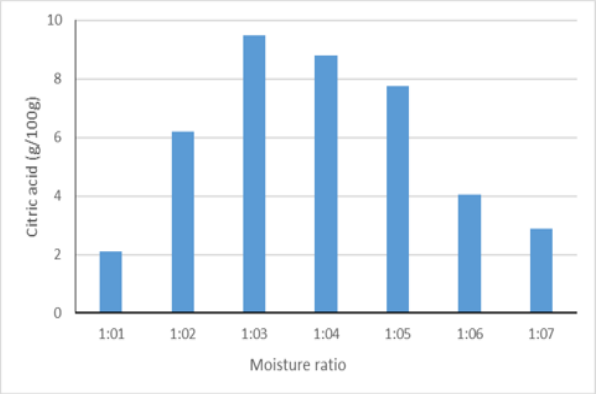

Fig. 5. Effect of moisture ratio on citric acid production

Many literatures confirmed the importance of moisture in the production of citric acid via solid state fermentation. Yadegary *et al.* (2013) reported that raising moisture content increased in SSF process increased the yield of citric acid produced by A. niger, where the maximum production achieved at 65% moisture. Also, Kumar *et al.* (2003) achieved the maximum production of citric acid by A. niger via solid-state fermentation at ratios 55, 65, 75 and 85% moisture, respectively.

Effect of carbon sources:

Several decades of research have revealed that there is a direct impact on citric acid production by microorganisms due to the type of carbon source. Microorganisms prefer mono and di-saccharides as carbon sources, as it is more quickly metabolized by fungi, resulting in a higher yield. On the other hand, polysaccharides are unsuitable as a raw material because of the long time it takes to hydrolyzed to achieve the required rate of sugar to produce citric acid, this slow rate of polysaccharide hydrolysis may be interpreted because of the decrease of enzymatic activity, which make changes in the pH of the fermentation medium (Mattey 1992). Soccol *et al.*, (2006) mentioned that the type of carbon source strongly affected citric acid production, also, it is very necessary to supply fermentation medium with rapidly metabolized carbohydrates by microorganisms for high production of citric acid.

Data in Fig. (6) show various effects on citric acid production by *A. flavus* via SSF due to the supplementation of fermentation medium with different sugars as an additive carbon source. Data showed that only fructose increased citric acid production compared to control (without addition of carbon source). Furthermore, data show that lactose addition makes the largest decrease in citric acid production. The citric acid production increased by 29.67 %, resulting 11.93 g/100g. In contrast to our results, according to previous research, sucrose is the best carbon source among easily metabolized carbohydrates, followed by glucose, fructose, and galactose (Dasgupta *et al.*, 1994 and Vandenberghe *et al.*, 1999). Hossain *et al.*, (1984) mentioned that the effect of sucrose in decreasing citric acid production is superior to other sugars as a carbon sources such as fructose, glucose and lactose. Angumeenal & Venkappayya (2013) pointed out that the superiority of sucrose is attributed to the powerful *A. niger* mycelium-binding extracellular invertase, which at low pH, sucrose rapidly hydrolyzes (Kubicek-Pranz *et al.*, 1990).

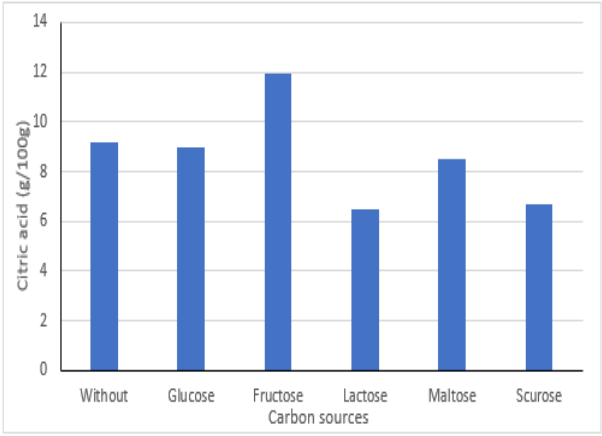

Fig. 6. Effect of carbon sources on citric acid production by *A. flavus* via SSF on sugarcane bagasse medium

Effect of Nitrogen sources:

Results in Fig. (7) show that replacing ammonium nitrate with other organic and mineral nitrogen sources decreased citric acid production, although this decrease was little with using ammonium sulphate. The nature of the nitrogen source clearly influences the production of citric acid. Researchers have concluded that ammonium salts are preferred for citric production because their consumption lowers the pH required for citric acid fermentation (Kumar *et al.*, 2003; Soccol *et al.*, 2006 and Yadegary *et al.*, 2013). The effect of nitrogen sources on citric production explained by Papanikolaou, *et al* (2008), they proved that adding different nitrogen sources to the fermentation media obviously affected the production of citric acid by affecting the pH of the medium**.** Data in Fig. 7 show that using ammonium nitrate as a nitrogen source gave the highest production of citric acid (12.32 g/100g) by *A. flavus*.

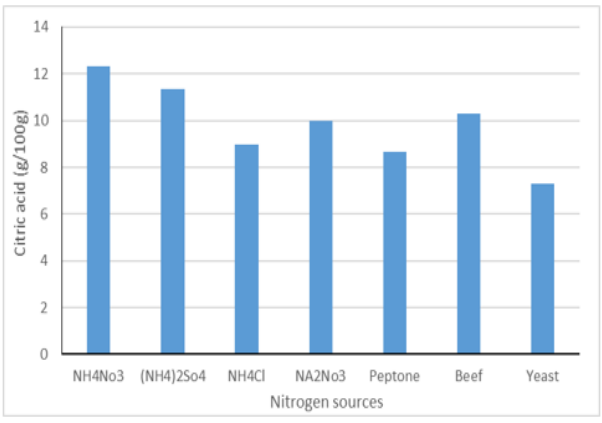

Fig. 7. Effect of nitrogen sources on citric acid production by *A. flavus* via SSF on sugarcane bagasse medium

Effect of Incubation temperature:

Hang and Woodams (1986) have explained that the incubation temperature is one of the critical factors that have a profound effect on the production of citric acid from grape pomace by *A.niger* via solid-state fermentation. They recorded that *A. niger* and the *A. flavus* were produced higher citric acid at the temperature 30°C. Data in Fig. (8) shows the effect of incubation temperatures on the production of citric acid by *A. flavus*. Data showed that 25°C was the optimum incubation temperature for citric acid production via SSF by *A. Flavus*, where citric acid production reached 11.77g/100g after 7 days of incubation. Also, data showed that *A. flavus* produced the best yield of citric in the temperature range between 20-30°C, which demonstrated the fungus' mesophilic nature**.** On the other side, the decrease in citric acid production above 30°C might be explained by the way that high temperature slows the growth of fungi.

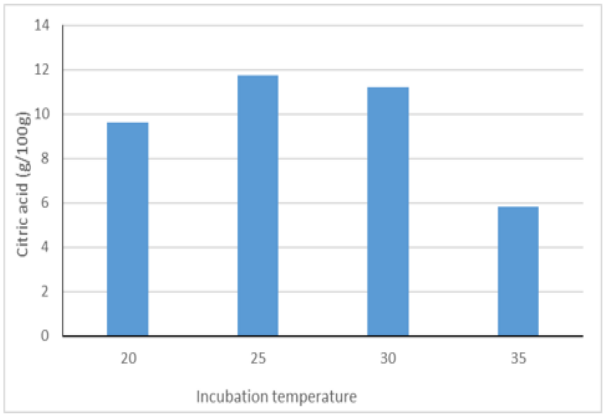

Fig. 8. Effect of incubation temperature on citric acid production by *A. flavus* via SSF on sugarcane bagasse medium

Effect of pH:

pH is an important factor affecting citric acid production. The importance of pH of the medium becomes clear in two stages of treatment. All fermentation begins with germs, and germination requires a pH above 5.0 for ammonia absorption. Protons are released as a result of the vegetative spores, thus lowering pH and improve citric acid production, low pH during production reduces the fermentation media from pollution by other microorganisms, also prevent the production of unwanted organic acids which makes citric acid recovery easier (Max *et al.*, 2010). Data in Fig. (9) show the effect of different pH values on citric acid production by *A. flavus*. The production of citric acid gradually increased by raising pH value from 4.0 to 6.0 which recorded highest citric acid production 14.41g/100g, then citric production decreased with raising the pH of the medium over 6.0, where the lowest yield of citric was recorded at pH 8.0. El-Kady, (2003) mentioned that pH affected citric acid production by *A. niger* and explained that may be due to the pH affected the enzymes activity in degrading the substrate and the cell membrane permeability of sucrose and citric acid. These results come according to Kumar *et al.*, (2003); Soccol *et al.*, (2006) and Yadegary *et al.*, (2013).

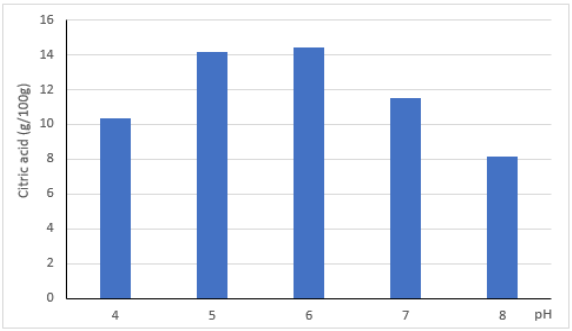

Fig. 9. Effect of initial pH of fermentation medium on citric acid production by *A. flavus* via SSF

CONCLUSION

The production of citric acid under solid-state fermentation by isolated local strain of *A. flavus* can be improved after 7 days of incubation where production of citric acid reached 14.41g/100g bagasse, by using sugarcane bagasse as a raw material under moisture adjustment at 1:3, supplemented with fructose as carbon source and ammonium nitrate as nitrogen source under fermentation conditions at pH value of 6.0 and an incubation temperature of 25°C.

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إنتاج حامض الستريك من اسبيرجيلس فلافس باستخدام تفل القصب بطريقة التخمر الصلب

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الملخص

يزداد الطلب على حامض الستريك نظرًا لانخفاض سميته عند مقارنته بالمواد الأخرى التى يشيع استخدامها كمحمضات فى الصناعات الغذائية والصيدلانية. تهدف الدراسة الحالية إلى تحديد المتغيرات الفعالة لإنتاج حامض الستريك عن طريق تخمير الحالة الصلبة باستخدام تفل قصب السكر عن طريق ميكروب اسبيرجيلس فلافس. تم عزل سلالة فطر محلية وتم تعريفها أنها اسبيرجيلس فلافس وتم اختبار قدرتها على إنتاج حامض الستريك بالتخمير الصلب واستخدامها لزيادة إنتاجيتها من حامض الستريك باستخدام تفل قصب السكر المتخلف عن صناعة السكر ومحلات عصير القصب كمادة رخيصة التكلفة قابلة لعملية التخمير. وقد كان أقصى إنتاج من حامض الستريك 14.41 جم ستريك /100 جم من تفل القصب بعد 7 ايام من التحضين على 25م مع تعديل رطوبة البيئة لنسبة 3:1 ومضاف إليها الفركتوز كمصدر للكربون بنسبة 1% ونترات الأمونيوم كمصدر للنيتروجين بنسبة 1% مع ضبط الأس الهيدروجينى على 6.