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Naturally Occurring Microorganisms of Industrial Waste for Citric Acid Production by Solid State Fermentation

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ABSTRACT

The solid state fermentation method with naturally occurring *Aspergillus niger* MAF3 might be feasible in a practical system for citric acid production from industrial solid potato waste (SPW) under static condition. Solid potato wastes excluded in the potato processing industry (chips), used as a substrate for citric acid production. Maximum production of citric acid (29.24 g kg^{-1} SPW) followed by the lower reducing sugar and pH of 0.25 g L^{-1} and 2.22 respectively, was obtained when *A. niger* MAF3 was grown in a medium containing 65 g SPW, initial pH 5, after fermentation at 25°C for 5 days. The addition of 3.0% (v/w) methanol of potato waste was known as the most influential treatment to increase citric acid concentration (32.68 g kg^{-1} solid potato waste).

Key words: Naturally occurring microorganisms, industrial solid potato waste, citric acid, solid state fermentation

INTRODUCTION

Citric acid is of industrial importance because; it is widely used in dairy, medicine and biochemical industries. Considerable interest has been shown in using agricultural wastes for citric acid production (Tongwen and Weihua, 2002). Potato is one of the most important crops grown in Egypt for local consumption, export and processing. The area cultivated with potatoes about 212,000 acres producing about 2.2 million tons, with an average of 10.5 tones per acre (Hegazy, 2009). Potato annual world production is around 300 milliontons, and areas planted cover more than 18 millionha. Major producing countries (and the world's share of production) are China (20%), Russia (12%), India (8%) and United States (8%) (Miranda and Aguilera, 2006).

The food, agricultural and forestry industries produce large volumes of wastes that can be used as raw materials under SSF conditions. Example of them include sweet potato residue, corn cob, cassava bagasse, sugar cane bagasse, wheat bran, rice bran, carob pod, spent brewery grains, among others (Rodríguez-Couto, 2008).

As the microorganisms in a solid substrate are growing under the conditions similar to their natural habitat, they can produce certain enzymes, metabolites, proteins and spores more efficiently than in submerged fermentation (Ellaiah *et al*., 2004).

Citric acid is produced by submerged fermentation of starch or sucrose-based media, using the filamentous fungus *A. niger* (Jianlong, 2000). Citric acid production in solid state fermentation (SSF) has been a subject of interest (Roukas, 1999; Shojaosadati and Babaeipour, 2002) because SSF offers numerous advantages for production of bulk chemicals and enzymes. Koji fermentation

was conducted using the peels of banana (*Musa acuminata*) as an inexpensive substrate for the production of citric acid using *Aspergillus niger*.

The key physico-chemical parameters influencing the growth of *A. niger* on a solid substrate and its production of citric acid are: nutrient balance, solid substrate composition, moisture content and particle size distribution, incubation temperature, pH and inoculum density (Lee and Yun, 1999; Ellaiah *et al*., 2004). Among many environmental factors and growth conditions, the accumulation of citric acid is strongly affected by medium composition. Besides optimization of sugar and mineral levels in solid substrate, higher production of citric acid was recorded by applying additives, such as methanol, ethanol, phytate, vegetable oil, oximes, *n*-dodecane, fluoroacetate and chelating agents (Jianlong, 2000). Also, the production of citric and gluconic acids from fig by *Aspergillus niger* ATCC 10577 in solid-state fermentation was investigated by Roukas (2000) and the results pointed out that the maximal citric and gluconic acids concentrations were obtained at a moisture level of 75%, initial pH 7.0, temperature 30°C, and fermentation time in 15 days. Organic solvents, including ethanol and methanol, stimulate the production of citric acid by increasing the permeability of the cell membrane, decreasing cell growth or changing the activity of citrate synthetase and aconitase in TCA cycle (Pazouki *et al*., 2000).

Industrial solid potato waste produced in huge amount in Egypt is either used as an animal feed or disposed to the soil. Since industrial potato waste is rich in carbohydrate and other nutrients, it can serve as a substrate for citric acid production using SSF. Solid potato waste which is excluded from potato processing industry (chips) and used as a substrate for amylase production, consists of (fresh matter w/100 g w) was as follows: Moisture content, 77.0; crude protein, 2.52; crude fat, 0.13; crude fiber, 3.50; Ash, 5.31; carbohydrate, 88.54. While, the determination of micro and macro element were widely varied (mg kg^{-1} Dry Mater) Fe, 87.75; Mn, 5.25; Cu, 11.45; Zn, 15.9; Na, 1350; K, 11002; Ca, 2800; Mg, 1560; S, 2295; P, 2050 (Darwish *et al*., 2009).

Selection of a suitable microorganism is one of the most important stages in SSF. *Aspergillus niger*, for example, is a microorganism able to produce as many as 19 types of enzymes and several other value added compounds, such as citric acid and alcohols, by SSF of agro industrial residues (Schuster *et al*., 2002; Rodríguez-Couto, 2008).

The objective of this study was to utilize solid potato waste in a fermentation medium for production of citric acid by naturally occurring *A. niger* via solid state fermentation as well as to study the effect of various fermentation parameters such as substrate concentration, incubation period, temperature, pH, and the addition of ethanol and methanol concentrations on citric acid production.

MATERIALS AND METHODS

Materials: Solid potato waste samples were obtained from the Chips Company for food industries in 2009, Assiut, Egypt. The other components of the culture media were obtained from Merck and Sigma in the highest purity available.

Isolation and identification of microorganism: *A. niger* MAF-3 used in this study was isolated from potato waste according to Nakayama (1981). Taxonomic identification of filamentous fungi were identified by using mature cultures on standard Potato Dextrose Agar (PDA) in order to ensure a good development of taxonomically relevant features, and following the identification keys provided by Von-Arx (1981) Domsch and Gams (1993). *A. niger* MAF-3 culture was maintained on PDA slants, incubated at 28°C, stored at 4°C and subcultured every 2 weeks.

Methods: Micro-organism: Eight strains of *A. niger* were screened for citric acid production in liquid culture which contained g L^{-1} glucose 120, $(NH_4)_2SO_4$ 3, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot 7H_2O$ 0.014, and $FeCl_3 \cdot 6H_2O$ 0.01 according to Kirimura *et al.* (1987). Based on the results on citric acid yields, one strain, *A. niger* MAF3, was used for production of citric acid.

Inoculum: *A. niger* MAF3 spores were produced in PDA Broth with (50 mL) in a 250 mL Erlenmeyer flask, incubated at 28°C for six days. A spore suspension was prepared by adding 25 mL distilled water with Tween-80 (0.1%) and stored at 4°C for two weeks according to Vandenberghe *et al.* (2000). It contained 1.2×10^7 spores mL^{-1}, and used as an inoculum in each experiment.

Fermentation: Liquid nutrient medium containing g L^{-1} $ZnSO_4 \cdot 7H_2O$ 0.2 and $FeCl_3 \cdot H_2O$ 0.014 (Vandenberghe *et al.*, 2000). Thirty grams of solid substrates in a 500 mL Erlenmeyer flask, and mixed with 20 mL^{-1} of the nutrient medium. Reducing sugars 1.25 g L^{-1}, and initial pH 3.8, were measured of the culture medium, sterilized at 121°C for 15 min, and inoculated with 1 mL of inoculum containing 1.2×10^7 spores mL^{-1}. The flasks were incubated at 28°C in an incubator under stationary conditions. Then the total volume made to 100 mL distilled water in each sample. The mixture was vigorously stirred for about 15 min and filtered. The filtrate was then used as the solid culture extract for analysis.

Analysis: The concentration of citric acid in culture filtrate was measured by titration with 0.1 N NaOH (Imandi *et al.*, 2007; Khosravi-Darani and Zoghi, 2008). After titration, citric acid was determined spectrophotometrically at 420 nm by the acetic anhydride-pyridine method (Marrier and Boulet, 1958; Imandi *et al.*, 2008). The pH of the substrate was measured by a pH-meter equipped with a glass electrode, using a solid-liquid ratio of 10% (w/v) with distilled water.

RESULTS AND DISCUSSION

Effect of initial concentration of potato waste on citric acid production: Potato waste concentrations of 15, 30, 65, 125, 150, 200 and 250 g L^{-1} were examined for citric acid production by *A niger* MAF3 using static flask technique in 500 mL Erlenmeyer flask (Fig. 1). At substrate concentration of 65 g L^{-1}, the maximum amount of citric acid produced was 10.94 g kg^{-} ¦potato waste with reducing sugar 0.20 g L^{-1} and pH 2.14.

Figure 1 Indicated that lowest concentration of both residual sugar and pH value were accompanied with the greatest concentration of citric acid. When the substrate concentration was higher than 65 g L^{-1}, citric acid production decreased gradually. Tsay and To (1987) reported that maximum citric acid production was obtained at 140 g L^{-1} of sucrose. When the sucrose concentration was higher than 140 g L^{-1}, citric acid production decreased, due to polyalcohol formation (Gutierrez-Rozas *et al.*, 1995). Citric acid production decreased at lower sucrose concentration because of oxalic acid formation (Honecker *et al.*, 1989). The higher initial sugar concentration (18% w/w) results to increase citric acid yield and productivity (Khosravi-Darani and Zoghi, 2008) 14-22% in industrial fermentations (Rohr *et al.*, 1983).

Effect of fermentation period on the citric acid production: The concentration of citric acid increased with the increasing in fermentation time till four days (Fig. 2). The maximum citric acid concentration 32.22 g kg^{-1} solid potato waste accompanied with reducing sugar 0.22 g L^{-1} and pH

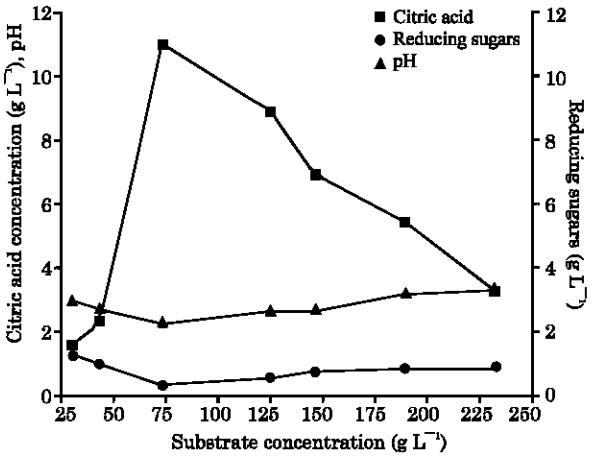

Fig. 1: Effect of substrate concentration on citric acid production. The initial conditions were pH 3.8; temperature, 30°C; incubation period 6 days

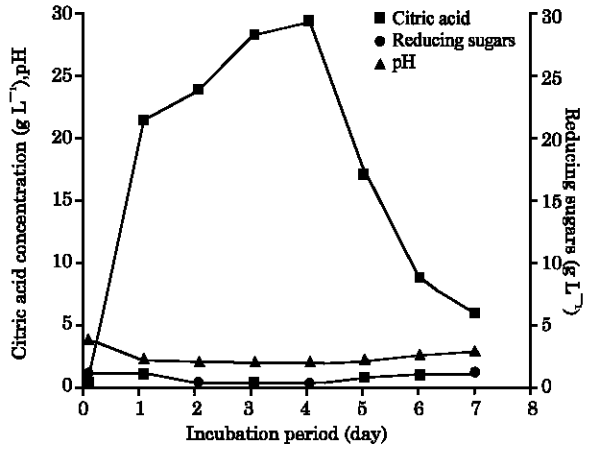

Fig. 2: Effect of fermentation period on citric acid production. The initial conditions were pH 3.8; temperature, 30°C; substrate concentration, 65 g

2.04, was obtained after four days of fermentation and then reduced on the fifth day as found by (Demirel *et al*., 2005). As a result of the rapid production of citric acid at the first day of incubation, the pH value strongly shifted from 4.12 to 2.26. However, it was almost constant after the first till the last day of incubation.

Interestingly, the lowest reducing sugars concentration (0.22 g L^{-1}) was observed at the time of maximum production of citric acid. After four days of incubation, the amount of citric acid produced was strongly decreased. In contrast to this result, Xie and West (2006) studying on citric acid production by *Aspergillus niger* on corn distillers` grains with solubles and pointed out that citric acid production by *A. niger* ATCC 11414 or ATCC 201122 after 240 h was higher on untreated grains than on autoclaved grains or acid-hydrolyzed grains.

The concentration of reducing sugars decreased during the fermentation, coinciding with an increase in citric acid production (Fig. 2). The lowest, concentration of residual sugars (0.22 g L^{-1}), and value of pH (2.04), was observed after 4 days of incubation. Similar results were observed by Roukas (1999) which reported the lowest concentration of residual sugars (63 g kg^{-1} wet substrate) was observed after 12 days of incubation. At this time, 55% of sugars consumed were converted to

citric acid while the total amount of utilized sugars was 64%. The pH value was decreased as shown in Fig. 2, this was may be due to the citric acid production during fermentation of sugars.

Effect of initial pH on citric acid production: As shown in Fig. 3, citric acid production increased with increasing initial pH value in the medium up to 5. At initial pH value lower and higher than 5, citric acid production was reduced. The maximum concentration of citric acid, 18.22 g kg^{-1} potato waste, with reducing sugar of 0.23 g L^{-1} and pH value of 2.33, was obtained at initial pH value 5. Similarly, Al-Shehri and Mostafa (2006) study on citric acid production from date syrup using immobilized cells of *Aspergillus niger* and pointed out that the highest value of citric acid was obtained at pH 5.5.

In contrst to this result, Lodhi *et al.* (2001) pointed out that *Aspergillus niger* showed maximum citric acid production at pH 4.0.

The lower value of pH was accompanied with the higher concentration of citric acid, and the pH value increased due to oxidation of citric acid by the fungus (Hang *et al.*, 1975).

As shown in Fig. 3, the citric acid concentration increased with the increase in initial pH from 2.5-5.0. On the other hand, the reducing sugar and pH value were decreased by increasing of citric acid. Roukas (1999) reported that the citric acid yield and concentration increased with the increase in initial pH from 3.5-6.5. Khosravi-Darani and Zoghi (2008) reported that the initial pH of 5.5 caused an increase in citric acid yield and productivity.

Effect of incubation temperature on citric acid production: Temperature has a profound influence on the fungal production of citric acid using solid potato waste. The optimum fermentation temperature for citric acid production by *A. niger* MAF-3 grown on potato waste was found to be 25°C. The effect of temperature on solid potato waste fermentation is shown in Fig. 4. The maximum citric acid concentration (29.44 g kg^{-1} potato waste), with reducing sugar (0.25 g L^{-1}) and pH (2.22), was obtained in culture grown at 25°C.

The citric acid concentration increased obviously with the increase in fermentation temperature from 20-25°C and decreased above 25°C. At temperature higher than 25°C, the decreasing in citric acid concentration was accompanied with increasing in both pH and reducing sugars. Roukas (1999) studied on citric acid production from carob pod by solid-state fermentation found that the

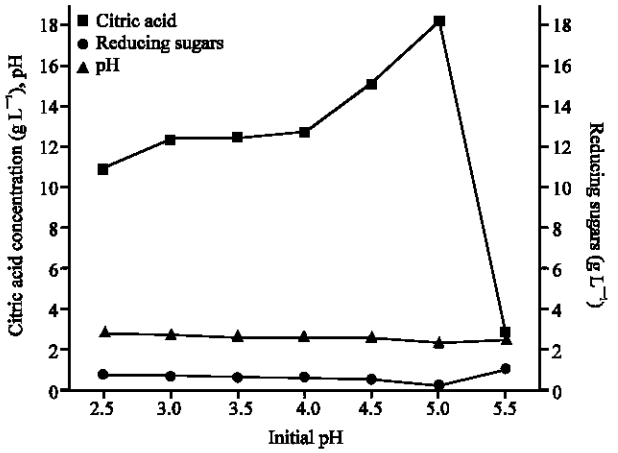

Fig. 3: Effect of initial pH values on citric acid production. The initial conditions were substrate concentration, 65 g; incubation period, 4 days; temperature, 30°C

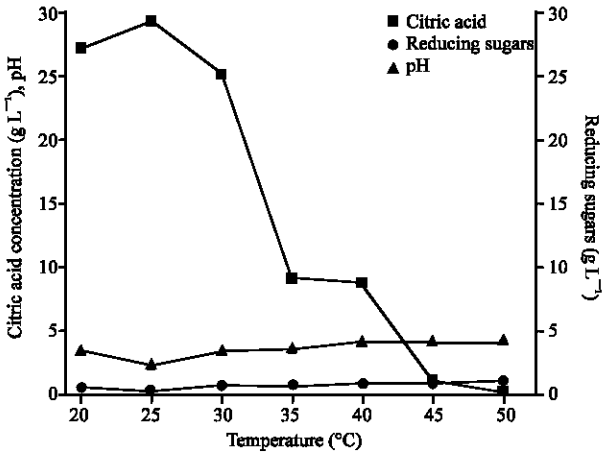

Fig. 4: Effect of incubation temperature on citric acid production. The initial conditions were substrate concentration, 65 g; incubation period, 4 days; pH, 5.0 and temperature, 25°C

citric acid concentration increased significantly with the increase in fermentation temperature from 25-30°C and decreased above 30°C. Also, Asad-ur-Rehman *et al.* (2002) studied on the temperature optima for citric acid accumulation by *Aspergillus niger* and indicated that, the maximum amount of anhydrous citric acid obtained during the course of study was 65.96 g L^{-1} at 30°C.

The sugar consumption and pH decreased slightly with the increase in fermentation temperature from 0-25°C. Figure 4 shows temperature in the range of 35-40°C does not influence citric acid production. Szewczyk and Myszka (1994) found that the temperature did not strongly affect the growth rate in SSF in the range of 28-34°C.

Khosravi-Darani and Zoghi (2008) reported that the optimum fermentation temperature for citric acid production by *A. niger* ATCC 9142 grown on crude sugarcane bagasse were found to be 30°C. The maximum citric acid concentration (176±4 g kg^{-1} dry pod) and citric acid yield (55±2%) were obtained in culture grown at 30°C while the biomass dry weight and the sugar utilization were maximum at 40°C (Roukas, 1999). Ali *et al.* (2001) studied on the biosynthesis of citric acid by locally isolated *Aspergillus niger* using sucrose salt media and found that the cultural conditions such as pH (3.5), temperature (30°C), incubation period (8 days) and sugar concentration (15%), were optimised.

Effect of ethanol and methanol concentrations on citric acid production: As shown in Fig. 5 and 6, citric acid production increased with increasing ethanol and methanol concentrations in the medium up to 2 and 3% (v/w), respectively, while, ethanol and methanol concentrations higher than 2 and 3% (v/w), citric acid production were reduced.

The maximum concentration of citric acid, 15.70 and 32.68 g kg^{-1} potato waste, with reducing sugar of 1.0 and 0.21 g L^{-1}, and pH values of 2.42 and 2.37, were obtained in the presence of 2 and 3% (v/w) ethanol and methanol concentrations, respectively. Hang and Woodams (1984, 1985) and Hang (1988) reported that similar stimulatory effects of methanol on citric acid production by SSF using *A. niger* NRRL 567.

Similarly, Hamissa (1987) and Dasgupta *et al.* (1994) reported that moderate concentrations (1-3%) of methanol decreased the iron and manganese uptake by the fungus and doubled the citric acid yield. Hang *et al.* (1977); Roukas and Kotzekidou (1987) reported that the addition of methanol at concentrations of 1-4% (v/w) resulted in a marked increase in the amount of citric acid formed by *A. niger* on spent grain liquor and brewery wastes, respectively.

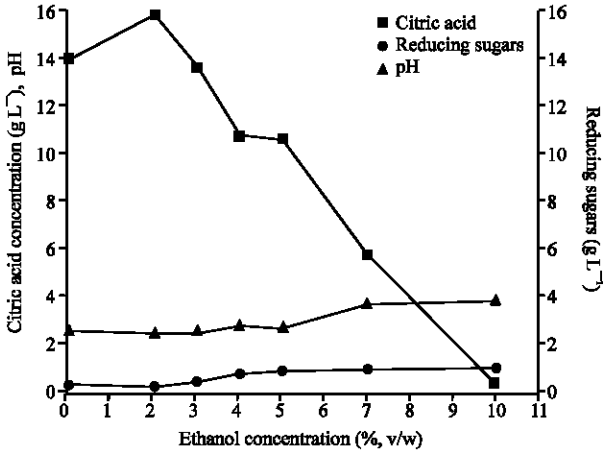

Fig. 5: Effect of ethanol concentration on citric acid production. The initial conditions were substrate concentration, 65 g; incubation period, 4 days; pH, 5.0; temperature, 25°C

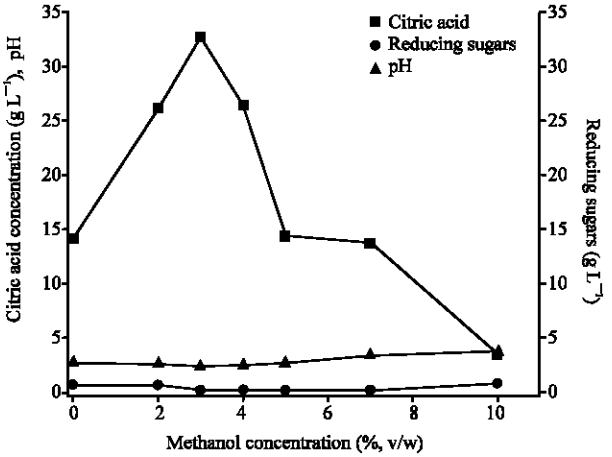

Fig. 6: Effect of methanol concentration on citric acid production.The initial conditions were substrate concentration, 65 g; incubation period, 4 days; pH, 5.0; temperature, 25°C

Khosravi-Darani and Zoghi (2008) reported that the citric acid and biomass concentration, sugar consumption, citric acid yield and productivity increased in the presence of methanol. The higher amount of methanol (4%, v/w) has increased the yield and productivity in all treated and untreated bagasse.

Citric acid production continuously increased by adding up to 4 mL methanol and 3 mL ethanol per 100 mL fermentation media. Maximum citric acid production was observed at these methanol and ethanol concentrations (Demirel *et al.*, 2005). Methanol supplementation to the grains was only capable of stimulating citric acid production by *A. niger* strains ATCC 26550 and ATCC 11414 (Xie and West, 2006).

CONCLUSION

Industrial solid potato wastes are an organic waste that is highly rich in basic nutrients that could support microbial growth. The potential economic benefits which may occur from the use of this cheap nutrient material as a source of both microorganisms and microbiological research

medium, and it has utilized as microbial substrate for production of valuable citric acid. In the present study, production of citric acid using industrial solid potato wastes by solid culture method and naturally occurring strain *A. niger*-MAF-3 produced citric acid 32.68 g kg^{-1} industrial solid potato waste.

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