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Citric Acid Production from Date Syrup using Immobilized Cells of *Aspergillus niger*

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Abstract: A total of 14 isolates of *Aspergillus niger* were obtained from soil samples of two different locations in Saudi Arabia. These cultures were screened for their ability to produce citric acid in date syrup medium under free and immobilized cells systems. Maximum productivity was about 1.44 times higher in the immobilized system compared to free cells. *A. niger* j4 isolated from Jazan region was selected as a good producer for optimization of the citric acid fermentation in immobilized cells technique. Citric acid production was increased gradually and reached to maximum value (30.6 g L^{-1}) after 6 days of cultivation. Optimum production was achieved at 15% sugar concentration with consumed sugar of 49.5%. The highest value of citric acid (42.5 g L^{-1}) was obtained at pH 5.5 with increasing the consumed sugar to 61.7%. A positive relationship between citric acid production and incubation temperature was also observed up to 30°C.

Key words: Citric acid, date syrup, immobilized cells

INTRODUCTION

Date palm plantations in Kingdom of Saudi Arabia cover an area around 140,000 ha with a total production of approximately 830,000 tons (Al-Beez, 2004). Date syrup called dibs or molasses is probably the most common derived date product, produced in three different ways: as an accidental by-product in the storage of bagged, humid dates, at the home or village level and on a semi-and full industrial scale. Date syrup can be used as a sweetener and flavoring for human consumption and in microbial fermentations for ethanol, vinegar and single cell protein production (Barreveld, 1993). Organic acids are important microbial products used in a variety of applications. Among the various organic acids, citric acid occupies predominant position as a commercial biochemical used as flavoring and preservative in food and beverage and as antioxidant agent when acting with ascorbic acid. The buffering properties of citrates are used to control pH in household cleaners and pharmaceuticals. The estimated world production of citric acid was reported as 1,000,000 tons/year and the world market demand in increasing day by day (Soccol *et al*. 2003). It is mainly produced from sucrose and glucose by surface and submerged fermentations. Recently, considerable interest has been shown in using agricultural products and their wastes for citric acid production by *Aspergillus niger*. Date syrup is an economical source of carbohydrates for conversion to citric acid because it is readily available and relatively low priced (Roukas and Kotzekidou, 1997). Citric acid has been produced by conventional submerged culture in which mycelial growth and metabolic activity related to the accumulation of citric acid are affected by a change in the composition and physical properties of the culture media (Kubicek and Rohr, 1986). The fungal fermentations have serious disadvantage of rising viscosity during growth, leading to poor oxygen supply to the cell. The fermentation method using immobilized cells provides ease of separation for the product, thus continuous production can be achieved without affecting the viscosity. The productivity obtained using immobilized cells is considerably higher, process can be more easily controlled and the immobilized cells are more stable than the free cell (Bayraktar and Mehmetoglu, 2000). The objective of this study was to investigate the optimum conditions for citric acid production by *Aspergillus niger* isolates using date syrup medium and immobilized cells system.

MATERIALS AND METHODS

Microorganisms: Wild strains of *A. niger* were isolated from soil samples collected from two different locations of Saudi Arabia, Asser (Abha) and Jazan. The cultures were maintained on PDA slants at 4°C. The identification was made according to Roper and Fennell (1965). These

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isolates were screened for their ability to produce citric acid in date syrup medium under free and immobilized cells systems.

Date syrup medium: Date syrup was obtained from Wadi Al-dawaser region, Saudi Arabia as an accidental by-product in the storage of bagged, humid dates called Alsari. Date syrup (70% total sugars), was diluted with distilled water in order to obtain 15% (w/v) total sugar concentration.

Immobilization: A loopful of *A. niger* spores was inoculated in PDA slants and incubated at 30°C. After 7 days of incubation, 5 mL of sterilized distilled water was added to each slant. The conidia suspension was then collected. Sodium alginate at 1.33% was mixed with conidia solution and then dropped into 0.27 M $CaCl_2$ solution by using 2 mL pipets in order to prepare the immobilized conidia. Conidia of *A. niger* were entrapped in Ca alginate pellets of approximately 3 mm in diameter. The pellets were left 1 h at 20°C, washed with distilled water and stored in 0.027M $CaCl_2$ solution at 4°C. The amount of conidia was 5.6×10^5 conidia g^{-1} of pellets (Bayraktar and Mehmetoglu, 2000).

Cultivation conditions: Conventional and immobilized cultures experiments were carried out in 250 mL Erlenmeyer flasks containing 50 mL of date syrup as a growth and fermentation medium (pH 6). The flasks were inoculated with 3 mL of spore suspension or 4 g of immobilized spores. The cultures were incubated for 8 day at 30°C on a shaking incubator (150 rpm). The fermentation broth was filtered and used for citric acid and consumed sugars determinations. Biomass was washed twice with distilled water and dried.

Analytical techniques: The fermentation liquid was used for the calorimetrically determination of residual sugars by phenol sulfuric acid method (Taylor, 1995). Anhydrous citric acid was estimated by pyridine-acetic anhydride method (Marrier and Boulet, 1958) using a double beam UV/Vis scanning spectrophotometer (Model: Shimadzu, 1601PC). Culture dry weight (Biomass) was determined by drying filtered cake or pellets at 70°C until constant weight (Mashhoor *et al*., 1987).

RESULTS AND DISCUSSION

Screening of *A. niger* isolates for citric acid production: A total of 14 isolates of *Aspergillu niger* were obtained from soil samples of two localities of Saudi Arabia, 6 of which (1-6 A) were isolated from Abha region and

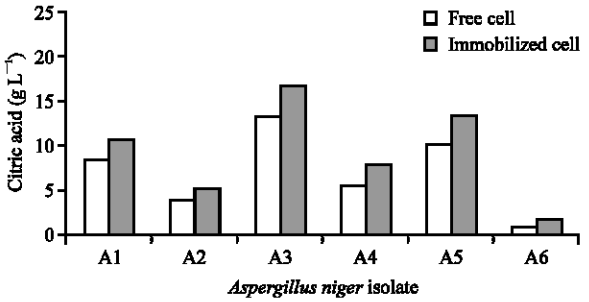

Fig. 1: Screening of *A. niger* isolates (from Abha) for citric acid production

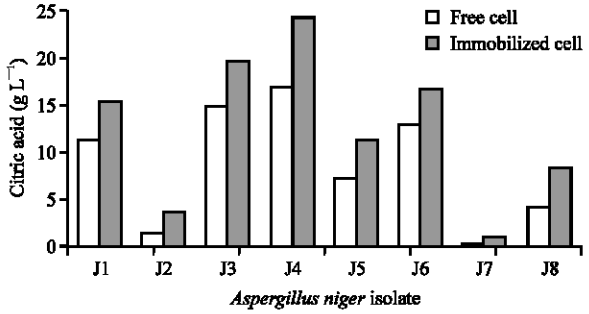

Fig. 2: Screening of *A. niger* isolates (from Jazan) for citric acid production

8 (1-8 J) isolated from Jazan region. The occurrence of isolates in the examined localities may be affected by the prevailing environmental conditions, nature and component of the soil and plant activates as reported by Hashem and Al-Faraj (1995). Most of the tested isolates were able to produce citric acid under free and immobilized systems (Fig. 1 and 2). The production of citric acid in the immobilized technique was about 1.44 times more than that obtained using free cell system. Similar observations were reported by Sankpal *et al.* (2001) and Ates *et al.* (2002) they reported that the presence of cells inside the alginate beads may be protect them from physiological and physical damage. *A. niger* j4 was produced the highest amount of citric acid under free (16.9 g L^{-1}) and immobilized (24.3 g L^{-1}) systems. The differences in the citric acid production by isolated cultures were also observed. Isolates A1, A3, A5 from Abha and J1, J3, J5, J6 from Jazan were capable to produce moderate amount of citric acid, while isolates A2, A4, A6 and J2, J7, J8 from Abha and Jazan, respectively, were produced slight amounts of citric acid. Highest citric acid obtained was not only dependent upon isolated organisms, but also the beneficial characters of date syrup as a fermentation medium. From the previous results, *A. niger* j4 was selected as a good producer for optimization of the citric acid fermentation in immobilized cell system.

Effect of fermentation period: Citric acid production was increased gradually during the fermentation period reached to maximum value (30.6 g L^{-1}) after 6 days, it was almost constant up to 8 days and then decline (Fig. 3). These findings were in agreement with the results of Kahlon *et al.* (1992) and Khare *et al.* (1994). They reported that faster processing, higher volumetric productivity and ease of applied the continuous fermentation mode are the major advantages of the immobilized cells system. At the optimum fermentation period, biomass and consumed sugar were 26.1 g L^{-1} and 49.4%, respectively. Increasing of fermentation period did not improve the citric acid production. This decrease in productivity may be due to the inhibitory effect of high concentration of citric acid, decay in enzymes system responsible for biosynthesis of citric acid, reduce the amount of nitrogen available in fermentation medium and depletions of sugar contents as reported by Kristiansen and Sinclair (1979), Alvarez-Vasquez *et al.* (2000) and Arzumanov *et al.* (2000).

Effect of sugar concentration: The effect of sugar concentration on citric acid production was examined with date syrup media containing 5 to 30%. Maximum citric acid was obtained at 15% with consumed sugar of 49.5% (Fig. 4). These results confirmed the findings of Roukas (1998) and Papagianni *et al.* (1999) they stated that the advantages of high fermentable sugars in the fermentation medium may be due to the suppression of osmosensitive contaminates and reduced the dilution water requirements. They also observed that initial sugar concentration in the fermentation medium had a marked effect on the rate of citric acid production and the morphology of the producer microorganism. The specific rate of citric acid formation increased with increasing the initial glucose concentration. The glucose level in the medium were shown to lead to a significant non catalyzed entry into the mycelium, which accounted for the citric acid productivity and growth rate changes. Biosynthesis of citric acid was reduced other than optimum value reached to 50.2% at 10% and 17.8% at 30% sugar concentration. Pazouki *et al.* (2000) recorded that the high sugar concentration leads to the decreasing of consumed sugars, making the process uneconomical, while a lower sugar concentration leads to the accumulation of oxalic acid in the culture broth.

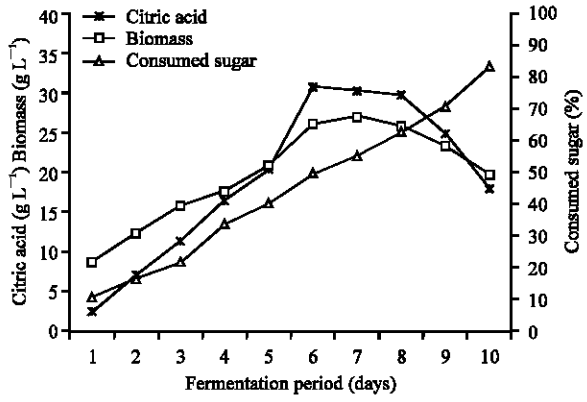

Fig. 3: Effect of fermentation period

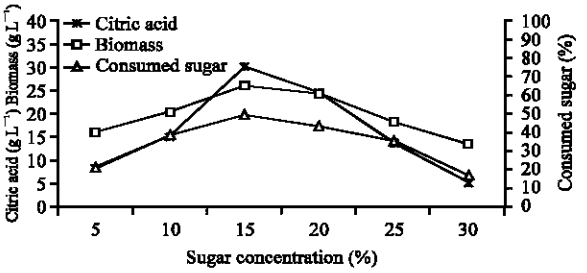

Fig. 4: Effect of sugar concentration

Effect of initial pH: To investigate the effect of initial pH on the citric acid production, initial pH of the medium was varied between 4.0 and 7.0 by addition of either HCl or NaOH. Optimum citric acid production (42.5 g L^{-1}) was achieved at pH 5.5 with increasing the consumed sugar to 61.7% (Fig. 5). Biosynthesis of citric acid was decreased at low and high pH value reached to 17.4 g L^{-1} at pH 4 and lost about 75.8% at pH 7. These results were consistent with findings of Kahlon *et al.* (1992) they found that maximum citric acid yield was obtained from a sugar cane molasses medium using *A. niger* entrapped in alginate gel at pH 5.5. The highest initial pH leads to the accumulation of by products such as oxalic acid (Ali *et al.*, 2002). Otherwise, Kristiansen and Sinclair (1979) mentioned that the initial pH had no effect on citric acid production through any direct influence on the Krebs

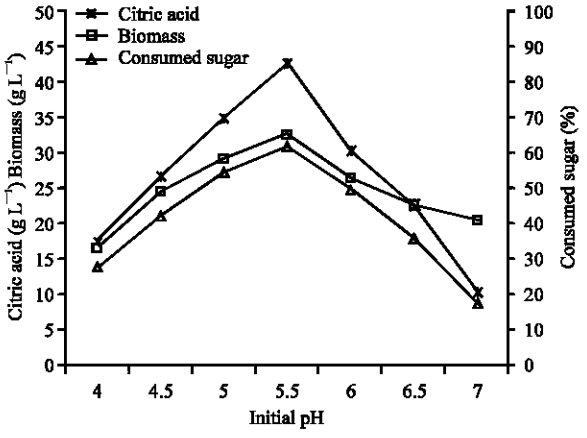

Fig. 5: Effect of initial pH

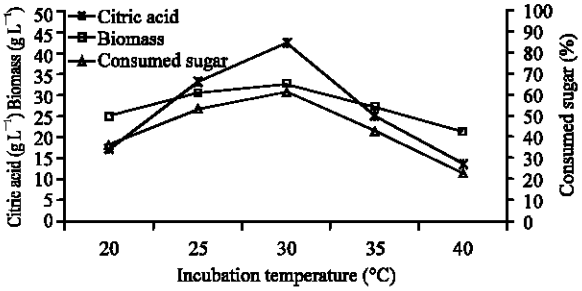

Fig. 6: Effect of incubation temperature

cycle, it is possible that pH affects the enzymes that are active in degrading the substrate and the permeability of the cell membrane to sugar and citric acid.

Effect of incubation temperature: To elucidate the effect of incubation temperature on the citric acid production, shake flask experiments were run at various temperatures between 20 and 40°C. A positive relationship between citric acid production and incubation temperature was observed up to 30°C (Fig. 6). These results were in agreement with those obtained by Arzumanov *et al.* (2000) and Ali *et al.* (2002) They also reported that when the incubation temperature was low, the enzymes activity responsible for citric acid production was also low, giving no significant impact on the enhancement of citric acid production. Higher temperature resulted in progressive decline in citric acid production reached to 31.9% at 40°C. It may be due to that the high temperature can cause denaturation of citrate synthase and accumulation of by-products such as oxalic acid. According to Hang and Woodams (1998), the temperature of 40°C was most favorable for oxalic acid production while, citric acid accumulation completely inhibits at this temperature.

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