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Effects of Fermentation Conditions on Citric Acid Production from Beet Molasses by *Aspergillus niger*

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The effects of initial sugar concentration, pH and temperature on citric acid production from beet molasses by *Aspergillus niger* were investigated in shake cultures. The maximum citric acid production (52.3 g L^{-1}) was achieved at the condition where the initial sugar concentration was 140 g L^{-1} at pH 5.0, 30 ºC. When the fermentation was performed in the treated medium the citric acid concentration increased from 14.3 to 33.2 g L^{-1}. Beet molasses is a suitable substrate for citric acid production by *A. niger* and this organism is sensitive to pH, temperature and sugar concentration in the medium.

Key Words: *Aspergillus niger*, Beet molasses, Citric acid, Fermentation conditions.

INTRODUCTION

Citric acid (a tricarboxylic acid) is one of the few bulk chemicals produced by fermentation with *Aspergillus niger*. It is widely used in the food, pharmaceutical, cosmetic, beverage and other industries[1,2]. Among the organic acids industrially produced, citric acid is an important commercial yield with a global production more than 1 million tons per year and its demand is increasing day by day which requires a much more efficient fermentation process for higher yield product[3,4]. A number of carbon sources such as molasses, carob pod extract, rape seed oil, corncobs and brewery wastes are used to produce citric acid[5]. The most widely used industrial substrate for citric acid production by *A. niger* is beet molasses, a by-product of beet sugar production[6-8]. It is necessary to use inexpensive and readily available raw materials in industrial production processes[9]. Due to its availability and low price, molasses is a convenient raw material for the industrial production of citric acid by fermentation[10].

It has been shown that citric acid production by *A. niger* is markedly influenced by a number of environmental conditions and some trace elements[10-12]. To develop a process for the maximum production of citric acid, standardization of media and fermentation conditions are crucial[13]. The complexity of this process has attracted numerous biotechnological investigations. Although conventional citric acid production by submerged culture of high-yielding mutant strains of *A. niger* has been

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optimized, there is still interest in redesigning the traditional manufacturing process to increase yield and subsequently to minimize overall operating costs[14].

The objective of this investigation is to examine the potential of beet molasses as a source of citric acid production by *A. niger* using batch fermentation as well as to study the effects of fermentation parameters such as temperature, pH, initial sugar concentration and treated medium.

EXPERIMENTAL

Microorganism and inoculum: The original culture of *A. niger* was supplied by Prof Dr Salih Maden, Department of Soil Plants, Ankara University, Turkey. It was maintained on potato dextrose agar (PDA, Difco) slants at 4 °C and sub-cultured at intervals from 15-30 d. The cultures were incubated on PDA petri dishes at 30 °C for 8 d. The sporulated culture was scraped off and suspended in 5.0 mL of sterile-distilled water to prepare the inoculum.

Pre-treatment of molasses: The beet molasses samples used as the carbon source in the present study were kindly provided by the Kirsehir Sugar Mills, Turkey. Prior to fermentation, crude molasses was pretreated by $K_4Fe(CN)_6$ at pH 4.5, 90 ºC for 15 min and then precipitations were removed by filtration. The molasses was diluted with distilled water to adjust the sugar concentration[8,15].

Fermentation medium and culture conditions: The initial fermentation medium for citric acid production contained, in g L^{-1}: molasses (total sugar), 140; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.5; $NH_4Fe(SO_4)_2.12H_2O$, 0.9; $ZnSO_4.7H_2O$, 0.5; $CuSO_4.5H_2O$, 0.25. The pH of the substrate was adjusted to 5.0.

Fermentation experiments were performed in 250 mL conical flasks containing 100 mL of media with untreated or treated beet molasses. The medium was autoclaved at 121 ºC for 20 min and inoculated with 5 mL of *A. niger*' broth cultures under aseptic conditions. The flasks were incubated at 30 ºC on a rotary shaker (200 rpm) and air-flow rate was 0.51/min. All experiments were carried out in duplicate.

Initial sugar concentration: In order to determine the optimum initial sugar concentration, a series of conical flasks containing medium were supplemented with molasses at different concentrations of 55, 80, 100, 120, 140, 160 and 180 g L^{-1}. The flasks were inoculated with 5 mL of inoculum and incubated at 30 ºC on a rotary shaker (200 rpm) for 8 d.

Initial pH: A series of conical flasks each containing 100 mL of the production medium (140 g L^{-1} initial sugars) adjusted at different initial pHs (3.5, 4.0, 4.5, 5.0 and 5.5) were inoculated with 5 mL of inoculum and incubated at 30 ºC for 8 d. Desired pH was adjusted with 0.1 N NaOH or 1 N HCl to avoid the change of the initial sugar concentration.

Temperature: Likewise above, separate experiments were carried out for each temperature values. The medium (140 g L^{-1} initial sugars molasses and pH 5.0) was inoculated with the fungus and incubated at different temperatures (25, 30 and 35 ºC) for 8 d.

Heavy metals: The effects of heavy metals on citric acid production were compared with treated by using $K_4Fe(CN)_6$ and untreated beet molasses and analyzed by various experiments. During these comparisons, citric acid, sugar utilization and biomass concentrations were determined at certain times of fermentation.

Analytical methods: Approximately 10 mL volume of fermentation broth was removed after every 24 h under aseptic conditions and transferred to a weighed Whatmann filter paper (no. 541) to remove mycelium. The filtrate was washed three times with distilled water, dried at 105 ºC to a constant mass and weighed as the biomass. Citric acid was estimated by the colorimetric method of Marrier and Boulet[16] and sugar was analyzed by the aniline method[17].

RESULTS AND DISCUSSION

Fermentation period: The effect of fermentation period, during citric acid fermentation by *A. niger* in beet molasses (initial sugar conc. 140 g L^{-1}, pH 5.0, 30 ºC) is shown in Fig. 1. The figure also shows the concentration of citric acid was increased with the increase fermentation time up to 168 h (7 d) and then decreased.

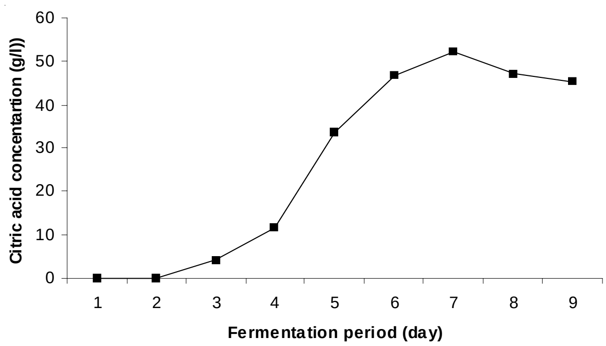

Fig. 1. Effect of fermentation period on citric acid production by *A. niger* in beet molasses

It is observed that fermentation time had a significant effect on the production of citric acid from beet molasses by *A. niger*. Maximum production of citric acid (52.3 g L^{-1}) was achieved after 7 d of fermentation at 30 ºC. However, after 7 d, there was a significant reduction in the citric acid production. It might be due to the decreased available nitrogen in fermentation medium, the age of fungi and depletion of sugar contents[3].

The biomass dry weight increased by the increasing of fermentation period until reached the maximal level after 8 d (15.8 g L^{-1}).Total sugar reduced from an initial concentration of 140 g L^{-1} to about 12 g L^{-1} after 192 h (8 d) of fermentation.

As expected, the fermentation of consumed sugars increased during fermentation, coinciding with an increase in citric acid and biomass. The concentration of consumed sugars decreased rapidly from 48 to 168 h of fermentation. This was due to rapid increase of citric acid concentration and biomass observed at the same time[18].

Initial sugar concentration: The experiments were performed with initial sugar concentrations of beet molasses (pH 5.0) to determine the optimum sugar concentration which gives the maximum citric acid production.

Fig. 2 shows that the citric acid production increased with the increase of sugar concentration from 55 to 140 g L^{-1}. The maximum production of citric acid (52.3 g L^{-1}) was obtained at a sugar concentration of 140 g L^{-1} after 144 h fermentation. Tsay and To[19] observed that maximum concentration of citric acid was obtained at 140 g L^{-1} sucrose containing medium. Similar types of works have been reported by Garg and Sharma[20] and Yaykasli *et al.*[21]. Our findings are in line with these results.

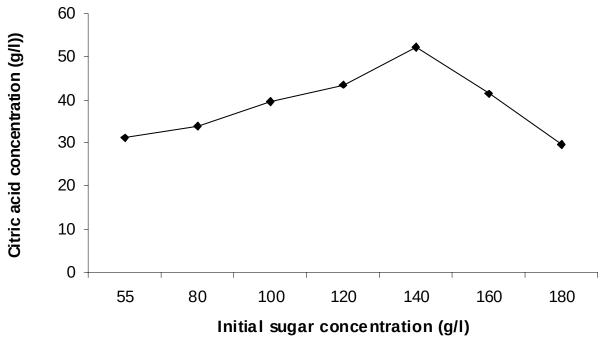

Fig. 2. Effect of initial sugar concentration on citric acid production

In line with micro-organism type and fermentation substrate, optimum sugar concentration also changes accordingly. Considering at amounts below 50 g L^{-1} sugar concentration, citric acid biosynthesis is not realized[22] and therefore, experiments always started from the threshold of 50 g L^{-1} and upwards. With initial sugar concentration higher than 140 g L^{-1}, citric acid production decreased gradually because of the polyalcohol forming[23].

It was observed that pH value strongly decreased from 5.0 to 1.8 by increasing the production of citric acid. Also progressive increase of biomass dry weight (from 2.8 to 13.9 g L^{-1}) was obtained with the increase of sugar concentration. This finding is an agreement with Saad[24].

Effect of initial pH: The effect of different pH (3.5-5.5) on the citric acid production with *A. niger* over 8 d fermentation period was carried out under constant-temperature of 30 ºC. The experimental result is shown in Fig. 3.

Initial pH is a critical environmental factor that has a profound effect on citric acid production. As shown in Fig. 3, the maximum citric acid production (52.3 g L^{-1}) was obtained when initial pH of fermentation medium was kept at 5.0. Similar results were observed by Ambati and Ayyanna[13] and Adham[12] who found that the highest values of the fermentation parameters (citric acid concentration, citric acid yield and consumed sugars) were achieved at an initial pH of 5.35-5.7. Decrease in pH caused reduction in citric acid production. This decline be due to that at low pH,

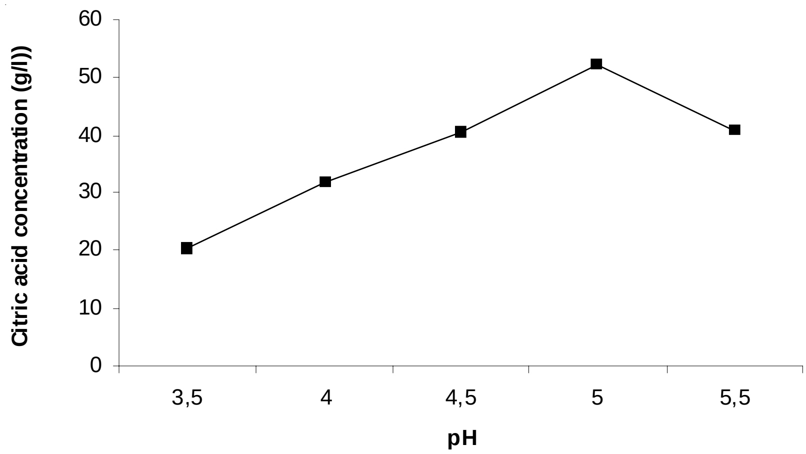

Fig. 3. Effect of initial pH on the citric acid production

the ferrocyanide ions were more toxic for growth of mycelium[3]. The highest biomass dry weight was obtained at pH of 1.8 (14.1 g L^{-1}).

Effect of temperature: The effect of temperature on the citric acid production with *A. niger* is shown in Fig. 4. The aim of this experiment was to determine the optimum initial temperature of beet molasses that would result in the highest citric acid concentration. A temperature of 30 ºC was found to be the best for citric acid fermentation (52.3 g L^{-1}) in the present study.

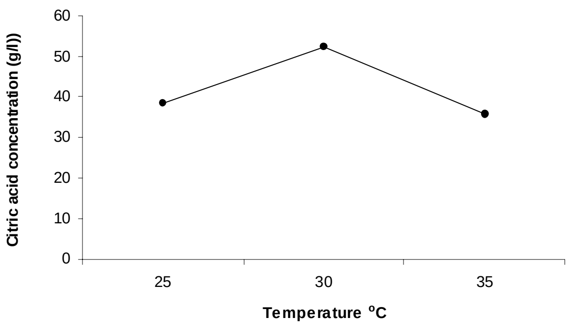

Fig. 4. Effect of temperature on citric acid concentration

It is determined that incubation temperature influenced the growth and product formation. Fig. 4 shows the citric acid concentration increased with the increase in fermentation temperature from 25 to 30 ºC and decreased above 30 ºC. Several workers[7,25] have also used values higher or lower than 30 ºC as the cultivation temperatures and obtained different amounts of citric acid. The biomass dry weight and the sugar utilization increased slightly with the increase in fermentation temperature from 25-30 ºC and remained constant between 30-35 °C. When the incubation temperature was low, the enzyme activity was also low giving no impact on the citric acid production. But at higher temperatures than 30 ºC, fermentation process is very rapid with extensive mycelial growth and the biosynthesis of citric acid was decreased. It might be due to the accumulation of by-products such as oxalic acid and gluconic acid. Therefore, an optimum range temperature is essential for higher yields of citric acid[13]. It is important to note that the findings in present conducted experiments support this result.

Effect of treatment of beet molasses on citric acid production: It is well known that citric acid fermentation is greatly affected by presence of some trace metals in toxic concentrations and it can be a significant problem during the fermentation of crude substrates into useful chemical products[5]. It is observed that there are evidences as a function of the fermentability improvement in the fermentation of the treated medium for the metals removal in comparison to the results obtained in not treated medium.

Beet molasses was treated by using $K_4Fe(CN)_6$ to precipitate the heavy metals which affected citric acid production. The results in Figs. 5 and 6 show that most treatments gave a remarkable increase in citric acid concentration, biomass dry weight productivity and consumed sugars in comparison with untreated beet molasses. The highest value of citric acid concentration (33.2 g L^{-1}) was obtained in culture grown in potassium ferrocyanide treated medium, while citric acid production from untreated beet molasses by *A. niger* was 14.3 g L^{-1}. However, the maximum biomass dry weight (13 g L^{-1}) was obtained in culture grown in potassium ferrocyanide treated medium, while biomass dry weight from untreated beet molasses by *A. niger* was 10.8 g L^{-1}. Consequently, the consumed sugar by *A. niger* during fermentation with untreated beet molasses increased from 58 to 72 % in comparison with treated beet molasses. Al-Obaidi and Berry[26] reported that heavy metals such as iron, zinc and copper which are present in great amounts in date syrup can cause a

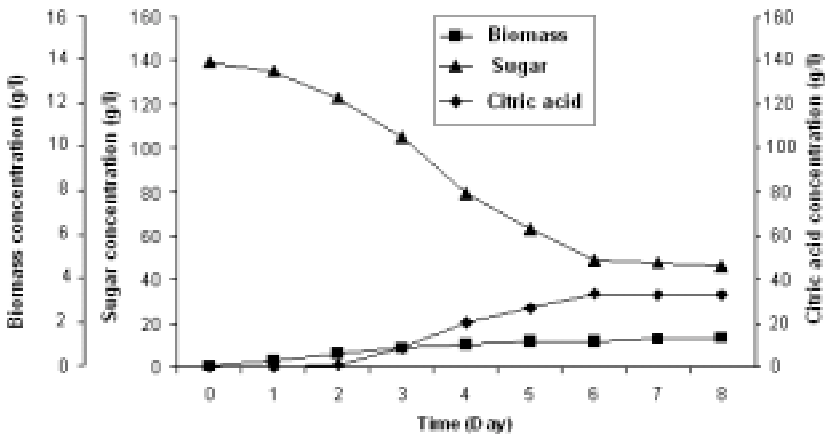

Fig. 5. Concentration of citric acid, biomass and sugar from treated beet molasses

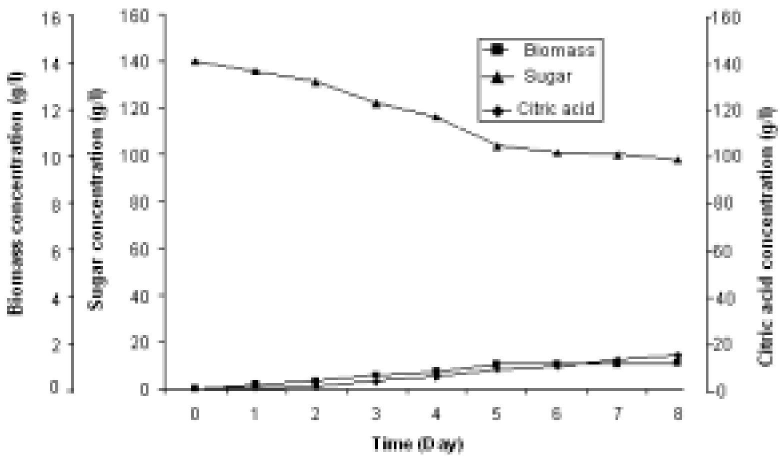

Fig. 6. Concentration of citric acid, biomass and sugar from untreated beet molasses

critical problem during citric acid fermentation. This means that beet molasses contained undesirable substances, which affected citric acid production. This is accordance with the findings of Saad[24].

Conclusion

The results from the present work demonstrate that some important aspects of citric acid production from beet molasses by *A. niger* in erlen culture. The optimal time of incubation for maximum citric acid production varies with fermentation conditions. The maintenance of a favourable temperature is noteworthy for the successful production of citric acid. When obtaining citric acid is in question, molasses with adequate sugar content can be used as an alternative carbon source by fermentation. Another point worth emphasising is that when heavy metals were removed from molasses, negative undesired effect of heavy metals to citric acid was also discontinued. Maximum citric production is obtained over 7 d fermentation with 140 g $L^{-1}$of initial sugar at pH 5.0 and 30 ºC.

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