OnLine Journal of Biological Sciences 2 (2): 70-72 2002


# Enhancement in Citrate Production by Alcoholic Limitation

Sikander Ali, Hamad Ashraf and Ikram-ul-Haq
Biotechnology Research Laboratories,
Department of Botany, Government College, Lahore, Pakistan

**Abstract:** Citric acid fermentation by *Aspergillus niger* GCB-47 was carried out in a 15L stainless stirred fermentor. Among the alcohols tested as stimulating agents, 1.0% methanol was found to give maximum amount of anhydrous citric acid (76.02 g/l), 24 hours after inoculation. This yield of citric acid is 1.76 fold higher than the control. The sugar consumed and %age of citric acid on the basis of sugar used were 108 g/l and 70.39 %, respectively. The mycelia were small round pellets having dry cell mass 14.5 g/l. Addition of ethanol, however, did not found to enhance citric acid production, significantly.

**Key words:** *Aspergillus niger*, citric acid, stimulatory effects of alcohol

## Introduction

Citric acid has wide range of applications and its world-wide demand is increasing day by day. It is solid at room temperature, melts at 153°C and decomposes at higher temperatures into other products (Hang and Woodams, 1986). Citric acid is a primary product of *Aspergillus niger* metabolism. A successful process depends both on an appropriate strain and on optimization of fermentation parameters. Cane-molasses is a desirable raw material for citric acid fermentation because of its availability and relative low price (Haq *et al.*, 1995). A variety of stimulants have been tested for improving citric acid yield by *Aspergillus niger*. The use of alcohols such as methanol and ethanol as stimulant, in citric acid production was found to enhance yield of citric acid (Majolli and Agirre, 1999).

The citric acid fermentation is very sensitive to the components of the medium, especially to iron, manganese and zinc. However, it has been shown that the addition of slightly toxic concentrations of low molecular weight alcohols to the medium increases the tolerance level of these trace metals in the fermentation (Maria and Waldslaw, 1989; Pazouki *et al.*, 2000). The purpose of the present investigation was to determine the value of alcohols, especially methanol ($CH_3OH$), in stimulating the production of citric acid by *Aspergillus niger* GCB-47 from crude carbohydrate cane-molasses by submerged culture in stirred fermentor.

## Materials and Methods

*Aspergillus niger* strain GCB-47, maintained on PDA slants, was used in this study. Vegetative inoculum prepared by taking 100 ml of fermentation (sugar 15%, pH 6.0) with glass beads in 250 ml Erlenmeyer flask, seeded with small amount of conidia and incubated at 30°C in rotary shaker (200 rpm) for 24 hours. A stirred fermentor was employed for citric acid fermentation. The fermentation medium i.e., clarified cane molasses was sterilized at 121°C for 20 minutes. Vegetative inoculum at a level of 5% (v/v) was used at 30°C for 6-days. The medium was agitated at 200 rpm (aeration rate 1.0 $ll^{-1}$ $min^{-1}$) and silicone oil was added to control the foaming.

The dry cell mass (DCM) was determined by filtering the mycelium through Whatman filter paper No. 44 and drying at 110°C for 6 h. Total acid was determined by titrating 1.0 ml of fermented broth against 0.1N NaOH using phenolphthalein as an indicator (Saha *et al.*, 1999).

$$\%\text{age Total acid} = \frac{\text{Titre x normality of alkali x Eq. wt. of acid}}{\text{Volume of sample}} \times 1000$$

Anhydrous citric acid was determined by using the method of Srivasta and Kamal (1979) while residual sugars were estimated by DNS method (Tasun *et al.*, 1970). The kinetics was studied after Sanjay and Sharma (1994).

## Results

**Effect of different alcohols**: The effect of different alcohols ($CH_3OH$ & $C_2H_5OH$) on the production of citric acid was studied (Fig. 1). Maximum amount of citric acid (72.55 g/l) produced when methanol was added into the molasses medium. The sugar consumption and dry cell mass of the medium were 110 and 13.0 g/l, respectively. The maximum amount of citric acid on the basis of sugar was 65.95%. The mycelia were intermediate (4-6 mm in diameter) rounded pellets. Addition of ethanol did not enhance citric acid production and 56.25 g/l citric acid was obtained in the fermented broth. Therefore, methanol was optimized as the best stimulant for citric acid production in the stirred fermentor.

**Effect of different methanol concentrations**: Fig. 2 shows the effect of different methanol concentrations (0.5 - 2.0%) on citric acid production by *Aspergillus niger* GCB-47 in stirred fermentor. The amount of citric acid production at 0.5% concentration of methanol, was 50.70 g/l. There was an increase in the production of citric acid with increase in methanol concentration. The maximum amount of citric acid (74.55 g/l) was obtained when 1.0% methanol was added in the fermentation medium. The sugar consumption was 100 g/l while dry cell mass was observed to be 12.5 g/l, having small round pellets. The maximum amount of citric acid produced on the basis of sugar used was 74.55%. Beyond 1.0% methanol concentration, the production of citric acid was gradually decreased. Hence, 1.0% methanol was found to be optimum.

**Effect of time of methanol addition**: The effect of the time of addition of methanol on the production of citric acid is shown in Fig. 3. The time interval was ranged from 0-48 hours, after inoculation. The production of citric acid was gradually increased when the time of addition of methanol was increased but up to a certain extent. The highest yield of citric acid (76.02 g/l) was obtained when 1.0% methanol was added in the production medium, 24 hours after inoculation. The mycelia were small round pellets, having dry mass 14.5 g/l. The consumption of sugar and %age of citric acid on the basis of sugar used were 108 g/l and 70.39%, respectively. Further increase in time of addition, did not enhance citric acid accumulation.

**Effect of partial replacement of methanol to ethanol concentration**: The data represents the effect of partial replacement of ethanol to methanol ratio on citric acid production by *Aspergillus niger* GCB-47 in SS fermentor (Table 1). The maximum amount of citric acid (62.76 g/l) was achieved when $C_2H_5OH$ $CH_3OH$ ratio was maintained at 0.4 : 0.6, 24 hours after inoculation. The %age of citric acid on the basis of sugar used was 56.03%, having 112 g/l sugar consumption. The mycelia were lax fluffy pellets in their

Table 1: Effect of partial replacement of ethanol to methanol concentration on citric acid production by *Aspergillus niger* GCB-47 in stirred fermentor

| Ethanol to methanol ratio(%) | Total acid(g/l) | Anhydrous citric acid (g/l) | Dry cell mass (g/l) | Sugar (g/l) | | | % of citric acid on the of basis sugar added | acidon the basis of sugar used | Mycelial morphology |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Added | Used | Residual | | | |
| 0.2 : 0.8 | 62.15 | 55.02 | 14.5 | 150 | 116 | 34 | 36.68 | 47.43 | Small pellets |
| 0.4 : 0.6 | 66.52 | 62.76 | 14.5 | = | 112 | 38 | 41.84 | 56.03 | Lax fluffy pellets |
| 0.6 : 0.4 | 58.26 | 48.55 | 13.5 | = | 127 | 23 | 32.37 | 38.23 | = |
| 0.8 : 0.2 | 51.04 | 39.29 | 12.0 | = | 122 | 26 | 26.19 | 32.20 | Elongated-Mycelium |

Temperature = 30°C Initial pH = 6.0 Aeration rate = 1.0 $ll^{-1}min^{-1}$

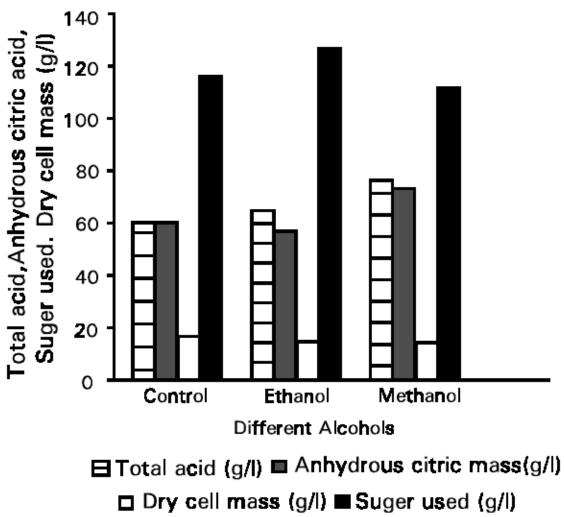


Fig.1: Effect of different alcohols on the production of citric acid by *Aspergillus niger* GCB-47 in stirred fermentor

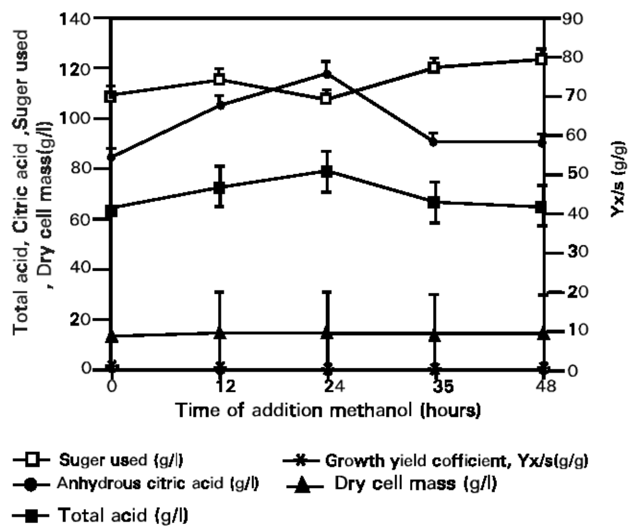


Fig.3: Effect of the time of addition of methanol on citric acid production by *Aspergillus niger* GCB-47 in stirred fermentor

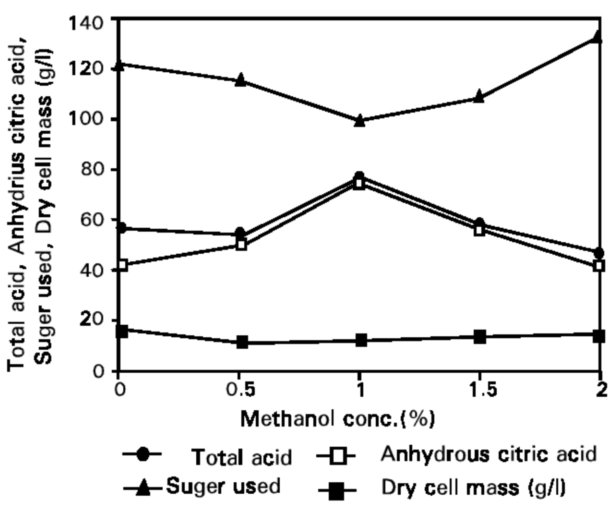


Fig.2: Effect of different concentration of methanol on the production of citric acid by *Aspergillus niger* GCB-47 in stirred fermentor

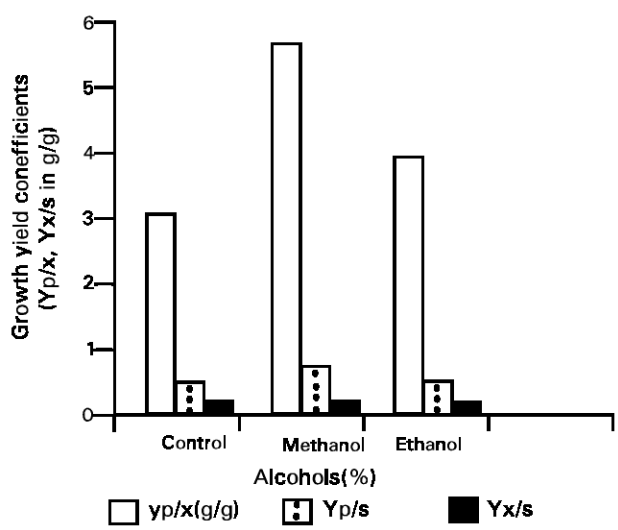


Fig. 4: Comparison of growth yield coefficients (Yp/s,Yx/s in g/g) for citric acid production by *Aspergillus niger* GCB-47 in stirred fermentor

morphology. The dry cell mass was 14.5 g/l. Any change in ethanol to methanol ratio resulted in the decreased secretion of citric acid from the mycelial cells. From the data it is clear that the partial replacement of ethanol to methanol concentration was not found economically feasible due to lower yields of citric acid and higher rate of energy consumption.

**Kinetic parameters**: The comparison of growth yield coefficients (Yp/x, Yp/s and Yx/s in g/g) for citric acid production by *Aspergillus niger* GCB-47 in stirred fermentor was studied (Fig. 4). The maximum value of Yp/x (i.e., 5.526 g/g) was observed when methanol (1%, v/v) was used as a stimulating agent, 24 hours after spore inoculation. This value is 1.86 folds higher than the control (without alcohol). The kinetic studies also revealed that ethanol also enhanced the acid production (Yp/x = 3.84 g/g) but to a less extent as compared to methanol addition. The ratio between citric acid production and substrate utilization by methanol (0.492 g/g) is much improved with respect to ethanol and control. The very low value of Yx/s (0.183 g/g) in case of methanol provoked the better excretion of citric acid in the fermented broth.

## Discussion

One of the main problems for achieving bioreactor performance in stable conditions with filamentous fungi involves limiting hyphal growth as well as avoiding diffusional restrictions. Different alcohols have stimulatory effect on the biosynthesis of citric acid in the fermented broth (Roukas and Kotzekidou, 1987). The effect of methanol and ethanol on citric acid production by *Aspergillus niger* GCB-47 is in the excretion of citric acid more efficiently. It may be due to that methanol increases the permeability of cell membrane, resulting in better excretion of citric acid from the mycelial cells. Similar kind of work has also been reported by Pazouki *et al.* (2000). The effect of methanol in increasing citric acid yield appears to be a general phenomenon in strains of *Aspergillus niger* (Saha *et al.*, 1999).

Methanol at 1.0% concentration was found to give maximum excretion of citric acid in this study. Methanol markedly depressed the synthesis of the cell protein in the early stages of the cultivation and also increased the metabolic activity of enzyme. From these facts it was concluded that methanol induced enzyme activities to become suitable for citric acid fermentation in the early stage of the cultivation. When the level of methanol concentration was further increased, it results in decreased production of citric acid. This is because the higher methanol concentration in the medium, disturbs the fungal metabolism, resulting in decreased citric acid production. Similar type of work has also been carried out by Hang and Woodams (1986) and Haq *et al.* (1995). Addition of methanol after 24 hours or later has not been found to be beneficial. Earlier it was suggested that methanol increases the tolerance of fungi to trace elements such as $Fe^{+2}$, $Mn^{+2}$ $Zn^{+2}$, etc. (Tauro, 1977). It is likely that methanol also affects the permeability properties and enable greater excretion of citric acid in the fermentation medium. The improvement of citric acid production due to methanol addition can be explained in terms of pellet size control by means of air or oxygen. The proper control on pellet size increased the active surface of mycelia and allowed a better oxygen availability, which is a critical point in citrate synthesis during the secondary metabolism of *Aspergillus niger*, but also bed compaction and consequently, improving mass transfer limitations and substrate conversion which ended in an increased production (Moreira *et al.*, 1996).

**Acknowledgments**: Authors acknowledge Pakistan Science Foundation (PSF) for financial support, for the research work.

## References

Hang, Y. D. and E. E. Woodams, 1986. Utilization of grape pomace for citric acid production by solid state fermentation. A. M. J. Enol. Vitric., 37:141-142.

Haq, I., M. H. Ashraf, S. Malik and M. A. Qadeer, 1995. Production of citric acid of *Aspergillus niger* by solid state fermentation Biologia, 41: 1-10.

Majolli, M. V. I. and S. N. Agirre, 1999. Effect of trace metals on the cell morphology, enzymic activity and citric acid production in a strain of *Aspergillus wentii*. Rev. Argent. Microbiol., 31: 65-71.

Maria, K. and L. Wladyslaw, 1989. Effects of medium purity on submerged citric acid fermentation yield. Acta Aliment., 15: 97-105.

Moreira, M.T., A. Sanroman, G. Feijoo and J. M. Lema, 1996. Control of pellet morphology of filamentous fungi in fluidized bed bioreactors by means of a pulsing flow: Application to *Aspergillus niger* and Phanerochaete chrysosporium. Enzyme and Microbial Technol., 19: 261-266.

Pazouki, M., P. A. Felse, J. Sinkha and T. Panda, 2000. Comparative studies on citric acid production by *Aspergillus niger* and *Candida lipolytica* using molasses and glucose. Bioprocess Eng., 22: 353-361.

Roukas, T. and P. Kotzekidou, 1987. Influence of some trace metals and stimulants on citric acid production from brewery waste by *Aspergillus niger*. Enzyme Microbiol. Technol., 9: 291-294.

Saha, M. L., Y. Sakai and F. Takahashi, 1999. Citric acid fermentation by magnetic drum contactor: use of methanol and ethanol for higher production. J. Biosci. Bioeng., 87: 369-394.

Sanjay, K. and P. Sharma, 1994. A highly performance fermentation process for production of citric acid from sugar-cane molasses. J. Microbiol., 23: 211-217.

Srivasta, K. and S. Kamal, 1979. Citric acid fermentation by *Aspergillus niger*. Ind. J. Microbiol., 19: 145-149.

Tasun, K., P. Chose and K. Ghen, 1970. Sugar determination of DNS method. Biotech Bioeng., 12: 921.

Tauro, P., 1977. Unpublished data. Haryana Agric. Univ. Hissor, India.