Pak. J. Sci. Ind. Res. 2008 **51**(5) 245-249

Metabolic Inhibitors as Stimulating Factors for Citric Acid Production

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(received July 21, 2007; revised July 3, 2008; accepted July 10, 2008)

Abstract. The effect of some metabolic inhibitors on citric acid (CA) production by *Aspergillus niger* in cane molasses medium was investigated. Addition of 0.01-0.1 mM iodoacetic acid and sodium arsenate, 0.05-1.0 mM sodium malonate, 0.01 mM sodium azide, 0.01-0.05 mM sodium fluoride, 0.1-1.0 mM EDTA stimulated CA production (5-49%). Higher concentrations (10 mM) of iodoacetic acid, sodium malonate and 0.5 mM sodium azide caused a complete inhibition of fungal growth. Iodoacetic acid, sodium arsenate and sodium fluoride (0.2 mM) caused a remarkable inhibition of CA production. The implications of those preliminary functions was discussed.

Keywords: citric acid, cane molasses, surface culture, metabolic inhibitor, *Aspergillus niger*

Introduction

Many strains of *Aspergillus niger* are well known for their capacity to produce citric acid under suitable conditions. By careful selection of strains and improving conditions, 80 to 85% of initial sugar substrate can be converted into citric acid (Kiel *et al*., 1981). Citric acid (CA) is a chemical commodity, widely used in different industries. Inexpensive and readily available raw materials remain in demand in industrial processes. Molasses is a desirable raw material for citric acid fermentation due to its availability and relatively low price (Lotfy *et al*., 2007; Haq *et al*., 2001; Pazouki *et al*., 2000).

Many investigators have tried to improve the production of CA by various additives. Moyer (1953) found that methanol, ethanol and isopropanol decreased growth but increased CA production from cane and beet molasses. Since that time, a lot of work had been done to study the effect of alcohol on citric acid fermentation (Lotfy *et al*., 2007; Roukas, 1999; El-Batal *et al*., 1995; Maddox *et al*., 1986; Szczodark and Ilczuk, 1975; Hamissa, 1966).

Millis *et al*. (1963) increased CA yield by about 20-50% when some natural oils with a high content of unsaturated fatty acids were added. Also, supplementation of surface culture with some oil increases CA yield, using molasses medium (Adham, 2002). Moreover, Barrington and Kim (2008) showed statistically that olive oil has significant positive effect on citric acid production.

Specific inhibitors such as fluoroacetate and iodoacetate, are particularly useful (Peters, 1957; Racher and Krimsky, 1952). Addition of some metabolic inhibitor to synthetic medium stimulated citric acid production (Agrawal *et al*., 1983). Ali and Haq (2005) discussed the role of different additives and metabolic microminerals on the enhancement of citric acid production by *A. niger* using different carbohydrate materials. They found that both ethanol and coconut oil in 3.0% (v/w) concentration increased citric acid production. Fluoracetate at a concentration of 1.0 mg/ml bagass increased the yield of citric acid significantly. Also, addition of copper sulphate and molybdenum sulphate remarkably enhanced the production of citric acid using molasses medium.

The present study was undertaken mainly to determine the effect of some metabolic inhibitors on citric acid production by *A. niger* in cane molasses medium.

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Materials and Methods

Microorganisms and culture conditions. Strains of *A. niger* Van Tieghem 595, 599 were provided by the Centre of Culture Collection of Northern Regional Research Laboratory (NRRL), USA. *A. niger* A10 and A20 were provided by the Center of Culture Collection National Research Center (NRC), Egypt. *A. niger* EMCC III, EMCC103 and EMCC 147 were obtained from the Cairo Mercen (CAIM), Egypt.

The slants of *A. niger* were incubated in potato dextrose agar (PDA) at 30 °C for 7 days. Inoculum was prepared from spore suspension (10^5-10^6 spore/ml) in 0.01% v/v tween 80.

Cane molasses. The cane molasses samples used in the present study were kindly supplied by the cane sugar factory of Egypt.

Citric acid fermentation. Fermentation media were prepared by diluting cane molasses (CM) with tap water to approximately 15% sugar concentration. Preparation of molasses was undertaken according to Mohamed and Adham (2003). Stationary cultures were grown on cane molasses media containing different concentrations of the inhibitors and incubated in slanting position (surface fermentation) at 30 °C

for 15 days. Aliquots of the fermentation medium were withdrawn on 4th, 8th and 12th day then analyzed for the total titratable acidity and citric acid content. Maximum citric acid content was reached on the 12th day of incubation.

Analytical techniques. Citric acid was photometerically determined at 420 nm as described by Lowenstein (1969). Citric acid content was calculated as mg/ml sample with reference to the standard solution. Total titratable acidity was estimated by titrating 1 ml aliquots of the fermentation media against 0.1 M of NaOH solution and calculated as anhydrous citric acid; 1 ml of 0.1 M NaOH is equivalent to 6.4 mg anhydrous citric acid.

Growth was measured in terms of grams of dry weight of mycelium per flask. At the end of the incubation period, on the 15th day, mycelial pads were separated, washed and dried at 60 °C for 24 h.

Results and Discussion

Seven different strains of *A. niger* were screened for citric acid production (*A. niger* NRRL 599 and 595; *A. niger* EMCCIII, EMCC102 and EMCC147; *A. niger* A10 and 20) using cane molasses. *A. niger* NRRL 599 exhibited the highest production capacity and was selected for this study to get a preliminary idea of the metabolic reactions involved in the accumulation of citric acid in cane molasses medium.

It should be reported that addition of 0.2 mM iodoacetate inhibited fungal growth, total titrable acidity and citric acid production, while 1.0 mM concentration strongly inhibited citric acid production and total titrable acidity by 85 and 67%, respectively (Table 1). On the other hand, presence of 10 mM iodoacetic acid completely inhibited fungal growth. Lower concentrations (0.01 and 0.1 mM), however, stimulated citric acid production by 34 and 41%, respectively, without markedly affecting fungal growth and titratable acids.

Agrawal *et al*. (1983) demonstrated that addition of 0.001 to 0.1 mM iodoacetate to stationary cultures of *A. niger* grown on a synthetic medium stimulated citric acid production but not total titratable acids and noted that the reason for the enhancement of citric acid production at lower concentration of iodoacetate is not clear. Iodoacetate has been reported to be a rather specific inhibitor of glyceraldehyde-3-phosphate dehydrogenase, especially at concentration ≤ 1 mM. At higher concentrations, other enzymes with sulphydryl groups at the active sites are also affected (Webb, 1966). Hence, it is likely that the interruption of the glycolytic cycle due to iodoacetate inhibition might be responsible for inhibition of fungal growth and consequently, of citiric acid production (Agrawal *et al*., 1983).

Addition of sodium malonate (Table 2) to the fermentation medium inhibited the mycelial growth which in turn was completely inhibited at 10 mM concentration and stimulated citric acid production (up to 27%) without affecting the total titratable acidity. Berk *et al*. (1957) demonstrated that *A. niger* possessed the ability to metabolize malonate. It is thus possible that at low concentrations, malonate is completely metabolized during the early period of fungal growth without adversely affecting citric acid production. At these levels precisely, malonate has been shown to inhibit succinate dehydrogenase, specifically in case of *A. niger* (Tissieres, 1951). Because of this interruption in the tricarboxylic acid cycle, further metabolism of the produced citric acid is probably reduced, thereby leading to an increase in citric acid accumulation in the medium (Agrawal *et al*., 1983). Barron and Ghiretti (1953) reported 73% inhibition of citric acid accumulation by yeast upon addition of high concentration of malonate. This could be due to depression of succinate oxidation and reduction in the rate of acetyl CoA entry into the tricarboxylic acid cycle (Webb, 1966).

Table 1. Effect of iodoacetic acid on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0	3.63
0.01	120.96	60.50	+33.73	3.23
0.10	119.04	63.80	+41.02	3.76
0.20	109.90	39.00	-13.80	2.90
0.50	80.12	15.90	-64.85	2.50
1.00	38.40	7.00	-84.52	2.32
10.0	0.0	0.0	-100.0	no growth

* = control; change in citric acid (CA) content (%) =

$$\frac{\text{CA in cotrol (mg)} - \text{CA in treated sample (mg)}}{\text{CA in control (mg)}} \times 100$$

Table 2. Effect of sodium malonate on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.30	45.24	0.00	3.63
0.05	102.99	53.58	+18.43	3.37
0.10	116.73	57.67	+27.47	3.20
1.00	117.00	47.70	+5.43	3.05
10.0	0.00	0.00	-100.0	no growth

* = control

The presence of 0.5 mM sodium azide (Table 3) in the molasses medium completely inhibited fungal growth. On the other hand, 0.1 mM, slightly inhibited citric acid production but inhibited total titratable acidity significantly. The least concentration of sodium azide (0.01 mM) slightly stimulated citric acid production (16%) and inhibited total titratable acid (11%) and dry weight (17%). It is noticeable that the citric acid secretion by *A. niger* is not always parallel to the biomass formation (Franz *et al*., 1993). Tissieres (1951) and Case and Mcllwain (1951) observed inhibitory effects of sodium azide at 1.0 M concentration. This may be attributed to the inhibitory effect of sodium azide on oxidative phosphorylation.

Table 3. Effect of sodium azide on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	104.4	52.68	+16.41	3.01
0.10	68.43	44.77	-1.04	3.22
0.50	0.00	0.00	-100.0	no growth
1.00	0.00	0.00	-100.0	no growth

* = control

Lower levels of sodium arsenate (0.01-0.1 mM) stimulated the production of the acid up to 32%. On the other hand higher levels (0.2-1.0 mM) inhibited citric acid production, fungal growth and the total titratable acidity markedly (Table 4). This inhibition may be due to the fact that sodium arsenate is an uncoupler of substrate-linked phosphorylation– for example, during the oxidation of D-glyceraldehyde-3-phosphate and alpha-ketoglutarate– leading to decreased energy production in the cell and, hence, decreased growth (Sanadi *et al*., 1954; Crane and Lipman, 1953). Glutarate-semialdehyde dehydrogenase (EC 1.2.1.20) is more sensitive to sodium arsenate; this could explain the stimulation of citric acid accumulation in the medium at lower concentration levels of sodium arsenate (Agrawal *et al*., 1983).

Addition of sodium fluoride to the molasses significantly stimulated citric acid production at 0.01 mM (49%) but was inhibitory at concentrations ≥ 0.2 mM (16-58%) (Table 5). It should be noted that the same concentration also suppressed the total titratable acidity. However, fungal growth was stimulated up to the concentration of 0.5 mM. At the highest concentration of 10 mM, there was marked inhibition of fungal growth, total titratable acidity and citric acid production (56, 61 and 58%, respectively). Agrawal *et al*. (1983) recorded 100% fungal inhibition in the presence of 10 mM sodium fluoride. This inhibition has been proposed to be due to the fluoride ions and their ability to form complexes with several metalloenzyme system.

Table 4. Effect of sodium arsenate on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	112.35	59.79	+32.16	3.50
0.02	128.61	58.74	+29.84	3.92
0.05	87.90	52.89	+16.90	3.79
0.10	71.40	50.58	+11.80	3.65
0.20	68.70	28.21	-37.64	2.90
0.50	66.30	18.84	-58.35	2.50
1.00	66.90	12.00	-73.47	2.15

* = control

Table 5. Effect of sodium fluoride on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	127.2	67.38	+48.93	3.87
0.05	109.2	63.37	+40.07	4.00
0.20	76.50	30.0	-33.68	3.77
0.50	94.86	37.8	-16.44	3.80
1.00	61.20	23.85	-47.28	2.19
10.00	45.90	18.75	-58.55	1.60

* = control

Addition of (0.1, 1.0 mM) of the sodium salt of ethylene diaminetetracetic acid (EDTA) stimulated citric acid production, dry weight and total titratable acidity (Table 6). We have came to a conclusion that any increase in the fungal growth may lead to parallel increase in the total titratable acids but at higher concentration (10 mM) inspite of the increase in the fungal growth (15%) there is considerable inhibition in the production of the total titratable acids. Since more of the glycolytic and tricarboxylic acid cycle enzymes are dependent on metal ions (particularly Mg^{2+}) for their activity, it is likely that at higher concentration (10 mM) EDTA chelate certain metals ions essential for the activity of enzymes (directly or indirectly) related to the synthesis and accumulation of citric acid in the medium. Compared with other fungi, *A. niger* can be affected, to a greater extent, by the presence of metal ions in the media (Franz *et al*., 1993).

Table 6. Effect of EDTA sodium salt on mycelial growth and citric acid production

Inhibitor concentration (mM)	Maximum total titratable acids (mg /ml)	Maximum citric acid content (mg/ml)	Change in citric acid content (%)	Dry weight after 15 day growth (g)
0.00*	117.3	45.24	0.00	3.63
0.01	90.71	39.00	-13.79	3.50
0.10	133.45	48.00	+6.2	3.78
1.00	173.47	56.00	+23.78	3.75
10.0	96.77	38.00	-16.00	4.16

* = control

As discussed above, most of the studied inhibitors caused inhibition of growth at concentrations, 1 to 10 mM, with the exception of EDTA. It is likely that some of these inhibitors may affect the growth of the fungus and citric acid production indirectly. Stimulation of fungal growth using different concentration of EDTA, may be due to the removal of excess mineral impurities from molasses by EDTA yielding more suitable molasses for fungal growth and citric acid production. However, 0.2 to 0.5 mM sodium fluoride could inhibit citric acid production slightly without adversely affecting the fungal growth. Also, there appeared to be a poor correlation between the inhibitory effects on total titratable acidity and on citric acid production, total titratable acidity being more or less susceptible to the inhibition.

In addition to citric acid, oxalic acid is a major contributor to total titratable acidity. At lower levels, most of the studied inhibitors stimulated citric acid accumulation in the medium, but not the production of total titratable acidity, thus increasing the proportion of citric acid among other organic acids formed. Perhaps lower levels of these inhibitors suppress the activity of enzyme more closely associated with the synthesis of other organic acids, rather than activity of those concerned with citric acid formation and accumulation in the medium. These findings with the great viability of cane molasses could have important implications in industrial processes.

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