Metabolic inhibitors as stimulating factors for citric acid production

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Abstract

The effect of some metabolic inhibitors on citric acid production by Aspergillus niger in cane molasses medium was investigated. Addition of 0.01-0.1 iodoacetic acid and sodium arsenate, 0.05 -1.0 sodium malonate, 0.01 sodium azide , 0.01-0.05 sodium fluoride , 0.1-1.0 EDTA stimulated citric acid production (5-49%), but not necessary total titratable acids. Higher concentrations 10mM of iodoacetic acid , sodium malonate and 0.5 mM sodium azide caused a complete inhibition of fungal growth. 0.2 mM of iodoacetic acid, sodium arsenate and sodium fluoride caused a remarkable inhibition of citric acid production. The implications of those preliminary functions was discussed.

INTRODUCTION

Many strains of Aspergillus niger are well known for their capacity to produce citric acid under suitable conditions. By carefully selection strains and improving conditions, 80 to 85 % of the weight of the initial sugar substrate can be converted into citric acid (Kiel et al., 1981). Citric acid is a commodity chemical, so it is necessary to use inexpensive and readily available raw materials in industrial process molasses is a desirable raw material for citric acid fermentation because its availability and relatively low price (Pazouki et al., 2000; Haq et al., 2002)

Many investigators have tried to improve the production of CA by various additives. Moyer (1953) found that methanol, ethanol and isopropanol decreased with parallel increase in CA production from cane and beet molasses media. Since that time , a lot of work had beeen done on the efectg of alcohol on citric acid fermentation (Hamissa, 1966; Szczodark and Ilczuk, 1975; Maddox et al., 1986; El-Batal et al., 1995 and Roukas, 1999)

Millis et al. (1963) increased CA yield by about 20-50% by the addition of some natural oils with a high content of unsaturated fatty acids, also supplementation of a surface culture with some oil makes a direct increase in CA yield possible in using molasses medium (Adham, 2002).

Specific inhibitors such as fluoroacetate and iodoacetattors, are particularly useful (Peter, 1957; Packer and Krimsky, 1952).

Addition of some metabolic inhibitor to synthetic medium stimulated citric acid production (Agrwal et al., 1983).

Ali and Haq, 2005 discussed the role of different additives and metabolic micro minerals on the enhanced citric acid production by Aspergillus niger using different carbohydrate materials. They found that ethanol and cocnut oil at 3.0 5(v/w) increased citric acid productivity fluoroacetate at a concentration of 1.0 mg/ml bagass enhanced theyield of citric acid significantly. Also, addition of copper sulphate and molybdenum sulphate remarkably enhanced the production of citric using molasses medium.

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

MATERIALS AND METHODS

MICROORGANISMS AND CULTURE CONDITIONS

A strains of Aspergillus niger Van Tieghem 595, 599 were kindly provided by the culture collection of Northern Regional Research Laboratory (NRRL), USA. Aspergillus niger A10 , A20 were kindely provided by the center of culture collection National Research Center (NRC), Egypt. Aspergillus niger EMCC III, EMCC103, EMCC 147 were obtained from the Cairo Mercen (CAIM), Egypt.

The slants (PDA) of A.niger were incubated at 30 C for 7

days. Inoculum prepared form 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, Egypt.

CITRIC ACID FERMENTATION.

Fermination medium were prepared by diluting cane molasses (CM) with tap water to approximately 15 % sugar concentration, preparation of molasses have been reported previously (Mohamed and Adham , 2003). Stationary cultures were grown on cane molasses media containing different concentration of the inhibitors and incubated in slating position (surface fermentation) at 30 C for 15 days.

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

ANALYTICAL TECHNIQUES

Citric acid was determinable colorimetric method of Lowenstein (1969). Total titratable acidity was estimated by titrating 1 ml aliquots with 0.1 M sodium hydroxide solution.

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

RESULTS AND DISCUSSION

Seven different strains of Aspergillus niger were screened for their potency in citric acid production, A. niger NRRL 599, 595, A. niger EMCCIII, EMCC102, EMCC147; A. niger A10, A20 using cane molasses. A. niger NRRL 599 exhibit high producing capacity and it was selected for this study which undertaken to get a preliminary idea of the metabolic reactions involved in the accumulation of citric acid in cane molasses medium.

Addition of 0.2 mM iodoacetate inhibited fungal growth, total titrable acidity and citric acid production. 1.0 mM strong inhibited citric acid production and total titrable acidity by 85 and 67 % respectively (Table 1). The presence of 10 mM iodoacetic acid completely inhibited fungal growth. Lower concentrations (0.01 and 0.1 mM), however, markedly stimulated citric acid production by 34 and 41 % respectively without markedly affecting fungal growth and titratable acids.

Figure 1

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

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

Addition sodium malonate (Table 2) to the fermentation medium inhibited the mycelial growth which was completely inhibited at 10 mM concentration but stimulated citric acid production (up to 27%) without affecting the total titrable acidity, Berk et al, have demonstrated that Aspergillus niger process the ability to metabolize malonate. It is thus possible that low concentrations added malonate are completely metabolized during the early period of fungal growth without adversely affecting citric acid production. It is precisely at these levels that malonate has been shown to inhibit succinate dehydrogenase, specifically in A. niger (Shimi et al., 1962) because of this interruption in the tricarboxylic acid cycle, further metabolism of citric acid already formed is probably reduced , thereby leadfing to increased citric acid accumulation in the medium (Agrawal et al., 1983). Barron and Ghiretti, 1953 have reported 73% inhibition of citric acid accumulation by yeasts upon addition of high concentration of malonate this could arise due to depression of succinate oxidation and reduction in the

rate of acetyl CoA entry into the tricarboxylic acid cycle (Webb, 1966).

Figure 2

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

The presence of 0.5 mM sodium azide (Table 3) in the molasses medium completely inhibited fungal growth, 0.1 mM, slight inhibit citric acid production but inhibited total titratabe acidity significantly, lowest concentration (0.01 mM) slightly stimulated citric acid production (16%) and inhibited total titrable acid (11%). Several workers (Tissieres, 1951; Case and McIlwain, 1951) has been observed the inhibition effects of sodium azide at 1 m concentration and this possibly be explained to the inhibitory effects of sodium azide on oxidative phosphorylation.

Figure 3

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	-	3.63
0.01	104.4	52.68	+16.41	3.01
0.10	68.43	44.77	-1.020	3.22
0.50	0.00	0.00	-100.0	no growth
1.00	0.00	0.00	0.00	no growth

Lower levels of sodium arsenate (0.01 to 0.1mM) stimulated the production of the acid up to 32% on the other hand higher levels 0.2-1.0 of arsenate inhibited the citric acid production. Fungal growth and the total titrable 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-glyceraldhyde -3-phosphate and alpha –ketoglutarate, thus leading to a decreased rate of energy production in the cell and , hence, decreased growth [Crane and Lipman, 1953; Sanadi et al., 1954]. If glutarate –semialdhyde dehydrogenase (EC 1.2.1.20) is more sensitive to sodium arsentare, this could explain the stimulation of citric acid accumulation in the medium at lower levels of sodium arsenate (Agrawal et al., 1983).

Figure 4

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

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%), the same concentration also suppressed the total titratable acidity. However, fungal growth was stimulated up to a concentration of 0.5 mM. At highest concentrations 10 mM, there was a markable inhibition of fungal growth total titratable acidity and citric acid production (56, 61 and 58%, respectively).

Agrawal et al., 1983 had recorded 100% fungal inhibition in the presence of 10mM sodium fluoride . This inhibition has been proposed to be due to the fluoride ions and their ability to form complexes with several of the metalloenzyme system.

Figure 5

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	370.8	-16.44	3.80
1.00	61.20	23.85	-47.28	2.19
10.00	45.90	18.75	-58.55	1.60

Addition of (0.1, 1.0mM) the sodium salt of ethylene diaminetetracetic acid (EDTA) stimulated citric acid production dry weight and total titrable acidity. We have came to conclusion that it may be increase in the fungal growth may lead to parallel increase in the total titrable acids but at higher concentration (10mM) inspite of the increase in the fungal growth to by 15% there are a considerable inhibition for the total titratable acids. Since more of the glycolytic and tricarboxylic acid cycle enzymes are

dependent on a metal ion, particularly Mg^{2+} for their activity it is likely that at higher concentration (10mM) EDTA is involved in the formation of a metal ion chelate with certain metals essential for the activity of enzymes (direct or indirectly) related to the synthesis and accumulation of citric acid in the medium.

Figure 6

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	-	3.63
0.01	90.71	39.00	-13.79	3.50
0.10	133.45	48.00	+33.33	3.78
1.00	173.47	56.00	+23.78	3.75
10.0	96.77	38.00	-16.00	4.16

As discussed above, most of the inhibitors studied caused a inhibition of growth when present at concentrations in the range 1 to 10mM, with the exception of EDTA. It is likely that the effect of some of these inhibitors may be on growth of the fungus, affect citric acid production indirectly. Stimulation fungal growth by using different concentration of EDTA, this stimulation may be due to the fact that EDTA act as remove excess mineral impurities from molasses and has resulted in molasses more suitable 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 only a poor correlation between the inhibitory effects on total titratable acidity and on citric acid production. Total titratable acidity 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 inhibitors studied stimulated citric acid accumulation in the medium, but not the production of total titratable acidity, thus increasing the proportion of citric acid to other organic acids formed. Perhaps lower levels pf these inhibitors suppress the activity of enzyme more closely associated with the synthesis of other organic acids, ruther 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.

References

r-0. Adham, N.Z. (2002) Attempts at improving citric acid fermentation by Aspergillus niger in beet molasses medium. Bioresources Technology 84:97-100.
r-1. Agrawal, P.K., Bhatt, C. S. and Viswanathan, L. (1983) Effect of some metabolic inhibitors on citric acid production by Aspergillus niger. Enzyme Microb. Techol. 5: 373-376.
r-2. Ali, S. and Haq, I.M. (2005) Role of different additives and metabolic micro minerals on the enhanced citric acid production by Aspergillus niger NNNG115 using different carbohydrate materials. J. Basic Micobiol. 45(1) :3-11.
r-3. Barron, E.S. G. and Ghiretti, F. (1953) The pathway of acetate oxidation. Biochem. Biophys. Acta. 12: 239-249.
r-4. Case, E. M. and McILwain, H. (1951). Respiration and phosphorylation in preparation from mammalian brain. Biochem. J. 48:1-11.
r-5. Crane, R.K. and Lipman, F.J. (1953). The effect of arsenate on aerobic phosphorylation. J. Biol. Chem. 201:235-243.
r-6. El-Batal, A.I., Khalil, A.H. , Mostafa, M.M. (1995). Citric acid production by gamma irradiated Aspergillus niger from treated beet molasses under different fermentation conditions. Egypt J. Rad. Sci. Appl. 8(2): 253-266.
r-7. Hamissa, F.A. (1966). Chemical studies on citric acid fermentation. M.Sc. Thesis, Faculty of Science, Cairo University, Egypt.
r-8. Haq, I., khurshid, S., Ali, S. Ashraf, H., Qadeer, M.A. Rajaka, M.I. (2002) Mutation of Aspergillus niger for enhanced citric acid production by blacks strap molasses. World J. Microbiol. Biotechnol; 17: 35-37.
r-9. Kiel, H, Guvrin, R. and Henis, Y. (1981) Citric acid fermentation by Apsergillus niger on low sugar concentrations and cotton waste. Applied Environ Microbiol. 42(1):1-4.
r-10. Lowenstein, J.M. (1969) Chemical methods for citric acid and aconitate. Methods Enzymol., 13: 513.
r-11. Maddox, I. S., Hossain, M. and Brooks, J. D. (1986) The effect of methanol on citric acid production from galactose by A. niger Appl. Microbiol. Biotechnol. 23: 203-205.
r-12. Millis, N.F., Trumpy, B.H., Palmer, B.M., (1963). The effect of lipids on citric acid production by an Aspergillus niger mutant. J. Gen. Microbiol. 30: 365-379.
r-13. Mohamed, K.A. and Adham, N.Z. (2003) Formations of autobiploid Aspergillus niger strains by collagen calcium treatment and their application to citric acid production from beet molasses, Egyptian Journal of Biomedical Science vol. 12:184-198.
r-14. Moyer, A.J. (1953). Effect of alcohols on the mycological production of citric acid in surface and submerged culture. I. the nature of the alcohol effects. Appl. Microbiol. 11: 1-7.
r-15. Pazouki, M., Felse, P.A., Sinha, J. and Panda, T, (2000). Comparative studies on citric aicd production by Aspergillus niger and Candida lipolytica using molasses and glucose. Bioprocess Engineering. 22(4): 353-361.
r-16. Peters, R. A. (1957). Mechanism of the toxicity of the active constituent of Dichapetalum cymosum and related compounds. Adv. Enzymol. 18: 113-157.
r-17. Racher, E. and Krimsky, I, (1952). The mechanisms of oxidation of aldehydes by Glyceraldehydes 3-phosphate dehydrocenenase. J. Biol. Chem. 198:731-743.
r-18. Roukas, T. (1999) Citric acid production from pod by solid state fermentation . Enzyme Microb. Technol. 24: 54-59.
r-19. Sanadi, D. R., Gibson, D.M., Ayengar, P. and Ouellet, L. (1954). Intermediates in the phosphorylation coupled to -ketoglutarate oxidation. Biochem. Biophys. Acta. 13:146-148.
r-20. Shimi, I. R. and El-Dein, M.S.N. (1962). A study of the cytochrome system and some other aspects of the respiration of Aerobacter aerogenes. Arch. Mikrobiol. 41:261-267.

r-21. Szczodark, J., Ilczuk, Z. (1975) Effect of alcohol on the synthesis of citric acid by the fungus Aspergillus niger. Przem Ferment. Rolny. 19(6), 20-21.
r-22. Tissieres, A. (1951). A study of the cytochrome system and some other aspects of the respiration of Aerobacter aerogenes . Biochem. J. 50:279-288.
r-23. Webb, J. L. (1966) Enzyme and metabolic inhibitors, Academic Press, New York Vol. 2

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