Inhibition of Citric Acid Accumulation by Zn^{2+}, Mo^{6+} and V^{5+} in *Aspergillus Niger* Mutant by Control of Aconitase Activity

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ABSTRACT

Aconitase activity of a mutant *Aspergillus niger* AB_{1801} was studied in presence of different amount of Zn^{2+},Mo^{6+} and V^{5+} citrate accumulation was seriously impended by Zn^{2+},Mo^{6+} and V^{5+}. They hundred growth of *A. niger* mutant without lowering aconitase activity.
[Key works : Aconitase, mutant, *Aspergillus niger*, citrate]

INTRODUCTION

Living organism store and transport transition metals both to provide appropriate concentrations of them for use in metalloproteins or cofactors and to protect themselves against to toxic effects of metal excesses. The normal concentration range for each metal in biological systems is narrow, with both deficiencies and excesses causing pathological changes. Zinc is relatively abundant in biological materials. Among the well known characterized Zn proteins are a variety of hydrolases involved in metabolism of sugars, proteins and nueleic acids [1]. Molybdenum proteins catalyse the reduction of nitrogen and nitrate as well as the oxidation of aldehydes, pweines and sulfites [2]. Vanadium is present in small amounts in most organisms. V^{5+} could act in vitro as phosphate analogue. In proteins, vanadium is a cofactor in algal bromoperoxidase and in certain prokaryotic nitrogenases [3]. Manganese plays a critical role in oxygen evolution catalyzed by the proteins of photosynthetic reaction center superoxide dismutase of bacteria and mitochondria are also Mn proteins.

Increasing the yield of citric acid, the organic acid of manifold usage may be done by enhancing the multifarious potentiality of the strain and / or alteration of raw material composition conducted to set optimal conditions. Presently, the influence of various metal ions supposedly in the industrial raw material (viz. molasses) or in the steel fermentor have been investigated with Ni^{2+}(10 µg/ml), Co^{2+}(10 µg/ml) and B^{3+}(0.05 gm/l) have stimulated fermentation by this mutant [4]. The role of other trace elements have been assessed.

History of ions in citrate synthesis reveals that $ZnSO_4$, $CuSO_4$, $MnSO_4$ and $CaCO_3$ have favored citric acid fermentation in anticipation of mimicking the events [5-7]. These ions were individually added to the medium along with Mo^{6+} and V^{5+} Nicolas and Fielding (1950) recorded 57 mg increase in mycelia output of *A. niger* when only 0.1 ng Mo was added to a 50 ml purified medium [8]. Work on relationship between vanadium and *A. niger* has been carried out by steinberg (1939) as both V^{5+} and Mo^{6+} are considered as botanical micronutrients [9].

The key point of citric acid accumulation and thus is supposedhy controlled by aconitase as it s the enzyme catalyzing that reaction step which is responsible for carrying the cycle forward. Hence the alteration in status of this enzyme upon ion addition has been quantitiated.

MATERIALS AND METHODS

Microorganism : The parent strain of *Aspergillus niger* ABX_1 was isolated from North Bengal soil, exposed to ultra violet rays from 12 cm distance for different periods from Hanovia germicidal lamp (15 watt). Sterile spore suspension (2.6 X 10^7 spores/ml) of the maximally producing strain was diluted to 1 : 3000, 1 : 5000 and 1 : 7000 by adding ethyleineimine. After one hour intervals, the spores were plated in CD agar medium. After sporulation, the colonies were transferred to malt extract and yeast extract slants for storage at 4^oC [4].

Preparation of inoculum : The spores were harvested, suspensions were prepared in 10 ml sterile triple distilled water and standardized to a concentration of 2.6 X 10^7 spores/ml. 5 ml of each such suspension was used as inoculum [4].

Fermentation medium and cultural conditions : 150 ml chemically defined medium (pH 3.0) was composed of the following : sucrose, 10%; urea, 0.2%; KH_2PO_4, 0.15%; $MgSO_4.7H_2O$, 0.01%. Each made free of other chemical impurities by chloroform extraction procedure. The required amounts of aqueous solution of each was mixed well with 0.1 gm of 8 hydroxyquinoline in chloroform in a separating funnel, first at pH 5.2, then at pH 7.2. The chloroform dissolved impurities were extracted out and the process repeated thrice. The solutions were made chloroform free by heating in a water bath [10].

Surface culture fermentations were then carried out in IL Roux bottle with 150 ml fermentation medium, at pH 3.0 for 8 days at $28^oC – 30^oC$.

Preparation of enzyme extracts : The mycelia mat was neutralized with KOH at pH 7.2 to 7.4 ground in borate buffer (0.1 N), the extract centrifuged at 500 g for 10 min and again at 21,600 g for 15 min. The supernatant was stirred with 1% protamine sulfate before centrifuging at 4,600 g for 10 min. the supernatant was used for aconitase assay. All steps of the experiment were carried out on ice [11].

Aconitase assay : The assay was carried out in Hitachi U2000 double beam spectrometer with the temperature set at 30^oC. The activity of the enzyme was determined by measuring the rate of appearance of cis-aconitase from Na-citrate. To a cuvatte were added : 0.3 ml Na-citrate (0.2 M) in 0.05 M potassium phosphate buffer (pH 7.4); enzyme and water to 3 ml. The increase in extinction was measured at 1 ml interval for 5 min. One unit of enzyme activity was defined as the initial rate of increase in A_{240} by 0.01/min/ml enzyme extract [12].

Citric acid assay : The citric assayed in the broth colorimetrically at 420 nm using acetic anhydride and pyridine. Citric acid was assayed from the mycelia similarly, using the supernatant of the extract prepared by centrifuging at 500g for 10 min [13].

Estimation of dry cell weight : Dry cell weight was measured according to the method as described by Shah et al (2002) [14].

Statistical analysis : All data were expressed as mean ± SEM, were n = 6. The data were analyzed by one way ANOVA followed by Dunett's post hoc multiple comparison test using "prism 4.0" software (Graph pad Inc., USA). A "p" value less than 0.05 was considered significant and less than 0.01 as highly significant.

RESULTS AND DISCUSSION

Figs 1 – 3 show the effects of different concentrations of Zn^{2+},Mo^{6+} and V^{5+} citrate accumulation by a mutant *Aspergillus niger* AB_{1801}. In all cases, citric acid production was remarkably depressed and aconitase activity bore an inverse relationship with it while cell growth was mildly hampered. Addition of different concentrations of $CaCO_3$ upto 5%, Cu^{2+} as $CuSO_4.5H_2O$, Mn^{2+} as $MnSO_4.4H_2O$ both upto 20 µg/ml resulted in gradual decrease of production. Aconitase activity varied inversely with the production parameters. Mycelial growth was variable. Addition of these metals exerted drastic inhibition on fermentation. Concentrations as low as 1 µg/ml poisoned the accumulation process as the aconitase activity was observed to be high which was congenial to isocitric acid formation and thus, continuation of the cycle.

Though the essentiality of Zn^{2+} has been claimed and confirmed by various investigators, the capability of *Aspergillus niger* to detect extremely negligible concentration of the element in the ppm level does not make the study unequivocal. Hanissa et al (1980), found that among $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $MnSO_4.4H_2O$, none was suitable for citrate over flow [15]. Akiyama et al (1970) observed cell autolysis on Mn^{2+} (2 ppm) addition Acid production was adversely inversely related to cell growth [16]. Addition of 500-600 mg Ca/Kg showed no interference in fermentation [17]. Vanadium and molybdenum failed to show any increase in production [18]. It may thus, be inferred from the experiments that exclusion of Zn^{2+},Mo^{6+} and V^{5+} is essential during citric acids fermentation by *Aspergillus niger* AB_{1801}. Ca^{2+}, Cu^{2+} and Mn^{2+} too failed to show any positive response.

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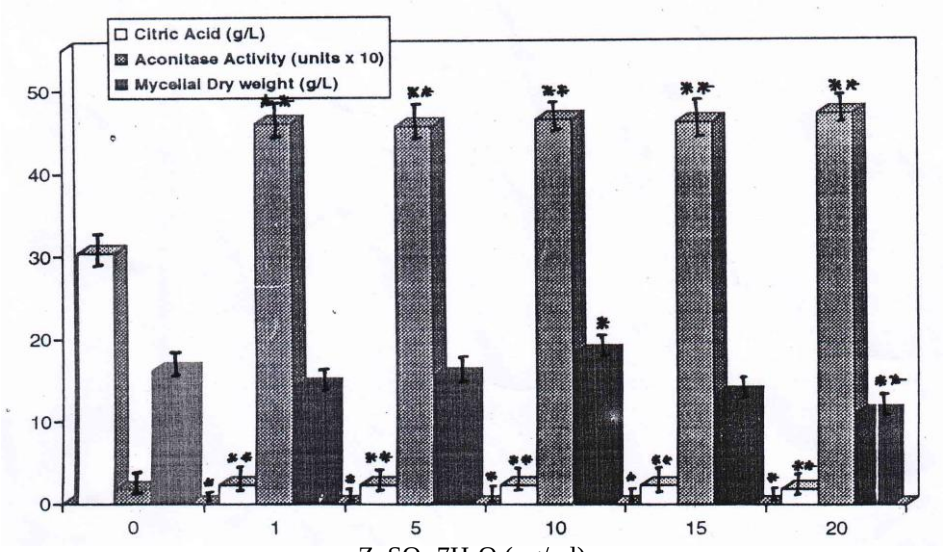

Fig. 1. Effect of different concentrations of Zn^{2+} as $ZnSO_4.7H_2O$ on citric acid fermentation by *A. niger* AB_{1801}.

(Values were expressed as mean ± SEM; where n-6; "O" stands for control. *p <0.05, **p < 0.01 when compared to control).

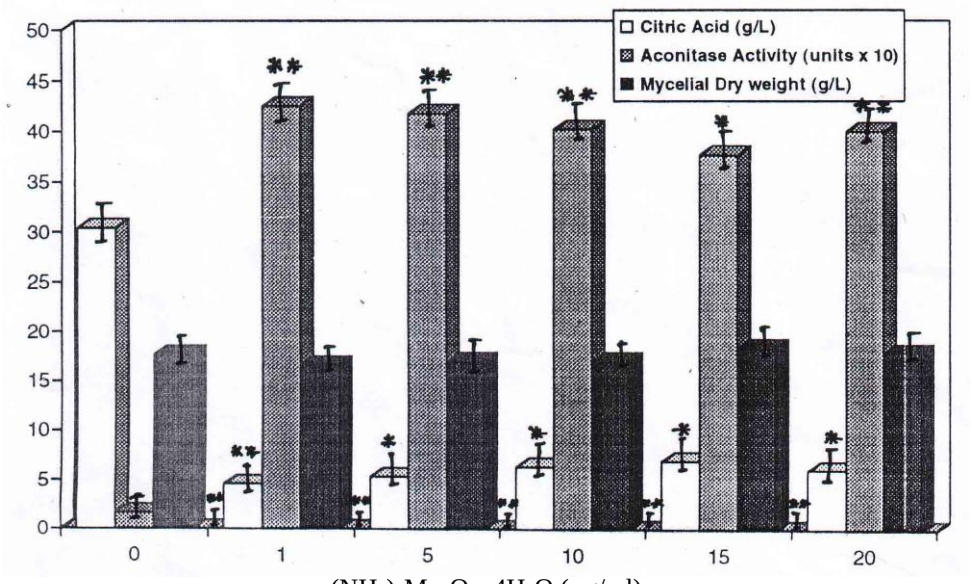

Fig. 2. Effect of different concentrations of Mo^{+6} as ammonium molybdate on citric acid fermentation by *A. niger* AB_{1801}.

(Values were expressed as mean ± SEM; where n-6; "O" stands for control. *p <0.05, **p < 0.01 when compared to control).

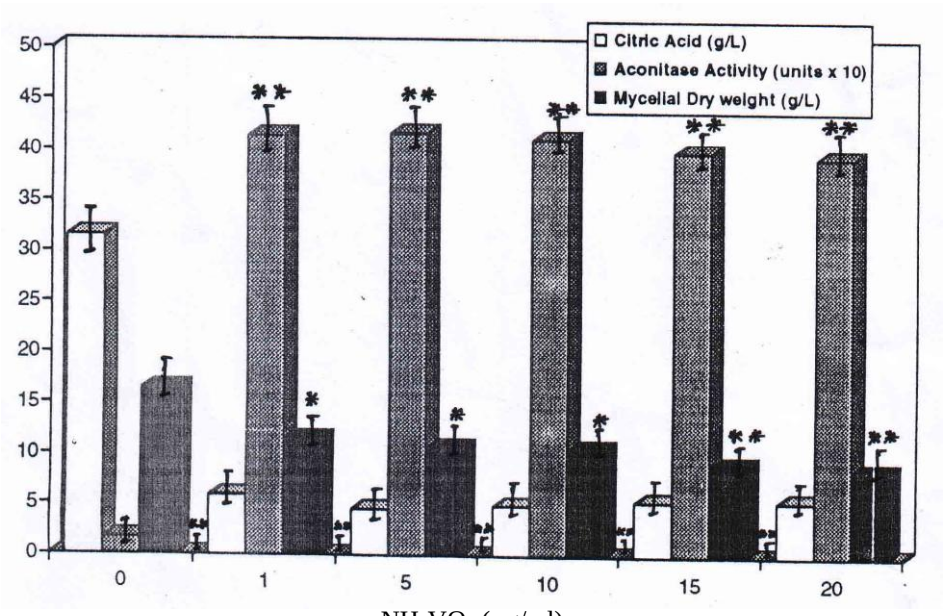

Fig. 2. Effect of different concentrations of V^{+5} as ammonium meta vanadate on citric acid fermentation by *A. niger* AB_{1801}.

(Values were expressed as mean ± SEM; where n-6; "O" stands for control. *p <0.05, **p < 0.01 when compared to control).