# Mycological Production of Citric Acid in India
# IV. Role of sulphur and phosphorus compounds

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Sulphur, though required in very small quantity, has an important role in metabolism of an organism. The utilization of sulphur compounds depends on the state of oxidation, and also the specific structure. In the citric acid fermentation sulphur is usually provided in the form of sulphates, generally magnesium sulphate. Magnesium probably has a role in activation of enzymes (Lehninger, 1950; and McElroy and Nason, 1954) as well es a role in transphosphorilation (Abelson and Aldous, 1950). Also, the magnesium ions have an antagonizing effect on the toxicity caused by metals as Al, Cu, Hg (Lockwood and Reeves, 1945; Lohrmann, 1940; and Marsh, 1945).

The concentration of magnesium sulphate is another controlling factor for citric acid production. There are two conflicting reports about the concentration of magnesium sulphate in the fermentation medium. Doegler and Prescott (1934) were of the opinion that more than 0.3 g/liter magnesium sulphate decreased citric acid yield and favoured sporulation. Wells and Herrick (1938) reported 0.01 to 0.05% $MgSO_4$ to be the optimum. On the other hand Shu and Johnson (1948) are of the view that the magnesium sulphate in concentration below 0.5 g/liter resulted in lower yields, while amounts from 0.5 to 2 g/liter did not effect the conversion efficiency. According to Karow (1942), the amount of magnesium sulphate used may be varied appreciably without seriously effecting growth or citric acid production, as long as sufficient is furnished to supply the basal requirement.

Assimilable phosphates are also one of the essentials for the growth and the metabolism of the moulds. It participates in the formation of cell wall, and also numerous intermediate compounds, and coenzymes, essential for metabolism and other intracellular processes. Phosphates are also important as buffers, helping to regulate the hydrogen-ion concentration of protoplasm and surrounding fluid.

There is a controversy regarding the importance of phosphate in citric acid fermentation. Molliard (1922) reported that citric acid accumulates when the culture is deficient in some essential nutrients and phosphate deficiency in surface cultures of *A. niger* was shown to enhance acid production. Some of the workers advocated the presence of phosphate to be essential during growth phase and its absence

during fermentation (Karow, 1942; Szücs, 1944 and 1948). Szücs (1944) was the first to show that the presence of a small amount of assimilable phosphate compound in the aqueous fermentation solution retarded but did not prevent citric acid formation by *Aspergillus niger* under submerged culture condition.

Shu and Johnson (1948) were of the opinion that the phosphate ions are essential for citric acid production in addition to their effect as a buffer and food constituent. They also noted that if the two stage process is used, phosphate must be added to the fermentation medium if the mycelium (grown on a complete medium, i. e., containing high phosphate) is carefully washed free of adhering phosphate. Working with another culture of *A. niger*, they had shown that it is not the phosphate alone which is important but a balance of manganese, zinc and phosphate.

The concentration of phosphate in the fermentation medium has also a paramount effect on citric acid yield. Doegler and Prescott (1934) found that addition of more than 1.5 gm/liter $KH_2PO_4$ inhibited citric acid yield and Wells and Herrick (1938) reported 0.03 to 0.1% $KH_2PO_4$ to be optimum for fermentation.

The present paper deals with the study of the effect of different sulphur and phosphorus compounds on citric acid producing capability of six strains of *Aspergillus niger* group.

## Materials and methods

The strains taken for the study were the same as in the previous studies (Mehrotra and Singh, 1971) $M_2$, $L_3$, $RK_2$, $A_2$, $SJ_1$ and $O_{40}$. The medium employed was that recommended by Doegler and Prescott (1934).

To evaluate the effect of different sulphur compounds on citric acid yield by six selected strains of *A. niger* the basal medium employed, consisted of the following compounds — Sucrose (commercial market sugar), 150 g; $NH_4NO_3$, 2.23 g; $KH_2PO_4$, 1.0 g; distilled water to make 1000 ml. The following sulphur compounds were added singly in the above basal medium in amounts so as to furnish 29 mg of sulphur per liter (present in 0.223 g $MgSO_4$):

Inorganic sulphur compounds: Sodium sulphate, potassium sulphate, magnesium sulphate, ammonium sulphate, sodium sulphite, sodium thiosulphate, sodium metabisulphite, potassium metabisulphite, and potassium persulphate.

Organic sulphur compounds: Methionine, cystine and thio-urea.

To observe the effect of different phosphorus compounds on the citric acid production the basal medium consisted of the following compounds — Sucrose (commercial market sugar), 150 g; $NH_4NO_3$, 2,23 g; $MgSO_4$ $7H_2O$, 0.223 g; and distilled water to make 1000 ml. To this different phosphorus compounds were added singly so as to furnish

0.226 g/liter phosphorus (present in 1.0 g $KH_2PO_4$). The phosphorus compounds used were ammonium phosphate, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, sodium dihydrogen phosphate, di-sodium hydrogen phosphate.

The method of preparation of medium, sterilization and inoculation were the same as described in the previous studies (Mehrotra and Singh, 1971). The pH of the fermentation medium was adjusted to 2.2—2,8 after sterilization. The experiments were run in triplicates and the cultures were kept at 28° C ($\pm$ 1) for 8 days.

Another experiment was set in which the different strains were grown on the above mentioned medium with high phosphate ($KH_2PO_4$) content, and after 4 days growth the mycelial mat was washed with sterilized distilled water and then transferred aseptically to the medium without any trace of phosphorus. These cultures were tested for citric acid after six days.

Sugar estimations were made by the method of Somogyi (1945), acidities were measured by titrating the 2 ml portion of aliquot with 0.1 N sodium hydroxide solution taking phenolphthalein as an indicator. Citric acid was determined by the method of Bernhauer (1926).

## Results and Discussions

(a) Effect of different sulphur compounds.

The results presented in Plate I, Figs. 1—6 show that magnesium sulphate was the best among sulphur sources tried and all other sources were poor for citric acid production. The growth of the strains was good on magnesium sulphate, ammonium sulphate and sodium thiosulphate; moderate on sodium sulphate, sodium sulphite and thio-urea; poor on sodium metabisulphite, potassium sulphate, potassium metabisulphite, potassium persulphate, methionine and cystine. The strains sporulated well only on magnesium sulphate and poorly on ammonium sulphate. No sporulation was observed on the rest of the sources except that $M_2$ and $A_2$ showed slight sporulation on sodium sulphate and $L_3$, $RK_2$ and $O_{40}$ on sodium thiosulphate. The acid formation only on magnesium sulphate shows that it is not only the $SO_4$ ion which is necessary for citric acid formation but the $Mg^{**}$ ions are also equally effective in this metabolism and it is the joint effect of both, the magnesium and sulphate ions which is responsible for acid production. To confirm this idea an experiment was conducted in which sodium sulphate was taken instead of magnesium sulphate as a sulphur source and magnesium chloride was added to the medium so as to furnish 22 mg magnesium (present in 0.223 g of $MgSO_4$). It was observed from the results that when magnesium chloride was added along with sodium sulphate all the strains yielded almost the same amount of acid as in the case of magnesium sulphate (and two strains, i. e., $M_2$ and $L_3$ produced even more acid).

It seems from the results that magnesium probably takes part in the activation of enzymes necessary for the normal growth and metabolism. Though the enzyme systems are activated also by manganese or other divalent ions, it is believed that magnesium is the physiologically most active metal (Lehninger, 1950; Mc Elroy and Nason, 1954). It is also evident from the results that magnesium is essential not only for growth and metabolism but also for sporulation. Rennerfelt (1934) analysed the vegetative mycelium and spores of *A. niger* for mineral content and found magnesium to be one of the most essential components of the mycelium, and the spores contained about five times more magnesium than the mycelium.

To find out the optimum concentration of $MgSO_4$ required for citric acid production an experiment was set in which different concentrations of $MgSO_4$ viz., 0.01%; 0.02%; 0.025%; 0.03%; 0.04% and 0.05% were supplied to the medium and all the six strains were tested for citric acid on each concentration. The results are presented in Plate I and Figs. 7—12.

From the results thus obtained it was observed that the optimum requirement of $MgSO_4$ varied with the strains. For $RK_2$ and $A_2$ it was 0.01% for $L_3$ and $O_{40}$, 0.02%; for $M_2$ and $SJ_1$ it was 0.025%. Above these concentrations there was a fall in the rate of the accumulation of citric acid though this effect on yield was not very marked upto 0.05% concentration. These results accord with the finding of Karow (1942) that the amount of magnesium sulphate used may be varied appreciably without seriously affecting the growth or citric acid formation, as long as sufficient amount is furnished to supply the basal requirement.

The optimum range of $MgSO_4$ concentration, however, was found to be 0.01—0.03%. This is similar to the finding of Doegler and Prescott (1934) and Wells and Herrick (1938) who reported 0.03 and 0.01—0.05% respectively to be optimum concentration of $MgSO_4$ in their case. The present case differs, however, from that reported by Shu and Johnson (1948). According to them magnesium sulphate in concentrations below 0.5 g/liter resulted in lower yields, while amounts from 0.5 g to 2 g/liter did not effect the conversion efficiency.

(b) Effect of different phosphorus sources.

The results are presented in Plate II Figs 1—6. Among the phosphorus compounds tried only $KH_2PO_4$ and $K_2HPO_4$ were found to be good sources for all the six strains. Even out of these two sources dipotassium hydrogen phosphate was a little better than potassium dihydrogen phosphate for all the strains except one, i. e., $SJ_1$. Other three sources were poor even for the growth of all the strains. Sodium dihydrogen phosphate and disodium hydrogen phosphate, however, favoured oxalic acid formation.

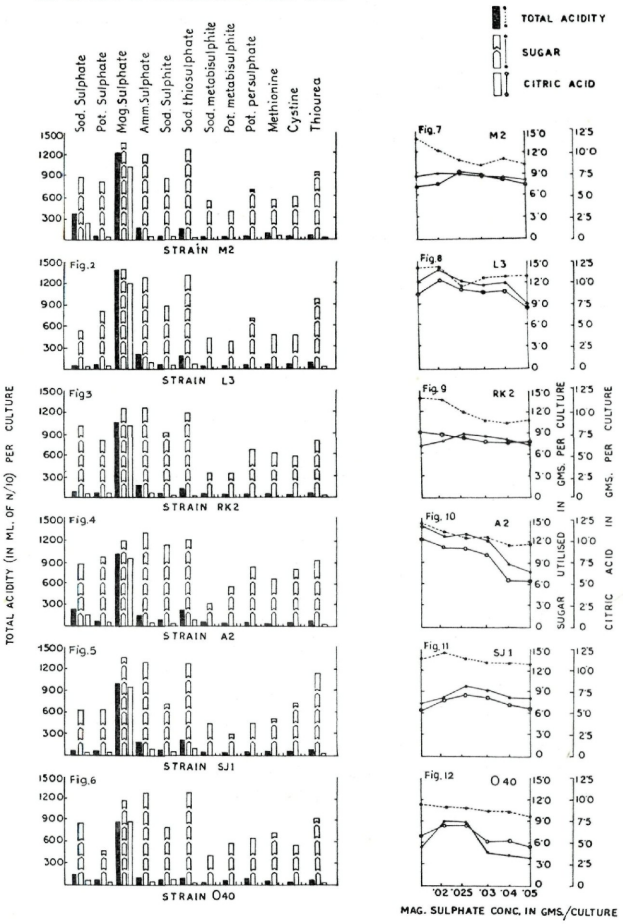
In the experiments in which phosphate was totally removed after

growth phase of the strains, the yield of the citric acid was very much retarded.

It is evident from the results that phosphorus is one of the essen-

Plate I

EFFECT OF DIFFERENT SULPHUR COMPOUNDS (Figs 1-6) AND DIFFERENT CONCENTRATIONS OF $MgSO_4$ (Figs. 7-12) ON CITRIC ACID PRODUCTION BY SIX STRAINS OF ASPERGILLUS NIGER GROUP



tials for the growth and metabolism of fungi. It is also clear that the phosphates have a specific effect on the formation of a particular metabolite. The maximum yield of citric acid, obtained in the case of $KH_2PO_4$ and $K_2HPO_4$ indicates that K* ion has also a role together with phosphorus in citric acid fermentation. Also, Rippel and Behr (1934) reported that this K* ion has an inhibitory effect on oxalic acid formation, Thus oxalic acid formation may be prevented by the use of $KH_2PO_4$ and $K_2HPO_4$ as phosphate sources. Oxalic acid formation in the presence of ammonium phosphate, sodium dihydrogen phosphate and dihydrogen sodium phosphate supported the finding of Wehmer (1892) that the presence of alkaline neutralizing agents such as $Na_2HPO_4$ and $(NH_4)_2PO_4$ are conducive to maximum yield of oxalate.

The inhibition of citric acid formation in the absence of the biologically available phosphate from the fermentation medium supports the opinion of Shu and Johnson (1948) that phosphate is concerned with citric acid production in addition to its effect as buffer and food constituent.

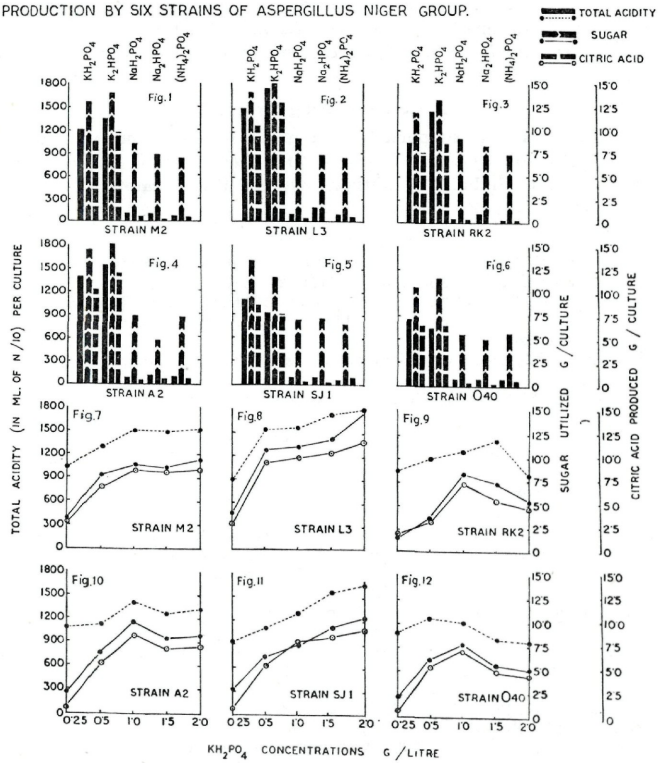
An experiment was set to find out the optimum concentration of $KH_2PO_4$ (the phosphate source generally used in citric acid fermentation) for our six selected strains. Different concentrations of $KH_2PO_4$ tried were — 0.025, 0.05, 0.1, 0.15, 0.2 per 100 ml. The results presented on page 241, Figs. 7—12 show, that at 0.025% concentration very little citric acid was formed. The acid formation started increasing with the increase in the concentration of phosphate from 0.05% to an optimum which was as follows: 0.1% for $RK_2$, $A_2$ and $O_{40}$; 0.15% for $SJ_1$; and 0.2% for $M_2$ and $L_3$. These results are similar to the finding of Doegler and Prescott (1934) and Wells and Herrick (1938). The optimum concentration of $KH_2PO_4$ for $M_2$ and $L_3$ was a little higher, i. e., 0.2%. This supports the idea that the optimum requirement varies with the organism. The decrease in citric acid yield at higher concentration of $KH_2PO_4$ support the idea of „Shunt metabolism", i. e., the enzymes normally involved in complete oxidation of the substrate become saturated and the substrate molecules then are excreted as such or shunted to a secondary or subsidiary enzyme system (Foster, 1949).

## Summary

The effect of different sulphur and phosphorus compounds was observed on the citric acid producing capability of six selected strains of *A. niger*, namely, $M_2$, $L_3$, $RK_2$, $A_2$, $SJ_1$, and $O_{40}$.

Among the different inorganic and organic sources of sulphur tested only magnesium sulphate supported acid production. All other sources were poor not only for acid production but also for growth. The optimum concentration of magnesium sulphate for acid production was as follows — 0.01% for $RK_2$ and $A_2$; 0.02% for $L_3$ and $O_{40}$; and 0.025% for $M_2$ and $SJ_1$. From a comparative study it was also confir-

EFFECT OF DIFFERENT PHOSPHORUS COMPOUNDS (Figs. 1-6) AND DIFFERENT CONCENTRATIONS OF POTASSIUM DIHYDROGEN PHOSPHATE (Figs. 7-12) ON CITRIC ACID PRODUCTION BY SIX STRAINS OF ASPERGILLUS NIGER GROUP.




med that it is not only the $SO_4^{**}$ ion which is necessary for citric acid formation but the $Mg^{**}$ ions are also equally effective in this metabolism and it is the joint effect of both the ions which is responsible for acid production.

From an experiment it was also confirmed that phosphate is as essential for growth as for citric acid formation. The yield was very much retarded in the absence of a phosphorus source from the fermentation medium.

It was observed that potassium dihydrogen phosphate and dipotassium hydrogen phosphate were good supporters of citric acid formation by all the strains. Ammonium phosphate, sodium dihydrogen

phosphate and disodium hydrogen phosphate were poor sources for citric acid formation. They, however, supported oxalic acid formation. Among the different concentrations of $KH_2PO_4$ tested for citric acid, 0.01% was optimum for strain $RK_2$, $A_2$ and $O_{40}$; 0.15% for $SJ_1$; and 0.2% for $M_2$ and $L_3$. Above these concentrations there was a decrease in the citric acid yield.

## Acknowledgement

This work was supported by a grant to the second author by the Ministry of Defence, Research and Development Organization, Government of India. The authors express their grateful thanks to the Director of the Organization.

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