Mycological Production of Citric Acid-III Effect of the different Nitrogen compounds

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Nitrogen is one of the most essential constituents of a nutrient medium for fungi. Besides the formation of a chitinous cell wall and protoplasmic material, nitrogen has an important role in the formation of nucleic acids.

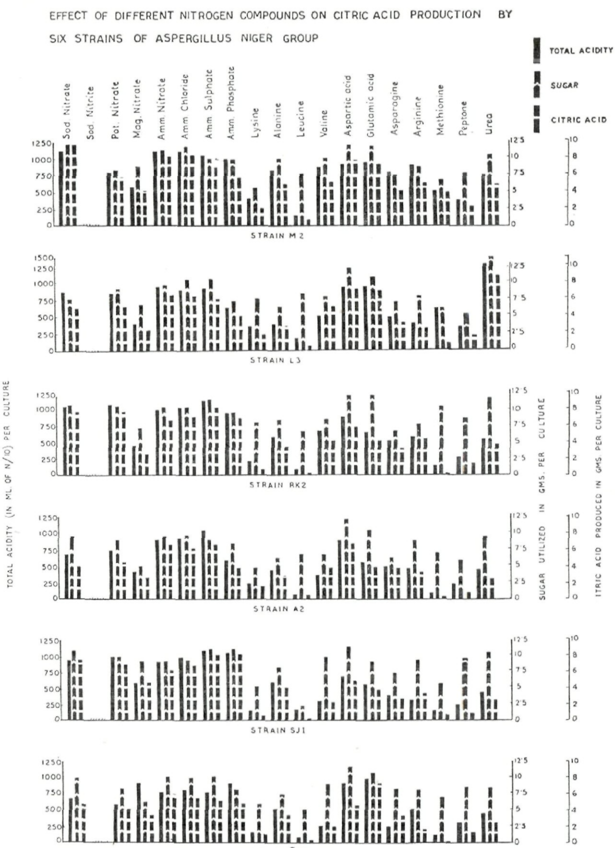
Wehmer (1892) found that the nature of a nitrogen compound in a medium is a critical factor in oxalic acid formation. Accumulation of oxalic acid was possible only when $Ca(NO_3)_2$, KNO_3, $NaNO_3$, NH_4NO_3, $(NH_4)_2PO_4$ and peptone were the nitrogen sources but NH_4Cl and $(NH_4)_2SO_4$ never allowed the formation of oxalic acid. High citric acid yields were found only when nitrate nitrogen (NH_4NO_3, KNO_3, and urea nitrate) was present in the growth medium (Bernhauer, 1928).

Loesecke (1945) reviewing citric acid word concluded that there seems to be no source of nitrogen which is best for all the strains of *Aspergillus niger*. It has also been shown that different strains used different sources of nitrogen for the same metabolic reaction. Butkewitsch and Osnitzkaya (1936) using one strain of *Aspergillus niger,* obtained a marked increase in the yield of citric acid from sucrose by the addition of 0,3% magnesium nitrate, but when using a second strain, the addition of magnesium nitrate actually caused a slight diminution in the yield.

Not only an excess of biologically available nitrogen in the medium be avoided (Shu and Johnson, 1948; Chrzaszez and Tikumov, 1931) but also the correct concentration of nitrogen be supplied for a particular metabolite. The concentration of the nitrogen source is also a controlling factor in the production of a metabolic product. Gluconic acid is formed almost exclusively from strains of *Aspergillus niger* when the mycelium is cultivated on a growth medium poor in 'N' content (Bernhauer 1928 b, d). On the other hand, citric acid is formed on a medium relatively rich in nitrogen, and at still higher concentration, there is increase in the growth and sugar consumption but decrease in the amount of citric acid produced (Karow, 1942). Doegler and Prescott (1934) reported that more than 2,5 g/lit. NH_4NO_3 favoured oxalic and less citric acid formation. Wells and Herrick (1938) reported 0,16 to 0.32% NH_4NO_3 to be the limit for good citric acid yield. Sodium nitrate in a 0,4% concentration was found to be better than ammonium nitrate or ammonium sulphate by Porges (1932) while Butkewitsch and Gaewskaya (1935) used potassium nitrate in 0,3% concentration in order to obtain highest yield of citric acid.

The present study was undertaken to evaluate the effect of different nitrogen sources on citric acid production by six selected strains of *Aspergillus niger* group.

Plate I



Materials and Methods

The six strains taken for the study were: M_2, L_3, RK_2, A_2, SJ_1 and O_{40}. The fermentation medium employed in this experiment was that recommended by Doegler and Prescott (1934) with the following composition: sucrose (commercial cane sugar) 150 g; KH_2PO_4, 1,0 g; $MgSO_4.7H_2O$, 0,223 g; and distilled water to make 1000 ml. Various nitrogen compounds were added singly so as to furnish 0.78 g nitrogen per liter (present in 2,23 g of NH_4NO_3). 100 ml. of this solution was taken in 300 ml. Erlenmeyer's Pyrex flasks. The flasks were steam sterilized for half an hour and for three successive days. The pH of the medium was adjusted to 2,4—2,8. Three flasks of each source were taken for each strain. The cultures were incubated at 30° C for 8 days (as these conditions have been found optimum for maximum yield). The method of inoculation, harvesting, estimation of total acidity, residual sugar and acid were the same as given in the first paper of the series.

The nitrogen sources taken for the study were as follows:

A. Inorganic: — sodium nitrate, sodium nitrite, potassium nitrate, magnesium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium phosphate.

B. Amino acids: glycin, DL-alanine, L-leucine, DL-valine, DL-aspartic acid, D-glutamic acid, asparagine, D-arginine, DL-methionine, peptone, urea.

Results

The results are given in the plate I, figs. 1—6.

Among the different inorganic nitrogen sources used both nitrate and ammonium compounds were found to be equally good for citric acid accumulation. Sodium nitrite was the only compound that showed inhibitory effect. The six strains showed the following degree of usefulness of individual inorganic compounds in relation to citric acid production:

M_2: sodium nitrate > ammonium chloride > ammonium nitrate > ammonium sulphate > potassium nitrate > ammonium phosphate > magnesium nitrate > sodium nitrite.

L_3: ammonium nitrate > ammonium chloride > ammonium sulphate > sodium nitrate > potassium nitrate > ammonium phosphate > magnesium nitrate > sodium nitrite.

RK_2: ammonium sulphate > potassium nitrate > sodium nitrate > ammonium chloride > ammonium phosphate > ammonium nitrate > magnesium nitrate > sodium nitrite.

A_2: ammonium nitrate > ammonium sulphate > ammonium chloride > potassium nitrate > sodium nitrate > ammonium phosphate > magnesium nitrate > sodium nitrite.

SJ_1: ammonium phosphate > ammonium sulphate > sodium nitrate > potassium nitrate > ammonium chloride > ammonium nitrate > magnesium nitrate > sodium nitrite.

O_{40}: ammonium nitrate > ammonium chloride > ammonium sulphate > ammonium phosphate > sodium nitrate > potassium nitrate > magnesium nitrate > sodium nitrite.

Among the organic nitrogen compounds tested aspartic and glutamic acids were of value. Urea in the case of one strain, i. e., L_3 showed an excellent result. However, the strains showed the following degree of usefulness of individual compounds:

M_2: aspartic acid > glutamic acid > valine > arginine > alanine > urea > asparagine > methionine > peptone > glycine > leucine.

L_3: urea > aspartic acid > glutamic acid > valin > asparagine > arginine > alanine > glycine > peptone > methionine > leucine.

RK_2: aspartic acid > glutamic acid > valine > arginine > urea > alanine > asparagine > peptone > glycine > leucine > methionine.

A_2: aspartic acid > glutamic acid > valine > asparagine > arginine > alanine > urea > glycine > peptone > leucine > methionine.

SJ_1: alanine > glutamic acid > arginine > asparagine > urea > valine > aspartic acid > methionine > peptone > glycine > leucine.

O_{40}: glutamic acid > aspartic acid > asparagine > alanine > urea > valine > arginine > peptone > glycine > leucine > methionine.

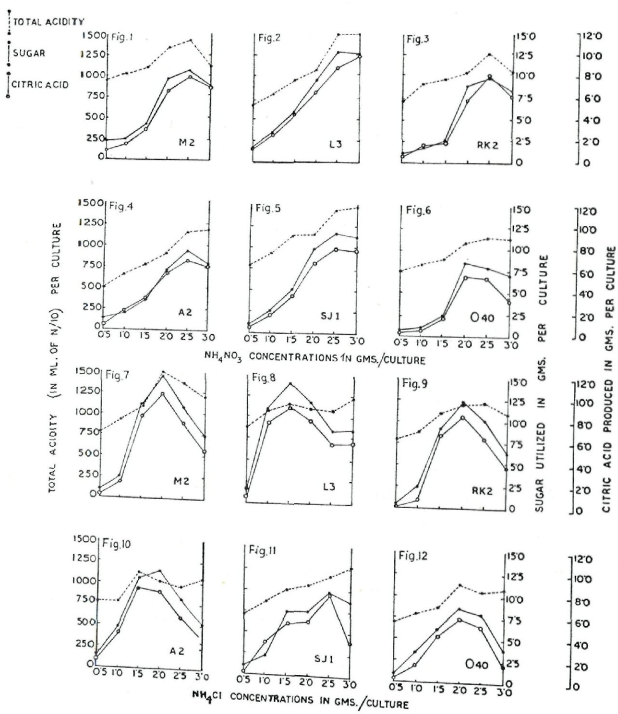
The choice of the strains for nitrogen sources differed from each other but ammonium nitrate and ammonium chloride were good in general. In view of this fact an experiment was set in which different concentrations of ammonium nitrate and ammonium chloride were tried for each strain to find out the optimum concentration of these compounds for citric acid production. The results are presented in plate II and figs 1—6. It was observed from the results that at 0.05% concentration the strains produced very little acid and from 0.1% the production started increasing with the increase in NH_4NO_3 and NH_4Cl concentration and reached an optimum. The optimum concentration of NH_4NO_3 for different strains was as follows: 0.2% for O_{40}; 0.25% for M_2, RK_2, A_2 and SJ_1; and 0.3% for L_3 after which there was a fall in the rate of acid production. In the case of NH_4Cl a slightly lower concentration was favourable. The results are given plate II and figs. 7—12. The optimum concentrations of NH_4Cl for different strains was a follows: 0.15% for L_3 and A_2; 0.2% for M_2, RK_2 and O_{40}; 0.25% for SJ_1. One noteworthy point is that none of these strains sporulated above 0.15% concentration of NH_4Cl.

Discussion

The present results show that the nature of a nitrogen compound exerts a considerable influence on the citric acid production. Among the different inorganic nitrogen sources used citric acid accumulated

Plate II

EFFECT OF DIFFERENT CONCENTRATIONS OF AMMONIUM NITRATE (Figs.1-6) AND AMMONIUM CHLORIDE (Figs. 7-12) ON CITRIC ACID PRODUCTION BY SIX STRAINS OF ASPERGILLUS NIGER GROUP.



in the presence of both ammonium and nitrate salts. However, none of the sources was best for all the strains. Maximum yield in the presence of sodium nitrate was obtained from only one strain, M_2. Porges (1932) also found sodium nitrate (in 0.4% concentration) to be better than ammonium nitrate and ammonium sulphate for his strains. Highest yield of acid in the presence of NH_4NO_3 from the strains L_3, A_2 and O_{40} obtained in the present investigation is in agreement with the finding of Bernhauer (1928 d) who found NH_4NO_3 to be an excellent source for citric acid production. Ammonium suphate, ammonium phosphate, and ammonium chloride proved to be equally good and for some even better sources of nitrogen. RK_2 und SJ_1 strains yielded maximum acid in the presence of ammonium sulphate and ammonium chloride. Ammonium chloride proved to be fairly good for all the strains. Further the use of ammonium chloride for citric acid fermentation is advantageous for two reasons. Firstly, the consumption of cation $(NH_4)^+$ gives rise to a physiological acidity which never allows the formation of oxalic acid. Secondly, the sporulation of the citric acid producing organism may be checked completely by the use of ammonium chloride which may prevent contamination in the laboratory with fungus spores.

$Mg(NO_3)_2$ reported to be a good source for citric acid production by Butkewitsch and Osnitzkaya (1936) gave poor results in the present case. The inhibitory effect of sodium nitrite on growth and citric acid production may be due to the fact that nitrous acid is generated as the organism generates organic acid from carbohydrate. This nitrous acid is toxic due perhaps to its rapid interaction with amino groups of cell proteins and amino acids (Foster, 1940).

The optimum range of NH_4NO_3 concentration for acid production in the present case was 0.15—0.3% which is similar to the finding of Wells and Herrick (1938) who reported that not more than 0.16—0.32% NH_4NO_3 should be added to get the highest yield. The fall in the rate of citric acid formation above 0.25% concentration of NH_4NO_3 in the case of all the strains except L_3 supports the idea of Doegler and Prescott (1934) that more than 0.25% NH_4NO_3 favours more of oxalic and less of citric acid. Ammonium chloride in 0.15 to 0.25% concentration being equally good for citric acid fermentation proves that NH_3—N is as efficient as NO_3—N for citric acid production.

Out of the eleven organic nitrogen compounds tested glutamic and aspartic acids were the most efficiently utilized ones. There may be so many explanations to this fact. The one may be that these sources cause physiological acidity in the medium which is favourable for citric acid fermentation. Secondly, the aspartic acid by reversible transamination gives rise to oxalacetic acid; and glutamic acid by reversible reductive amination gives rise to α-ketoglutaric acid which are intermediates of citrate cycle.

Asparagine, the amide of aspartic acid was not found as good as aspartic acid in the present investigation. Leonian and Lilly (1940) also pointed out that aspartic acid is an even better source of ‚N' than asparagine for numerous phycomycetes. The utilization of arginine, valine and alanine may be explained by the following metabolic links: glutamic acid can be formed from arginine (urea cycle); propyonyl co-enzyme A is a product of valine degradation; and alanine can enter the citrate cycle as acetyl co-enzyme A after preliminary transamination to pyruvic acid. Steinberg (1942 a) also reported alanine, arginine, aspartate and glutamate as equally good sources to inorganic nitrogen for *A. niger*. However, glycine, which was reported by him as a good source was not found to be so in the present study.

The inhibitory effect of the sulphur containing amino acids as methionine on citric acid production may be due to incapacity of these organisms to split the sulphide linkage of this compound.

Poor yield was obtained on peptone. This has been reported as a poor source for citric acid production also by Bernhauer (1928 d).

Urea also gave poor result except for one strain, i. e., L_3 possibly because enzyme urease was absent in all the strains except L_3. Bernhauer (1928 d) also reported urea to be a source and urea — NO_3 a good source for citric acid formation.

It has been concluded by this investigation that different organisms act differently on the same and also on different nitrogen compounds. Jennison et al. (1955) believed that intrinsic differences in the molecular structure may be involved in the differences in utilization of these nitrogen compounds.

Summary

Effect of different inorganic and organic nitrogen compounds on citric acid accumulation by six strains of *Aspergillus niger* was studied. Utilization of these compounds differed from strain to strain. Among inorganic nitrogen sources both NO_3^- and NH_4^+ compounds were found equally good for acid production. Included in this group are sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, and ammonium phosphate. Magnesium nitrate was not found good for acid production. Nitrite had an inhibitory effect on growth and metabolism. The optimum concentrations of ammonium nitrate and ammonium chloride for acid production were 0.15—0.3% and 0.15—0.25% respectively. Among organic nitrogen compounds aspartic acid, glutamic acid and urea for one strain, i. e., L_3 were found to be of value. Valine, arginine, asparagine and alanine were also found good for acid production. Methionine and peptone had an inhibitory effect on acid production.

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