SECTION - F

. GENERAL SUMMARY
. BIBLIOGRAPHY
. APPENDIX
GENERAL SUMMARY
The present work has been devised to investigate the metabolic activities of some important fungi with a view of citric acid yielding organisms. Citric acid is an important chemical needed in medicinal purposes, food technology and beverage industries. It is also used in various dyeing and printing branches and also in plastic industries. Formidable amounts of citric acid worth crores of rupees are imported every year in India. Very many moulds are known to produce citric acid and in this work an effort has been made to select out the promising ones.

In the mycological laboratory of Saugar University, a large number of organisms were available as stock cultures which were never tested before for their capacity to produce citric acid. These were mostly isolated from soil in a variety of studies. Besides these, organisms were also isolated directly from soil by soil plate method (Warcup, 1950) and dilution plate method (Waksman, 1927). These organisms which numbered 118 were first screened in a preliminary manner by "Direct agar culture selection method" in which bromocresol green was used as an indicator.

The preliminary selection of suitable microorganisms was made on the basis of the ability of the organism to produce organic acid without enquiring the nature of acids produced.
In this method, an indicator (bromocresol green) was added to the agar and when organisms were grown in this plate, yellow zones against blue backgrounds were produced, indicating the presence of organic acids. Organisms which were not active in this respect, grew without any zone.

From this preliminary studies, 66 organisms, mostly species of Aspergillii and Penicillia and some members of Mucorales were selected as promising ones producing organic acids.

Further selection, out of the 66 acid-producing organisms, has been made by chromatographic analysis of the organic acids produced. For this, descending paper chromatographic method was used. The solvent system employed was Butanol : Formic acid : Water (10:2:3 v/v). The Rf values were calculated and spots of citric acid were identified. By this method, 26 microorganisms were found to be producers of citric acid. Among these 26, seven organisms, viz. Aspergillus flavus, A. tamarii, A. fischeri, Penicillium javanicum, P. miczynskii, P. multicolor and P. thomii were reported for the first time to produce citric acid. Faint and very small spots (very weak-producers) were ignored and rest of the 21 microorganisms were selected for further testing.

These organisms were again put to bromocresol test and acid zones were measured to estimate the degree of acid production. This gave only a rough estimation. The exact
quantitative estimation of citric acid was made by the method of Marier and Boulet (1958), with the help of Beckman model DU-2 Spectrophotometer.

From these results, it could be concluded that black Aspergilli were the best producers of citric acid. Amongst them, *A. niger* and *A. awamori* gave uniformly higher yields of citric acid. *F. multicolor*, *F. luteum* and *F. thomii* proved to be better producers of citric acid amongst tested Penicillia. *A. flavus* and *A. tamarill* were found to be moderate in this respect. *A. clavatus*, *F. citrinum*, *F. miczynskii*, *F. javanicum* and *F. obscurum* were recorded as poor producers of citric acid.

A voluminous work has already been done with black Aspergilli but no attempt has been made so far to study the citric acid production by *A. tamarill*, *A. flavus*, *F. multicolor*, *F. luteum*, *F. thomii* and *F. citrinum*.

Keeping these facts in mind, only eight organisms, 4 Aspergilli and 4 Penicillia were selected. *A. niger* and *A. awamori* were taken as being heavy producers and the rest of the six organisms taken were: *A. tamarill*, *A. flavus*, *F. multicolor*, *F. luteum*, *F. thomii* and *F. citrinum*.

**Selection of the suitable fermentation medium:**

In the production of metabolites by the microorganisms, the medium plays an important role. In all 12 media which
included synthetic, natural and modified ones (fermentation media) were tried. The fermentation medium, without trace elements, proposed by Forges (1932) was found to be the best, almost for all the organisms and was finally selected for further studies.

THE EFFECT OF ENVIRONMENTAL FACTORS:

The environmental factors play an important role in the production of citric acid. Among the environmental factors, length of incubation, temperature and initial hydrogen ion concentration are important ones which greatly affect the production of citric acid and were studied in detail.

Length of incubation:

The production of the citric acid was studied in a wide range of incubation period, from 3 to 18 days, at intervals of 3 days.

In the beginning of the fermentation, i.e. up to 3 days, 20% to 30% of the sugar was consumed and very little of citric acid was accumulated in the culture filtrates of the tested moulds. This shows that in the beginning, most of the sugar was used up in the mycelial development. After 3 days, on further incubation, citric acid production rapidly increased and a sufficient amount of citric acid was accumulated within
a period of 9 to 12 days in all the moulds.

It is evident from the results that there are two distinct phases of fermentation. The first phase was initial growth phase, where the sugar was used up mainly for the mycelial production and second was an acid forming period, which started shortly after the mycelial production. When citric acid accumulates to a maximum concentration and starvation progresses, a fall in the acid production curve was noted in each organism. The disappearance of the citric acid in the culture filtrates was probably due to utilization of acid during the later period of incubation, as it is well-known that some CO₂ is always formed by the mould via oxidative metabolism.

This experiment on incubation period further revealed that the maximum production of citric acid was reached in 9 days in most of the Aspergilli (except A. niger, where the yield of acid continued to increase slightly up to 12 days) while on the other hand, in Penicillia, increase in the yield was recorded up to 15 days except in P. citrinum, although the major part of the acid produced within 12 days. It can, therefore, be concluded that the most suitable period of incubation is 9 and 12 days, in Aspergilli and Penicillia respectively and further studies were performed under these optimum conditions of incubation.
Effect of temperature:

Another important factor is temperature. In order to find out the optimum temperature for the fermentation of citric acid, the effect of seven different temperatures, ranging from 22 to 45°C, were studied. These were 22, 25, 28, 30, 35, 40 and 45°C.

The results revealed that the maximum production of citric acid was recorded at 30°C in all the organisms excepting A. flavus. In A. flavus, the maximum yield was recorded at 28°C and at 30°C it was slightly decreased.

It was clear that low temperature like 22°C and higher temperatures above 30°C, were detrimental to the growth and activities of the organisms and retarded the production of citric acid.

On the whole, 30°C temperature was found to be most favourable for citric acid production almost for all the organisms and this temperature was kept for further experimentation.

The effect of pH:

The maintenance of proper pH of the medium is important for the successful fermentation.
The effect of different pH values, viz. pH 2.0, 2.2, 2.5, 2.8, 3.0, 3.2, 3.5, 3.8, 4.0, 4.5, 5.0 and pH 6.0 were tried to study their effect on the production of citric acid.

Results of tests served to show that citric acid-producing moulds, possess the greatest tolerance to low pH values, particularly *A. niger* which could not only grow well but gave an excellent yield of citric acid under extremely low pH (viz. pH 2.0). In most of the tested species, like *A. awamori*, *A. tamarii*, *P. multicolor*, *P. luteum*, *P. thomii* and *P. citrinum*, citric acid production was started from the pH value 2.2.

In all the eight organisms, a sharp increase in the yield of the citric acid was noted up to pH 2.5 which reached its peak within pH range 2.8 to 3.2. The optimum pH was found to be different in different species. The optimum pH was 2.8 for *A. niger*, *A. awamori*, *A. flavus*; pH 3.0 for *A. tamarii*, *P. multicolor*, *P. citrinum* and 3.2 for *P. thomii*.

*A. tamarii* at pH 2.8 and *P. thomii* at pH 3.0, produced sufficiently good amount of acid. Therefore, on the whole, it could be concluded that pH 2.8 for species of *Aspergillus* and pH 3.0 for species of *Penicillium* could be considered to be optimum and were maintained in further studies.

As initial hydrogen ion concentration increased from the optimum pH, a tendency was noticed to form more mycelium accompanied by downward trend in the acid production.
STUDY OF THE NUTRITIONAL FACTORS:

It is well-known that proper maintenance of the levels of carbon, nitrogen, phosphorus, sulphur, chloride ions and lastly the trace elements, in the fermentation medium is essential to obtain good yields. Extensive work has been done on the nutritional requirements of *A. niger* and to some extent also on the *A. awamori*. However, no work has been reported on nutritional requirements of remaining six organisms under investigation. In view of this, present study was undertaken.

Single variable and factorial experiments were used to determine the effect of carbon, nitrogen, phosphorus, sulphur, chloride and trace elements (Fe, Zn, Cu and Mn) on the production of citric acid. Several experiments were laid out in which the concentration of one salt in the medium was varied over a comparatively wide range while the other salts were held at the same level.

These experiments were run under optimum conditions of environmental factors as concluded from earlier experiments.

**Effect of carbon sources:**

It is well-known that carbon occupies an important place in the metabolism of fungus, usually carbohydrate is the preferred source of carbon.
To determine the best suitable source of carbon and its optimum concentration five different monosaccharides and disaccharides namely, glucose, fructose, commercial sugar, pure sucrose (BDH), maltose and lactose were tried in different concentrations (5, 10, 15, 20 and 25%).

Pure sucrose proved to be superior as a carbon source amongst all the tried carbohydrates for all the eight moulds, under investigation. 15% concentration of pure sucrose was found to be optimum for the maximum accumulation of citric acid in Aspergilli while on the other hand, in the species of Penicillium, greater yield was recorded at 20% concentration of the same. However, any further increase in the concentration showed no increase in the yield of citric acid.

Commercial sugar also proved to be good for citric acid production but it was found to be inferior to pure sucrose as a carbon source. Low yield of acid with commercial sugar was probably due to the impurities of trace elements which were incorporated in it.

Glucose and fructose, both proved to be moderate in this respect and among them, fructose was found to be inferior to glucose.

Maltose acted as a superior source of carbon than its hydrolytic product, i.e. glucose in case of A. awamori, A. tamarii, P. multicolor, P. luteum, P. thomii and P. citrinum,
particularly in case of *i. multicolor*, maltose gave an excellent yield and proved to be equally good as sucrose. Only for *A. niger* and *A. flavus*, it was recorded as poor source of carbon.

Only lactose was found to be very inferior with regard to the citric acid production.

On the whole, it is concluded that pure sucrose (BDH) was found to be the best as a source of carbon and the optimum concentration of it seems to fall 15% (for Aspergilli) and 20% (for Penicillia). In further studies, these concentrations of pure sucrose were kept in experimentation.

**Effect of nitrogen sources:**

Next to carbon, nitrogen has been found to govern the ability of an organism to yield metabolic products of importance. Therefore, different inorganic nitrogen sources including nitrate, nitrite and ammonium salts were tried in 7 different concentrations i.e. 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 gm/litre. Besides these, such potential organic nitrogen sources as urea and amino acids have also been studied for their effect on the production of citric acid.

Sodium nitrate was found to be most suitable source of nitrogen for all the tested organisms, as they yielded appreciable amount of citric acid at a concentration of 0.4%
(4 gm/litre). Higher concentration of it was not found to be beneficial for the accumulation of citric acid.

Potassium nitrate was found to be slightly superior to sodium nitrate as a source of nitrogen only in the case of A. flavus and P. thromii; in others, it proved inferior.

In A. niger, ammonium nitrate was found to be slightly superior to NaNO₃. A. awamori was also able to produce enough amount of acid with NH₄NO₃ while in remaining moulds it turned out to be poor and obstructed the accumulation of citric acid. Therefore, it could be concluded that only A. niger has leanings towards the ammonium nitrogen supplied by NH₄NO₃.

Nitrite source of nitrogen (NaNO₂) was found to be inferior to sodium nitrate in all the moulds. In F. multicolor, P. luteum, P. thromii, P. citrinum, A. flavus and A. tamarii, it proved to be superior to NH₄NO₃.

Organic nitrogen sources were found to be less efficacious in this respect. Substantial yield of acid was obtained by A. niger and A. awamori when urea was used as nitrogen source. Arginine also proved to be equally good for A. niger. In F. multicolor enough of citric acid was accumulated only in the presence of histidine and urea. Methionine for F. luteum; aspartic acid for F. thromii; asparagine for A. tamarii; urea for A. flavus and histidine for F. citrinum could be said to
be satisfactory, amongst the tried amino acids. It was noted that nowhere the organic sources could compare well with the inorganic ones.

Effect of Phosphorus sources:

After nitrogen, phosphorus is an essential component of the medium. It is well-known that a suitable phosphorus source and its proper level in the fermentation medium was essential to obtain higher yield of acid. In view of this, different phosphorus sources, di-potassium hydrogen phosphate, potassium dihydrogen phosphate, di-sodium hydrogen phosphate and tri-sodium ortho phosphate were tried in six different concentrations. Both \( K_2HPO_4 \) and \( KH_2PO_4 \) were found to be equally good as sources of phosphorus. It seems to be apparent that maximum accumulation of acid was found at slightly lower level of \( KH_2PO_4 \), i.e. at a concentration of 0.75 gm/litre. While on the other hand, the optimum concentration of \( K_2HPO_4 \) was found to be 1 gm/litre almost for all the eight moulds.

Di-sodium and tri-sodium ortho phosphate were experimented for the first time to study their effects on the production of citric acid. Both the sodium salts were found to be inferior as a source of phosphorus in all the organisms except \( F. luteum \) and \( F. multicolor \).

Remarkable results were obtained with tri-sodium ortho
phosphate in case of *P. multicolor* and *P. luteum*. Both the moulds were able to produce citric acid abundantly when it was added to the basal medium at a rate of 0.5 gm/litre. *P. multicolor* was able to produce 2.131 gm/100 ml while *P. luteum* gave 2.699 gms/100 ml of citric acid, in the presence of Na$_3$PO$_4$. In this way, a difference of 0.523 gm/100 ml in *P. multicolor* and 1.264 gms/100 ml in case of *P. luteum* was recorded when obtained yield with Na$_3$PO$_4$ was compared with the potassium salts.

**Effect of sulphur sources:**

The effect of magnesium sulphate, potassium sulphate, sodium sulphate and ammonium sulphate was studied in different doses (0.25, 0.5, 0.75, 1.0 and 1.5 gm/litre).

Magnesium sulphate proved to be most suitable as a source of sulphur for all the tested Aspergilli and Penicillia. A concentration of 0.5 gm/litre was found to be sufficient in the fermentation medium for the accumulation of citric acid in a greater amount.

Sodium sulphate, potassium sulphate and ammonium sulphate were found to be inferior to magnesium sulphate in this respect.

**Effect of chloride ions:**

The effect of potassium chloride and sodium chloride was
studied on the production of citric acid. They were tried in different concentrations of 0.25, 0.5, 0.75, 1.0 and 1.5 gm/litre.

Results of experiment served to show that the presence of chloride ions (KCl or NaCl), in the fermentation medium was not found to be indispensable, as substantial amount of citric acid was accumulated by each mould even in the absence of KCl or NaCl.

A comparative study of both the sources of chloride ions revealed that potassium chloride proved to be superior to sodium chloride. 0.5 gm/litre of KCl was found to be sufficient to increase the yield in all the eight organisms, although this increase in the yield could not be said to be remarkable.

**Effect of trace elements:**

An effort has been made to determine the effect of the addition of known quantities of trace elements to a highly purified medium on the production of citric acid.

The four trace elements, Zn, Fe, Cu and Mn, were added separately in modified medium in order to study their effect on the production of citric acid.

The original medium was modified on the basis of the results obtained in earlier studies. *A. niger, A. awamori.*
\textit{A. flavus, A. tamarii, F. thomii and F. citrinum} were cultured on the modified medium, having following composition:

\begin{center}
\begin{tabular}{ll}
\textbf{NaNO}_3 & ... & 4 gm/litre \\
\textbf{KH}_2\textbf{PO}_4 & ... & 0.75 gm/litre \\
\textbf{MgSO}_4 & ... & 0.5 gm/litre \\
\textbf{KCl} & ... & 0.5 gm/litre \\
\textbf{Sucrose} & ... & 15\% for Aspergilli \\
& & 20\% for Penicillia \\
\end{tabular}
\end{center}

For \textit{P. multicolor} and \textit{P. luteum}, \textbf{KH}_2\textbf{PO}_4 was replaced by \textbf{Na}_3\textbf{PO}_4, as they gave enormous quantity of citric acid in its presence. For these two organisms original medium was modified and effect of the trace elements was studied on the medium, having following composition:

\begin{center}
\begin{tabular}{ll}
\textbf{NaNO}_3 & ... & 4 gm/litre \\
\textbf{Na}_3\textbf{PO}_4 & ... & 0.5 gm/litre \\
\textbf{MgSO}_4 & ... & 0.5 gm/litre \\
\textbf{KCl} & ... & 0.5 gm/litre \\
\textbf{Sucrose} & ... & 20\% \\
\end{tabular}
\end{center}

Fermentation was run under optimum conditions of incubation (such as length of incubation, pH and temperature) which were found to be optimum in earlier studies.

The medium was purified twice by the method prescribed by Noguchi and Johnson (1961). \textit{Amberlite IR-120 (Resinous}
products and chemicals Co., Philadelphia, U.S.A.) was used as cationic-exchange material.

The results of tests served to show that there was considerable reduction in the yield with the purified medium. The yield of the acid, obtained with the untreated medium, was greater and it was presumably due to the presence of trace elements like Fe, Zn and Pb, in form of impurities as given on the labels of the manufacturers.

Zn and Fe were added to the purified medium in the concentrations of 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.5 and 1.0 mg%.

It is well-known that Cu and Mn were required in minute quantity, therefore, the following concentrations of these two elements were selected:

0.001, 0.005, 0.01, 0.05, 0.1, 0.15, 0.2 and 0.25 mg%.

Inclusion of any trace element, Fe, Zn, Cu or Mn to the purified medium stimulated the citric acid production. Omission of any of them from the fermentation medium had a marked depressive effect in the yields of citric acid. The amounts of Cu and Mn were required in a smaller quantity (viz. 0.001 to 0.01 mg%) than the Zn and Fe in order to obtain the greater accumulation of citric acid. Maximum yield of the citric acid was noted only at the lower levels of Zn, ranging from 0.01 to 0.1 mg/100 ml. Iron was required in
higher amounts, i.e. 0.1 to 0.5 mg%, to obtain the maximum yield. Excessive amounts of any trace element, Zn, Fe, Cu or Mn to the fermentation medium was found to be inhibitory for the production of citric acid in all the tested moulds.

Amongst all the trace elements, Mn had not given any significant results in any of the eight moulds.

Copper was found to be essential component of the medium for the maximum citric acid production, only in the case of \( P. \text{ citrinum} \). In the presence of 0.095 mg% of Cu, \( P. \text{ citrinum} \) produced (0.7178 gm/100 ml), 1.4 times more citric acid than the control and it was 0.2 times more than the yield (0.631 gm/100 ml) obtained with 0.2 mg% of Fe.

With Zn, in all the four Aspergilli and four Penicillia, a substantial amount of citric acid was accumulated. Particularly for \( A. \text{ flavus} \), Zn was found to be most suitable, in which roughly 2.2 times more citric acid was produced (1.445 gm/100 ml) as compared with control.

In \( A. \text{ niger} \), \( A. \text{ awamori} \), \( A. \text{ tamarii} \), \( F. \text{ multicolor} \), \( F. \text{ luteum} \) and \( F. \text{ thomii} \), maximum yields of the citric acid were obtained only in the presence of Fe. \( F. \text{ thomii} \) (1.628 gm citric acid/100 ml) and \( A. \text{ tamarii} \) (1.4578 gm citric acid/100 ml), both were able to produce 2 to 2.3 times more citric acid over the controls in the presence of optimum concentration of Fe.
A drastic increase in the yield has been obtained by the addition of Fe in case of *F. multicolor* and *F. luteum*. *F. multicolor* was able to produce 4.232 g/100 ml of citric acid in the presence of 0.5 mg% of Fe and 0.2 mg of Fe was found to be sufficient to give 5.845 g/100 ml of citric acid, in the case of *F. luteum*. *A. niger* and *A. awamori* produced 3.463 and 3.788 gms of citric acid/100 ml respectively in the presence of optimum concentration of Fe.

When the yields of citric acid obtained by *F. luteum* and *F. multicolor* were compared with the yields of *A. niger* and *A. awamori* then *F. luteum* was found to be 1.3 times more superior to *A. awamori* and 1.7 times superior to *A. niger*, while *F. multicolor* was found to be roughly 1.2 times superior to *A. niger* and *A. awamori*, which were said to be high yielders of citric acid.

However, from the present investigations, it seems to be apparent that citric acid-producing capacity of all the tested moulds except *F. citrinum*, greatly improved only by providing the optimum environmental conditions and nutritional requirements.

On the whole, a remarkable capacity of producing citric acid has been achieved in the case of *F. multicolor* and *F. luteum* by controlling the environmental and nutritional factors. These species, which proved already superior to *A. niger* can be further improved by mutational studies which is in progress.