SECTION A

. GENERAL INTRODUCTION
. HISTORICAL RESUME
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The fungi as a group are highly active biochemically and there are a large number of metabolites produced in the course of their physiological processes.

There are a large number of organic acids, produced by the cultures of microorganisms as a result of dissimilation of sugars. Only a few of them have commercial importance and "Citric acid" is one of the most important acids produced (industrially) by fungi. Recently the production of the citric acid by fermentation, on a commercial basis, has been an important achievement in the field of industrial microbiology.

Citric acid, an unsaturated tricarboxylic acid of aliphatic series, was for the first time isolated from lemon juice and crystallised in solid form by Scheele in 1784 when its commercial utility was not known to anybody. It is found as a natural constituent of many fruits, either in its own (chief) or admixed with malic acid. It is found as mixed form with malic acid in cherry, strawberry and raspberry. While in cranberry, pine apples and all citrus fruits - limes, lemons, oranges and grapes, all the acidity is due to citric acid. The citric acid, extracted from these fruits is known as the "natural citric acid". Prior to the development of the mycological
methods, "natural citric acid" was the main source of supply. It was chiefly produced in Italy, especially in Sicily, California, Hawaii and West Indies. All of the fruits described above are not of equal importance as a source of natural citric acid but the bulk of it was extracted from cull lemons and in small quantity from limes and pine apples.

Properties:

Chemically, the citric acid is known as 2-Hydroxy propane-1,2,3-Tri carboxylic. It forms three series of salts and esters, hence it is a hydroxy tribasic in nature. It contains three COOH groups and one OH group in a molecule. Its chemical and structural formulae are as follows:

\[
\begin{align*}
\text{C}_6\text{H}_8\text{O}_7 & \quad \text{COOH} \\
& \quad \text{CH}_2 \\
& \quad \text{OH} - \text{C} - \text{COOH} \\
& \quad \text{CH}_2 \\
& \quad \text{COOH}
\end{align*}
\]

(Chemical formula of anhydrous citric acid) (structural formula)

Citric acid crystallises in large rhombic prisms, containing one molecule of water of crystallization and the formula is \( \text{C}_6\text{H}_8\text{O}_7\cdot\text{H}_2\text{O} \). The anhydrous form of citric acid which is available for the commercial purposes crystallises in monobasic prisms. It is readily soluble in water, moderately
in alcohol but only sparingly in ether. The water of crystallization of hydrated form is entirely lost at 130°C, it melts at 153°C, and on further heating above 175°C, decomposes into aconitic and itaconic acid, citraconic anhydride, CO₂ and acetone.

Uses:

Citric acid forms a vital part of our civilization and it is widely used for medicinal purposes, food technology and in various industries.

Most of the citric acid is used in drugs and medicines of various kinds. Its calcium salt is used as an alkalinizing agent.

About one-fourth of the total citric acid production is used in food technology and beverage industries. The free acid is to a large extent, used in the preparation of soft drinks and in artificial flavours. Sodium citrate is a valuable emulsifying agent for milk and milk products such as cheese, evaporated and condensed milk, jelly powder and ice-cream. In the field of technology, it is used as silvering agent, as an ingredient of engraving inks and supplementary chemical in dyeing and calico-printing. In Italy, it has been used for synthesis of antipyridine and of certain azodyes. Citrate has also been useful in stabilizing liquid detergents against colour
deterioration.

The esters of citric acid are of particular interest today because of their applicability in the rapid developing field of plastics and synthetic resins. Both tri-ethyl citrate and tri-butyl citrate are excellent solvent plasticizers for cellulose esters and ethers. As about plasticizers, these esters are added to increase the flexibility and extensibility of the plastic. As sequestering agents, citric acid is now being employed to sequester iron, an important factor in "plugging" of oil-wells.

Besides, there are more complicated derivatives of citric acid such as acetyl triesters, which have been prepared and found to have potential value in the manufacture of cellulose acetate, textile and resistance lacquers.

There are a few other related acids namely, aconitic, citraconic, itaconic, which can be prepared from the citric acid.

**Citric acid in world trade:**

Italy produced about 90% of the world's total supply of citric acid, chiefly from the low grade lemons of Sicily, up to 1922. So Italy being the largest producer and other countries like England, France, United States, were dependent on the Italian producers for the supply of calcium citrate.
Major part of the Italian production of calcium citrate was exported to United States. Much of this imported citrate was processed to yield citric acid. For this salt, United States paid about 53 cents per pound in the year 1914 but by 1919 it cost a bit more than a dollar per pound and the total supply for the year cost about 2-3 million dollars. With the increase in duties on both citric acid and calcium citrate, imports of these two commodities in United States began to decline after 1922 and has practically ceased since 1927. A more significant factor contributing to the decline in imports was the commercial development of fermentation method for citric acid production by the moulds.

In the year 1923, the price was reduced by 46 cents per pound and in 1935 it had declined to 28 cents. By the year 1929, the United States was producing about 7,000,000 pounds of citric acid per year. Expansion of the mycological method was so rapid that between 1929-1935, the United States came in a sound position to export large quantities of fermentation citrate, mainly to England. After 1935, however, with the opening of world war II, the export decreased. It was so because the fermentation process had been developed in England in 1936. By the year 1944, United States was producing calcium citrate around 13,000 tons per year, more than 90% of the total output from mould (Wells and Herrick, 1938). In 1948, the annual production of citric acid by fermentation in United States amounted approximately to 35,000,000 lbs. (Perlman, 1949).
These data show how the production of citric acid in United States of America has risen and in recent years, the annual production in U.S.A. exceeds 70 million pounds (Rose, 1961). Thus the former dominant position occupied by Italian producers of this commodity has been completely lost.

Today citric acid is produced industrially by moulds in several parts of the world - United States, England, Japan, Belgium and Czechoslovakia, although complete figures of the production are not available. An institute devoted to the development and improvement of this process is established in Russia also.

**Position of citric acid in India:**

As regards the country's requirements of citric acid, it is imported from abroad as practically, there are no manufacturing firms of this item in the country. The manufacturing process of citric acid is quite simple but proper technique of purification has not been developed in our country so far.

The figures of import, during the past few years from 1937-38 to 1948-49 are as follows (Madan, 1949).
### Import of citric acid

<table>
<thead>
<tr>
<th>Year (period)</th>
<th>Quantity (in tons)</th>
<th>Value Rs. (in thousands)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1937-38</td>
<td>129.15</td>
<td>139</td>
</tr>
<tr>
<td>1938-39</td>
<td>160.50</td>
<td>179</td>
</tr>
<tr>
<td>1939-40</td>
<td>194.25</td>
<td>273</td>
</tr>
<tr>
<td>1940-41</td>
<td>159.05</td>
<td>279</td>
</tr>
<tr>
<td>1941-42</td>
<td>223.95</td>
<td>434</td>
</tr>
<tr>
<td>1942-43</td>
<td>21.15</td>
<td>73</td>
</tr>
<tr>
<td>1943-44</td>
<td>111.80</td>
<td>281</td>
</tr>
<tr>
<td>1944-45</td>
<td>208.15</td>
<td>571</td>
</tr>
<tr>
<td>1945-46</td>
<td>206.45</td>
<td>282</td>
</tr>
<tr>
<td>1946-47</td>
<td>153.85</td>
<td>285</td>
</tr>
<tr>
<td>1947-48</td>
<td>244.35</td>
<td>570</td>
</tr>
<tr>
<td>1948-49</td>
<td>290.70</td>
<td>675</td>
</tr>
</tbody>
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The following figures of import of citric acid during the years 1969-70 to 1972-73 and the nine months (April to December 1973) are obtained from the Director General of Commercial Intelligence & Statistics, Calcutta-1.

<table>
<thead>
<tr>
<th>Year (period)</th>
<th>Quantity (in tons)</th>
<th>Value Rs. (in thousands)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1969-70</td>
<td>1,279.423</td>
<td>4871.037</td>
</tr>
<tr>
<td>1970-71</td>
<td>846.901</td>
<td>4406.707</td>
</tr>
<tr>
<td>1971-72</td>
<td>1,354.877</td>
<td>8401.230</td>
</tr>
<tr>
<td>1972-73</td>
<td>1,403.576</td>
<td>8719.017</td>
</tr>
<tr>
<td>1973 (April to December only)</td>
<td>526.375</td>
<td>3154.095</td>
</tr>
</tbody>
</table>
The figures of the import show a marked increase in the demand of citric acid. The import prices of the foreign product works to approximately Rs. 600 per quintal whereas it can be manufactured in India at a much lower cost. Further, there is a major scope for the manufacturing of the citric acid and its products in India.

The output of mould-products is known to be dependent upon the genetic qualities of the organism in question. Obviously this brings up to the fore the role of microbiologist in this endeavour. The problem before us is to search organisms which yield high in the production of the citric acid and with this aim in view the present investigations were undertaken.

Considering the above facts, an effort was made in the present work to search and evolve high-yielding citric acid producing organisms. The work has been planned in the following format:-

SECTION - A :: PRIMARY SCREENING

In all 118 microorganisms were tested initially.

For this purpose three sets of experiments were carried out:

I. The selection of the organic acid producing fungi by direct agar culture selection method by adding an indicator in the agar.
II. Screening of the organic acid producing fungi by chromatographic technique.

III. Preliminary estimation of citric acid by direct agar culture selection method.

SECTION - B :: SELECTION OF THE FERMENTATION MEDIUM

SECTION - C :: STUDY OF THE ENVIRONMENTAL FACTORS ON THE PRODUCTION OF CITRIC ACID

I. The effect of length of incubation period on the production of citric acid.

II. The effect of different temperatures on the production of citric acid.

III. The effect of pH on the production of citric acid.

SECTION - D :: STUDY OF NUTRITIONAL FACTORS ON THE PRODUCTION OF CITRIC ACID

In this section, the influence of different carbohydrates, nitrogen, phosphorus, sulphur sources, chloride ions, and the trace elements have been studied on the production of citric acid. These studies have been carried out by setting up the following experiments:

I. The effect of different carbon sources on the production of citric acid.

II. The effect of different nitrogen sources on the production of citric acid.
III. The effect of different phosphorus sources on the production of citric acid.

IV. The effect of different sulphur sources on the production of citric acid.

V. The effect of chloride ions on the production of citric acid.

VI. The effect of trace elements on the production of citric acid.
HISTORICAL RESUME
It was Louis Pasteur (1879) who said: "We are convinced that a day will come when moulds will be utilized in certain industrial operations on account of their power of destroying organic matter". Then he immediately proceeded to indicate more specifically what he had in mind by citing the acetification of alcohol in Vinegar process and the production of gallic acid by the action of fungi on wet-gall nuts.

The production of citric acid by fungi was first investigated as early as 1893 when a German Botanist, Car Wehmer, demonstrated that the citric acid was produced by microorganisms. The organisms first studied by him were *Citromyces pfefferianus* and *C. glaber* (later classified by Thom as Penicillia), now purely of historical interest. According to Wehmer, capability of producing citric acid was confined to the species *Citromyces* while the Aspergilli were considered to be oxalic acid producers. The belief in this specific ability of *Citromyces* to produce citric acid was held by several investigators (Maze and Perrier, 1904; Buchner and Wustenfeld, 1909) till it was discovered by Thom and Currie in 1916, who demonstrated the production of citric acid by several strains of *Aspergillus niger*. They showed that citric acid and oxalic acid production could not be exclusively attributed to the activity of any specific fungus but that the
production of these acids was determined by the conditions of fermentation and the property of strains of \textit{A. niger}. In the following year, 1917, Currie published his classic paper "The citric acid fermentation of \textit{A. niger}", describing the production of citric acid by this mould in detail. The work of Currie which was carried out in the department of Agriculture, United States, later forms the basis for the commercial development; it was a milestone in the development of the subject. Currie's work was confirmed by Forges (1932). He had fermented sucrose by using strains of \textit{A. niger} and carefully investigated the nutritional condition for the production of citric acid. Doelger and Prescott (1934) corroborated the findings of Currie and carried out further extensive studies on the techniques of fermentation with the object of determining the methods for maintaining uniformity of conditions which would result in consistent yield of citric acid in successive fermentations.

An enormous or rather bewildering literature has come up round the subject of fermentation of citric acid during the last forty years. Further, the progress in this field has been rapid and a number of reviews have appeared from time to time.

Investigations on optimum conditions and nutritional requirements, for the growth and maximum citric acid producing capacity of \textit{A. niger}, have been made by Steinberg (1935); Wells and Herrick (1938); Melnikova and Trofimova (1940); and recently by Trumphy and Millis (1963). Conditions which control
the citric acid accumulation in fermentation with *A. niger*
have been reviewed by Foster (1949); Johnson (1954); Perlman
and Sih (1960); and Prescott and Dunn (1959).

In early studies, pure sugars (glucose or sucrose) were
used as carbon sources but considerable progress has also been
made towards solving the problems of utilization of crude
substrates such as Gur, cane molasses, beet molasses, corn
starch and wheat starch etc. "Gur" and molasses have been
fermented by species of *Mucor* by Das Gupta, Saha and Guha (1938).
Perlman, Kita and Peterson (1946) studied the citric acid
production by using cane molasses as substrate. Later on,
studies made on the use of cane molasses for citric acid
production have also been reported by Karow and Waksman (1947),
Clark (1962a), Lin (1963), Sanchez (1964), Suzuki et al. (1967)

Considerable researches on the production of citric acid
from beet molasses have also been carried out. The production
of citric acid from beet molasses has been investigated by
Roberts and Murphy (1944); Gerhardt, Dorrell and Baldwin (1946);
Clément (1952); Steel, Martin and Lentz (1954); Steel Lentz and
Martin (1955); Clark (1962b); Clark and Lentz (1961, 1963);
Clark et al. (1965, 1966); Hortisu and Clark (1966); Qudeer
et al. (1969) and Janota et al. (1970).

An effort towards the utilization of brown unrefined sugar
and black strap molasses has also been made for the production
of citric acid with *A. niger* by Gardner *et al.* (1956); Yamada
and Hidenasa (1964) and Sanchez *et al.* (1970). Corn starch,
ground corn, wheat starch, high test beet and black strap
molasses were fermented to citric acid in the presence of a
concentration of 1 to 4 per cent methanol by Moyer (1953b).
Liang and Tung (1965) have used directly gelatinized corn starch
for citric acid production and obtained good yield by adding
two per cent methanol or three per cent ethanol to the
fermentation medium. The effect of alcohols on the production
of citric acid was studied by Moyer (1953a) and according to
him, methyl and ethyl alcohols in concentrations of 1 to 3
per cent and isopropyl alcohol in a concentration of up to 2
per cent increased the production of citric acid in *A. niger*.

These workers usually used ferrocyanide, chelating resins,
metal-chelating agents or alcohols for the treatment of the
crude substrates.

The removal of trace metals from molasses by formation of
complexes with ferrocyanide ions was suggested by Mezzadri
(1938) in order to make this cheap source of carbohydrate
suitable for the production of citric acid by *A. niger*. Since
then, potassium ferrocyanide has been extensively used for the
treatment of molasses, used as a carbon source in the medium.
Its effect is generally attributed for the removal of deleterious
trace metals such as Zn, Fe, Mn, Cu etc., present in the molasses.
Eighteen, out of twenty one metals, were identified in beet molasses with the help of spectrographic analysis by Clark et al. (1965). The use of ferrocyanide for the treatment of molasses, to improve the yield of citric acid, has been reported by many workers, like Gerhardt et al. (1946); Martin (1955); Clement (1952); Hortisu and Clark (1966); Qudeer et al. (1969). Perlman et al. (1946) found that the yield of citric acid by strain of A. niger from untreated solution of molasses was lower in comparison with the ferrocyanide treated molasses. One litre of medium containing 280 gm of molasses was treated with 1.2 gm of potassium ferrocyanide by them. The amount of potassium ferrocyanide used for the treatment of the medium depends upon the composition of the molasses. Clement (1952) used 0.6 to 0.8 gm of potassium ferrocyanide in a litre of beet molasses medium. Hortisu and Clark (1966) reported that potassium ferrocyanide at a concentration of less than 30 ppm had no measurable effect on the citric acid production or on the growth rate during the submerged fermentation but the concentration of 30 ppm of ferrocyanide, however, stimulated the acid formation and markedly inhibited the cell development in growing mycelium. A concentration between 10 to 40 ppm was found to be most suitable for citric acid production by Clark (1962). According to Qudeer et al. (1969), 30 ppm concentration of ferrocyanide in beet molasses medium greatly stimulated the citric acid production.
Most of the investigators, like Tomilson and Campbell (1951), Taha and Zainy (1958), Noguchi and Johnson (1961), found that commercial grade of sucrose or unrefined sugar gave low yield of citric acid when fermented by *A. niger*, owing to the presence of metallic ions in the medium. These workers have used cationic-exchange resins such as Amberlite IR-100, Dowex chelating resin A-1 etc. for the purification of the medium. Perlman, Dorrell and Johnson (1946) have found that the yield of citric acid increased three-fold with the resin treated medium. Sanchez et al. (1970), obtained better yield of citric acid with resin treated medium in comparison with ferrocyanide treated medium.

A new and little used method for the purification of the medium by adding metal-complexing or metal chelating agents was advocated by Choudhary and Pirt (1965a, b). In this method, metal-chelating agents were added to the medium to decrease the concentration of free metal ions which were present as impurities in the medium. Choudhary and Pirt (1966) have shown that an addition of chelating agents—ethylenedia-minitetra-acetic acid (EDTA), 1,2-diaminocyclohexane-N, N-tetra acetic acid (CTDA) or diethylenetriamine penta-acetic acid (DTPA) to the fermentation medium at about 1.0 mM concentration stimulated the citric acid production. They have obtained higher yield of citric acid with EDTA at 1.0 mM concentration.

With the work of Perquin, the submerged culture method of
Growing fungi has been successfully applied to many fermentations where formerly only the surface culture method was used for the production of citric acid. The submerged culture method was first described in detail by Kluyver and Perquin (1937); they grew the mould in a vessel which was agitated and aerated. Then, considerable research efforts were made to develop the submerged fermentation process. The use of ferrocyanide treated molasses to improve the yield of citric acid by submerged fermentation has been reported by Gerhardt et al. (1946); Perlman (1949); Clement (1952); Martin and Waters (1952); Martin (1957); Clark and Lentz (1961); Clark (1962) and Suzuki et al. (1967).

Most of the work has been performed with the small-scale equipments but large-scale studies have also been reported on the citric acid production by *A. niger*. Clark and Lentz (1963) fermented the ferrocyanide treated beet molasses in fully baffled mechanically agitated, 2.5 litre tank type fermenters using pellets of *A. niger* as an inoculum which were grown in shake flasks and thus obtained higher yield (70%) under selected conditions of stirring (agitator speed 400-700 rpm). Buelow and Johnson (1952) using an adaptation of Shu and Johnson (1948), studied the aeration requirements of 100 litre fermentation medium in 50 gallon tanks. Steel, Lentz and Martin (1954) have studied the production of citric acid by using 36 litre fermenters.
The trace elements, like zinc, iron, copper and manganese, play an important role in the production of citric acid and the beneficial effect of these has been shown by many workers. Forges (1932) found that the addition of iron in the fermentation medium increased the yield of citric acid Chrzaszcz and Peyros (1935) also arrived at the similar conclusion regarding the role of iron in the citric acid production. Perlman et al. (1946) and Tomilson et al. (1950) have studied the influence of these trace elements in combination. Citric acid production has been successfully studied in the presence of limited amounts of Zn, Fe, Cu and Mn by Taha and Zainy (1958); Noguchi and Johnson (1961); Agnihotri (1963); Trumphy and Millis (1963); Clark et al. (1966) and Sanchez et al. (1970).

Besides A. niger, the production of citric acid has also been reported by Bacteria, Streptococci and Lactobacilli (Campbell and Gunsalus, 1944) and Yeast, Candida brumptii (Matazo et al., 1970).

It is clear from the brief historical resume that a good amount of work has been done on the citric acid production by black Aspergilli but India is lagging far behind in this field.

Das Gupta et al. (1938, 1940) have studied the production of citric acid and oxalic acid from "Gur" and molasses under controlled conditions of fermentation. Damodaran and his associates (1948) investigated the various factors which govern the citric acid production by strain of A. niger which was
selected after an examination of 13 strains of this organism. Citric acid fermentation by \textit{A. fumaricus} from sugar was first studied by Sen and Sankhala (1953). Further investigations were carried out on \textit{A. fumaricus} by Verma and Sankhala (1954). Mechanism of formation and accumulation of citric acid in \textit{A. niger} was reported by Joshi and Ramakrishnan (1959, 1960). Bhattacharya \textit{et al.} (1962) and Vakil and Bhattacharya (1962) have studied the role of carbon dioxide in the production of citric acid. Studies on the citric acid metabolism, nutritional requirements and metabolic changes during the citric acid utilization by \textit{A. niger} were made by Shah and Ramakrishnan (1962, 1963). An evaluation of citric acid producing power of four Indian isolates — \textit{A. niger}, \textit{A. phoenicis}, \textit{A. luchuensis} and \textit{A. awamori} — has been made under similar culture conditions by Mehrotra and Laloraya (1962), in which \textit{A. luchuensis} was found to be superior to the other tested Aspergilli. Studies on the production of some useful acids namely citric, fumaric and itaconic acid by the species Aspergilli were made by Mehrotra and his associates (1968). According to them, strains of three species, viz. \textit{A. niger} (NRRL, 372), \textit{A. phoenicis} (A13, 186) and \textit{A. awamori} (A 13, 188) were found to be more promising for the citric acid production. Agnihotri (1963) studied the effect of the trace elements — iron, zinc, copper and manganese — on the production of citric acid and concluded that copper and manganese were required in smaller quantities than those of iron and zinc. A project for the development of
superior strains of *A. niger* was undertaken by Das and Nandi (1965a & b, 1969). The parent strain of *A. niger* was first treated with nitrogen mustard (0.01 to 0.1%). Then the most promising sub-strain was subjected to ultraviolet irradiation and gamma rays treatment and ultimately they have obtained three times more citric acid by one of the isolated substrains of *A. niger*.