Studies on the Mycological Production of Citric Acid in India

II. Effect of factors other than nutritional

B. S. Mehrotra & Jyoti Singh

Botany Department, University of Allahabad

Besides nutritional factors there are a number of other factors which govern the production of a metabolite. Chief of these are the temperature, length of incubation and pH of the medium.

The temperature range of 20—35° C has been reported to be most suitable for citric acid production (Doegler and Prescott 1934; and Cahn 1935) and above this range the effects of certain metallic ions on citric acid production were accentuated (Tomlinson et al. 1951). However, sometimes the fungus had been grown at one temperature to obtain abundant mycelium and then transferred to the other to get a maximum yield (Kovats 1946).

The length of incubation period is another controlling factor for the production of citric acid. The optimum time required to obtain highest yield has been reported to be 9—12 days by Doegler and Prescott (1934) and 10 days by Gerhardt et al. (1946). Shu and Johnson (1948) distinguished two distinct phases in citric acid production (i) the phase during which the mycelial growth of an organism occurs or "initial growth phase "; followed by the (ii) "fermentation phase". The maximum yield is obtained at some point, where the cell material is enough to convert the residual sugar into a product and this fermentation capacity generally decreases with the age.

Hydrogen-ion concentration effects the metabolism of fungi in two ways. Externally it controls the degree of dissociation of inorganic ions in the culture solution and since the dissociation plays a part in the movement of the ions into the fungus, degree of dissociation will effect the fungal growth and metabolism. Internally it can cause a change in the pH of the mycelium with the result the behaviour of the fungus is absolutely different at different pH values.

The role of hydrogen ion concentration in citric acid production was first realized by Currie (1917). A low pH generally favours citric acid formation, minimizes the danger of contamination and makes sterilization simple, while high pH favours the production of oxalic acid and other undesirable organic acids and encourages contamination. After the reports of Currie (1917) further emphasis on the important role of pH in the citric acid fermentation was made by Bernhauer

(1926); Challenger (1929); Doegler and Prescott (1934); and Shu and Johnson (1948) and they all were in favour of low initial pH range (1.6—2.4) of the fermentation medium. Oxford et al. (1949 a) used a higher initial pH, i. e., 5.0—5.8 for fermentation of Natal mill molasses by *Aspergillus wentii*. However, the best citric acid producing fungi possess the greatest tolerances to low pH values, and the most favourable pH will depend largely on the strain and the fungus used (Bernhauer 1929).

In this second paper of the series an attempt has been made to determine the optimum temperature, incubation period and pH of the medium for maximum production of citric acid by six selected strains of *Aspergillus niger* group yielding fairly high yields of acid.

Materials and Methods

Pure cultures of the six strains of *Aspergillus niger*, viz., M_2, L_3, RK_2, A_2, SJ_1, and O_{42} were grown on Czapek's solution agar slants containing the following constituents: sucrose, 30 g; $NaNO_3$, 3.0 g; KH_2PO_4, 1.0 g; KCl, 0.5 g; $MgSO_4$, 0.5 g; $FeSO_4$, 0.004 g; agar agar, 20 g; and distilled water to make 1000 ml. A heavy crop of spores was obtained at 25° C within 4—6 days.

The fermentation medium employed was that developed by Doegler and Prescott (1934) containing per liter *): sucrose, 150 g or glucose **), 150 g or Gur ***) to supply 15% invert sugar (these carbohydrate sources have been found economical and equally good as sucrose by the author in the previous experiment), NH_4No_3, 2.23 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.223 g and distilled water to make 1000 ml. 100 ml portions of this medium were poured in 300 ml. Erlenmeyer pyrex flasks as fermentation and 25 ml. in 150 ml. flasks as germination medium. The flasks were steam sterilized for half an hour and for three successive days.

A heavy spore suspension was obtained by adding an equal amount of inoculum in flasks containing 25 ml. of germination medium. These flasks were kept at room temperature 28° C ± 1 for 4 hours. After 4 hours 10 ml. of the germinated spore suspension from each flask were poured in flasks containing fermentation medium in triplicates. The experiments were run in surface culture.

Effect of temperature

The pH of the fermentation medium was adjusted to 2.4—2.8 by adding required amount of N/10 hydrochloric acid. The flasks were

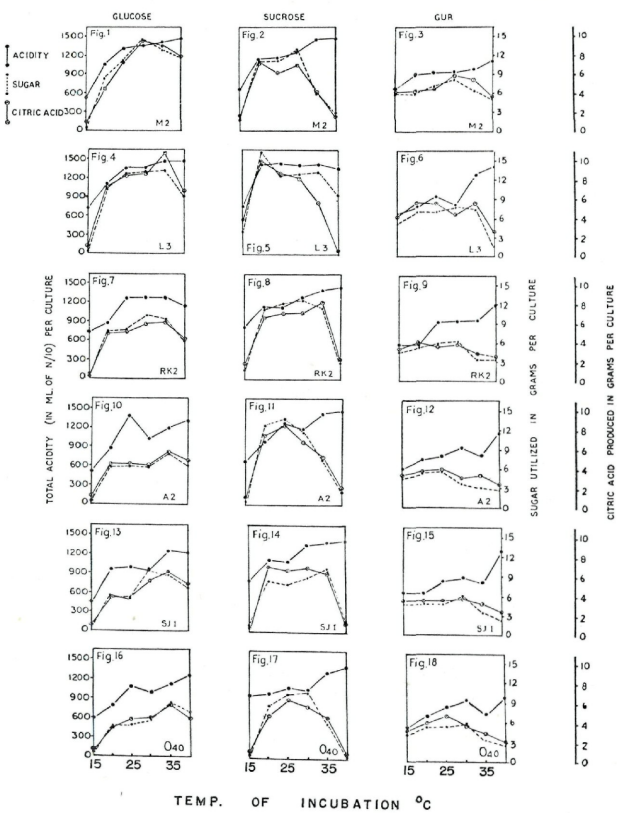
*) Commercial (market) sugar.

**) Dextrosal, a corn product, Corn product Co., India, Private Ltd., Bombay — 1.

***) A native product used as a substitute of sugar, prepared by concentrating sugar cane juice.

kept undisturbed at a temperature of which the effect was to be observed for one hour before inoculation to avoid the lag effect. Three flasks of each sugar (either sucrose or glucose or Gur) were inoculated with a heavy spore suspension of each strain and were kept at the following temperatures: 15°, 20°, 25°, 30°, 35° and 40° C in order to

EFFECT OF DIFFERENT TEMPERATURES ON THE CITRIC ACID PRODUCTION BY SIX DIFFERENT STRAINS OF ASPERGILLUS NIGER GROUP TAKING GLUCOSE, SUCROSE AND GUR SEPARATELY AS CARBOHYDRATE SOURCES.



ascertain the effect of temperature on the yield of citric acid in the presence of different sources of carbohydrate. The cultures were incubated for 10 days.

In one more experiment all the six strains were cultured on the above medium with sucrose as carbohydrate source and the temperature of incubation was changed after every 24 hours within the range of 20—35° C and the yield of citric acid was observed.

Effect of pH

The pH of the fermentation medium was adjusted to 2.4—2.8 after sterilization and a series of flasks with three different sugar sources were inoculated with each strain. The cultures were incubated at 30° C and three flasks from each sugar and of each strain were estimated daily from the second day till the 12th day.

Effect of pH

All the three sugar sources were tried to observe the effect of different pH values. The pH of the medium was adjusted after sterilization by addition of N/10 HCl or N/10 NaOH. Hydrochloric acid was used to adjust the pH values for the element chlorine has a distinct value as a constituent of the medium. The pH of the different lots of fermentation medium was adjusted within the range of 1.0 to 7.0 with differences of pH 1.0 but this difference was later narrowed down to 0.5 within the pH range showing maximum production of acid in order to determine the optimum pH. All the six strains were tested on each pH value and for each sugar source. The experiments were run in triplicates and the cultures were incubated at 30° C for 8 days as these have been found to be optimum conditions for maximum production of acid.

Estimations of citric acid were made by the method of Bernhauer (1926), sugar estimations were done by Somogyi's method (1945), and the total acidities were measured by titrating with 0.1 N sodium hydroxide using phenolphthalein as indicator.

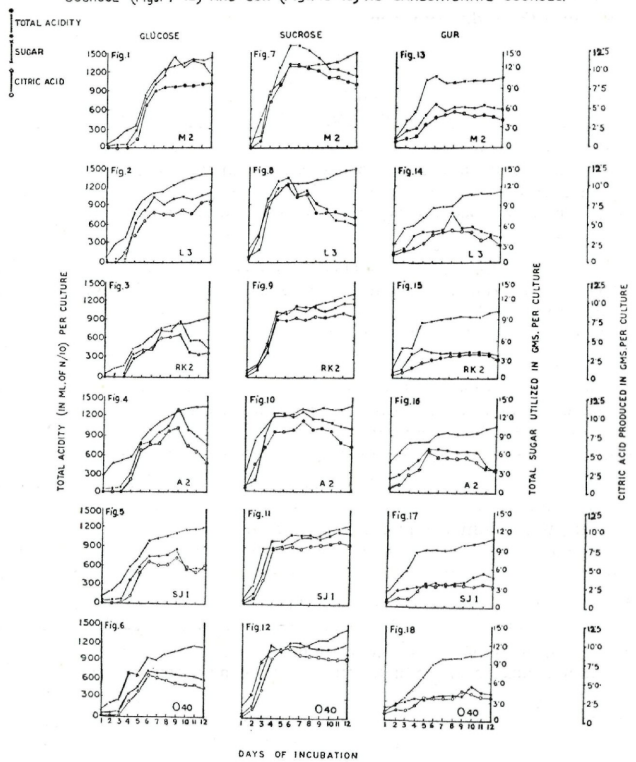
Results and Discussion

Effect of temperature

The results are presented in plate 1, figs. 1—18. At 10° C the strains did not grow and at 15° C the growth of the strains was late and poor, thus very little citric acid accumulated. At higher temperatures, the yield started increasing and the optimum temperature for citric acid production was 30° C for strains M_2, RK_2 and SJ_1 for all the three sources of sugar, 35°, 20° and 30° C for strain L_3; 35°, 25° and 25° C for strain A_2; and 35°, 30°, and 30° C for strain O_{40} on glucose (dextrosol), sucrose and 'Gur' respectively. At 40° C very little citric

acid was produced by all the strains and at still higher temperatures the oxalic acid formation was favoured. The yield of citric acid was not much effected by fluctuating temperatures provided the tempera-

EFFECT OF THE LENGTH OF INCUBATION PERIOD ON CITRIC ACID PRODUCTION BY SIX STRAINS OF ASPERGILLUS NIGER GROUP WITH GLUCOSE (Figs. 1-6), SUCROSE (Figs. 7-12) AND GUR (Figs. 13-18) AS CARBOHYDRATE SOURCES.



ture is not lowered or raised considerably from the optimum range which was 20° C — 35° C differing only slightly with the strain and the carbohydrate used.

The present results are in conformity with those of C a h n (1935) and T o m l i n s o n et al. (1951) who found the temperature range of

20°—35° C as the most suitable for citric acid production. However, Doegler and Prescott (1934); Karow and Waksman (1947); Oxford et al. (1949 a); and Steel et al. (1955) reported that the citric acid production decreased beyond 30° C. This difference may be due to the different cultural conditions and the strain used. According to the results obtained slight fluctuation of temperature has not much effect on the yield, a more economical production of citric acid is possible as maintaining constant temperature throughout the year may cost the industry more.

Effect of the length of incubation period

The results are summarized in plate II, fig. 1—18. It was concluded from the results that the citric acid formation took place after 2 or 3 days growth when glucose was the substrate, after that it started gradually increasing and reached a peak which was as follows: 6 days for strain O_{40}; 9 days for strain M_2, RK_2, A_2 and SJ_1; and 12 days for strain L_3. The acid accumulated from the second day in the case of sucrose and maximum yield was obtained after 5—8 days from M_2 and L_3; 6 days from O_{40}; 7 days from A_2; and 8—11 days from SJ_1. In the case of 'Gur' the acid formation started from the second day and the maximum yield was obtained on the 5th day from A_2; 6th day from SJ_1; 7th day from M_2; 8—11th day from RK_2; and 10th day from O_{40}. After that the rate of acid accumulation retarded.

It is evident from the results that the influence of incubation period on citric acid yield varies with the strain and the carbohydrate source used. It is also clear that there are two distinct phases in this fermentation, the first is the "initial growth phase" where the sugar split products combine with nitrogen, sulphur and minerals to build up the component of cell material, and near the end for maximum growth are maximum yields obtained due to diversion of carbohydrate dissimilation to acid formation channels. This is the second "acid formation phase" where the sugar split products cannot be further converted into protoplasmic material in conjugation with nitrogen, sulphur and minerals because the latter are absent (Foster 1949). This observation supports the finding of Shu and Johnson (1948) who distinguished clearly two phases in the citric acid fermentation.

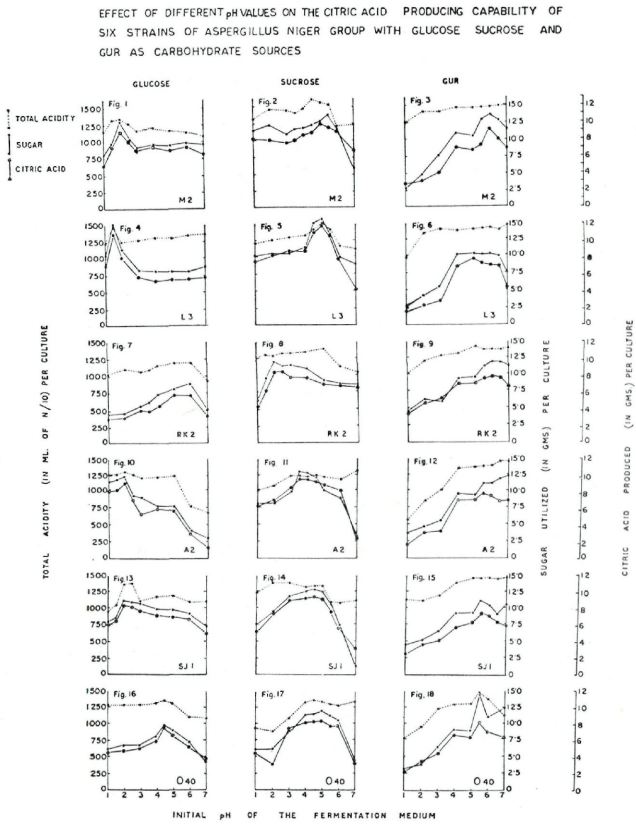
The decrease in the yield during later period of incubation may be due to the CO_2 formation at the expense of the citric acid formed, as it is well keown that some carbon dioxide is always formed by moulds via oxidative metabolism (Foster 1949).

It has also been observed that the citric acid accumulation starts from the second day in the case of sucrose and Gur while it takes place from the fourth day in the case of glucose. This may be explained by the fact that the fructose portion of sucrose is much more efficient in generating citric acid than glucose (Bernhauer 1928 c). Therefore,

as soon as sucrose is split into its hexose component, the fructose portion is utilized in the citric acid formation.

Effect of different pH values

The results presented in plate III and figs. 1—18, show that the citric acid yield was very poor below pH 1.0 and an increase in the acid production was observed as we go to higher pH range. Maximum yield was obtained at 2.0, 5.0 and 6.0 in the case of M_2; 1.5, 5.0, 5.0 in



the case of L_3; 5.0, 2.0 and 6.0 in the case of RK_2; 2.0, 4.0 and 5.5 in the case of A_2; 2.0, 4.5 and 5.5 in the case of SJ_1; and 5.5, 5.0, 5.5 in the case of O_{40} on glucose, sucrose and 'Gur' respectively. Above these pH values the citric acid production was very poor and the high value of total acidity was due to the oxalic acid formation.

It is also evident from the results that the initial low pH values for acid formation is needed only in the case of glucose. In the case of sucrose and 'Gur' the strains produced fairly good amount of acid even at pH 5.5 where there was no need of adjusting the pH of the medium by addition of extra mineral acid. Similar has been the experience of Bernhauer (1929). According to him a strain, able to tolerate and form organic acid in the presence of high initial acidity, might be a superior acid former in the absence of high initial acidity.

The need of extra addition of mineral acid in the case of glucose and not in the case of sucrose and 'Gur' may also be explained by the results of previous study (the effect of incubation period). The acid accumulation started from the very second day and lowered the pH to 1.8—2.2 when the substrate was sucrose and 'Gur' while fermentation started a little later, i. e., from the fourth day in the case of glucose, therefore, the addition of extra mineral acid is required at the initial growth stage so that the fungus may not form a heavy mycelial mat at the expense of substrate.

It was concluded from the result that an acid environment created either by addition of mineral acid or by organic acid fermented in the medium is necessary for citric acid production.

Summary

The effect of temperature, length of incubation period and hydrogen-ion concentration (pH) on citric acid production by six strains of *Aspergillus niger* group viz., M_2, L_3, RK_2, A_2, SJ_1 and O_{40} was determined. Three different sources of carbohydrate viz., glucose (Dextrosol, a corn product), sucrose (commercial market sugar) and 'Gur' (a native product used as a substitute of cane sugar prepared by concentrating the sugar cane juice) were tried.

The temperature requirement differed slightly with the strain and the carbohydrate used but the optimum range of temperature for acid production was 20—35° C. The citric acid yield was not much effected by the fluctuating temperatures provided the temperature is not lowered or raised much from the optimum range.

The optimum incubation period for conversion of sugar to citric acid was 6—12 days for glucose and 4—10 days for sucrose and 'Gur'. The amount of citric acid formed decreased on further incubation. However, there were two distinct phases of fermentation, first was the growth phase which extended upto 3—4 days with glucose; upto 2 days

with cane sugar; and 1—2 days for 'Gur'. Beyond that the fermentation phase started which lasted upto 10—12 days.

The optimum pH for citric acid production with glucose, sucrose and 'Gur' respectively was 2.5, 5 and 6 for strain M_2; 1.5, 5.0, 5.0 for L_3; 5.0, 2 and 6 for RK_2; 2.0, 4.0 and 5.5 for A_2; 2.0, 4.5, 5.5 for SJ_1; and 4.5, 5, 5.5 for O_{40}. However, it was concluded that too low a pH was required for fermentation in the case of glucose while maximum acid accumulated at pH 4.0—5.0 and 5.0—6.0 in the case of sucrose and 'Gur' respectively. Exceptionally for strain RK_2 and O_{40} higher pH was required for fermentation with glucose also and a low pH for sucrose only by RK_2.

Acknowledgements

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* Originals not seen.