Mycological Production of Citric acid in Indian

I. Utilization of some cheaper and readily available sources of carbohydrate

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One of the principal factors responsible for an economical mycological production of citric acid is the use of a cheaper, more readily available source of carbohydrate. A variety of carbohydrates having 2, 3, 4, 5, 6, 7 and 12 carbon atoms are known to produce citric acid but the maximum yields have been secured usually from sucrose and fructose. Occassionally, glucose under certain conditions has given high yields. Von Loesecke (1945) reviewed the literature dealing with the use of cheap carbohydrate sources, i. e., cane, beet or high test molasses, cellulose hydrolysate, and starch bi-products for citric acid fermentation in surface culture. Some of these materials have also been employed in submerged culture process (Karow, 1942; Sjolander, 1945; Waksman and Karow, 1946; and Moyer, 1953 b).

The object of the present investigation was to obtain citric acid on a large scale from our six selected strains. For this only cheaper and readily avaiable native carbohydrate sources have been tried.

Materials and Methods

The six good citric acid yielding strains of the *Aspergillus niger* group selected were: M_2, L_3, RK_2, A_2, SJ_1 and O_{40}. The medium employed for fermentation was that developed by Doegler and Prescott (1934) with the following constituents: Sucrose (commercial market sugar), 150 g; NH_4NO_3, 2.23 g; KH_2PO_4, 1.0 g; $MgSO_4 . 7H_2O$, 0.223 g; and distilled water to make 1000 ml. 100 ml. portions of this solution were taken in 300 ml. Erlenmeyer pyrex flasks as fermentation medium and 25 ml. in 150 ml. flasks as germination medium. The flasks were steam sterilized for half an hour and for three successive days. The pH of the fermentation medium was adjusted to 2.4—2.8 after sterilization. The inoculum for this purpose was prepared on Czakep's solution agar medium. This medium yielded a heavy crop of spores at 25°C within 4—8 days. The heavy crop of spores was dispensed in the germination medium (care must be taken for equal size of inoculum)

and was kept at room temperature for hours. After this period 10 ml. of the germinated spore suspension was poured into the flaks containing fermentation medium. The cultures were incubated at about 28° C (± 1) for 10 days. The experiments were run in triplicates in surface cultures.

At the end of 10 days incubation period the cultures were harvested by filtration. As the experiments were run in triplicates the filtrates of the three flasks were mixed and this volume of the aliquot was raised to 300 ml. by adding distilled water. The estimations were made for the residual sugar, titrable acidity and citric acid. Sugar estimations were made by Shaffer and Hartmann's method (1921). Titrable acidity was determined by neutralizing 2 ml. of aliquot solution against N/10 NaOH solution using phenolphthalein as indicator. For estimation of calcium citrate and citric acid the method of Bernhauer (1926) was adopted.

The following carbohydrates, in different concentrations, were tried:

(i) *Glucose:*

The glucose used was 'Dextrosol', a maize product of Corn Products Co., India, Private Ltd. Seven different concentrations of glucose used were: 10%, 12.5%, 15.0%, 17.5%, 20%, 22.5% and 25%.

(ii) *Liquid Glucose:*

This was a concentrated syrupy viscous liquid containing about 60—63% invert sugar. The liquid glucose tried was in amounts so as to furnish 15% of reduced sugar and all the six strains were tested.

(iii) *Sucrose:*

The sucrose used in this investigation was commercial crystalline cane sugar. Seven different concentrations, viz., 10%, 12.5%, 15.0%, 17.5%, 20.0%, 22.5%, 25.0% were tried for all the six strains.

(iv) *Sugar cane juice:*

Fresh sugar cane juice that contained 8.2% sugar was also tried for citric acid production by all the six strains.

(v) *Gur:*

It is a native product prepared by concentrating the sugar cane juice and contains nearly about 60—80% sugar mainly as sucrose, glucose, fructose along with some mineral impurities as phosphates and soluble ferrow salts. Seven different concentrations of 'Gur' were taken so as to supply 10%, 12.5%, 15.0%, 17.5% 20.0%, 22.5%, 25.0% of reduced sugars.

(vi) *Cane Molasses:*

The molasses taken for the investigation were the common commercial molasses of cane sugar industry. This molasse was diluted 2—3 times and was treated with potassium ferrocyanide (0.8 g per 250 g molasses) and then it was passed through an activated charcoal

column to remove colour and ash to some extent. In another method adopted to purify the cane molasses, it was diluted 2—3 times and then treated with lime to remove phosphate as calcium phosphate precipitate. This precipitate was filtered off and CO_2 gas was passed into the filtrate to acidify the solution. This solution was then passed through an activated charcoal column to remove colour and ash.

All the six strains were cultured in the diluted molasses' solution with 15% sugar plus mineral nutrients.

(vii) *Starch:*

Starch used in this experiment was maize starch and the constituents of the medium were those employed by Moyer (1953 b). 11% of starch was tried for all the six strains. The diastatic activity of the strains has also been tested by the method of Bose and Sarkar (1937), Bhargava (1943) and Mehrotra (1948) to substantiate the results.

In another experiment different concentrations of maize starch (10.0%, 11.0%, 12.0%, 13.0%, and 14.0%) were tested for the strain producing maximum acid. For the same strain different types of starch as BDH starch, Anil starch, Tapioca starch and dextrin in the concentration found to be most suitable in the previous experiment were tested.

(viii) *Malt:*

The malt used in this experiment was supplied by Mohan Meakin Breweries, Lucknow. It contained starch, soluble albuminoids, insoluble albumionids, fat, invert sugar, ash soluble, ash insoluble, gums and fibres. The amount of malt added to the medium was so as to supply 15% of total reduced sugar. In one more experiment malt was added to the medium in smaller quantities, i. e., 1.0 gm. and 2.0 gm./culture and the percentage of market sugar was reduced. All the six strains were tested again.

Results

The results are presented in the Plates I & II and Figures 1—18 and 1—6.

Glucose was found to be a good source of carbon for citric acid production for all the six strains. The citric acid yield increased with the increase in glucose concentration up to 15% in the case of L_3, RK_2 and SJ_1, up to 17.5 in the case of A_2 and up to 20% in the case of M_2 and O_{40}. The most favourable concentration range of glucose for citric acid production, however, was 12.5% to 20.0%. On liquid glucose the strains produced moderate amount of acid but less than in glucose.

Cane sugar also acted as a good source of carbohydrate, better than glucose for citric acid production by all the six strains. The acid accumulation increased with the increase in sugar concentration from 10 to 20% in the case of M_2, A_2 and O_{40}. In the case of L_3 increase

in the amount of acid was up to 22.5%. Maximum yield of citric acid was obtained on 15% sugar concentration in the case of SJ_1 and 17.5% in the case of RK_2 after which the rate of citric acid accumulation decreased. On sugar cane juice two strains, i. e., M_2 and L_3 produced fair, RK_2 and A_2 moderate, and SJ_1 and O_{40} poor amount of acid.

Gur also proved to be a good source of carbohydrate for acid production. It was inferior to sucrose but almost equivalent to glucose in acid production. The optimum concentration of Gur for acid production was 17.5% for M_2, RK_2 and O_{40}, 20% for L_3, 15% for SJ_1 and 12.5% for A_2.

Starch was observed to be a good source for acid production by M_2, L_3, A_2 and O_{40}. Especially M_2 produced a fairly good amount of acid (9.7 gm/11 gm starch) in case of a longer period of incubation, i. e., 15 days. RK_2 showed moderate and SJ_1 poor yield of acid on starch medium. It was also observed that 11% starch was best for acid production. Different varieties of starch tested for strain M_2 ranked in importance as follows:

BDH starch, Anil starch, dextrin and tapioca starch. The strains, however, showed the following relative degree of diastatic activity — $M_2 > A_2 > L_3 > O_{40} \geq RK_2 > SJ_1$, which is in correlation to their capacity to yield citric acid.

None of the strains produced acid on the medium containing malt as the carbohydrate source. Malt did not work even if it was partially replaced by cane sugar in the medium.

Molasses favoured poor growth, encouraged sporulation and oxalic acid formation. Both the methods of purifcation of molasses have been ineffective.

Discussion

Sucrose, which was found to be the best source of carbohydrate for citric acid production in the present case has been found the same by most workers (Prescott and Dunn, 1940). Bernhauer (1928 c) tested several carbohydrates for citric acid production and found sucrose and fructose to be the best for acid production. He explained the superiority of sucrose by ascribing it to the fructose portion of sucrose, responsible for acid production which is in the active gamma form and is much more efficient in generating citric acid, thus compensating for the low yielding glucose portion of the disaccharide. Another possible explanation is that the hexose may be phosphorilated when it is split in a manner similar to that in *Pseudomonas saccharophila* (Doudoroff *et al.*, 1943). Gur was also well utilized by these strains, the cause may be that it is mostly composed of sucrose and rest fructose and glucose which are good sources for citric acid production. The inferiority to sucrose may be due to the mineral impurities, for example,

iron and phosphates, as contaminents. The efficiency to produce citric acid in starch medium by four strains, less by RK_2 and very little by SJ_1, is due to the relative diastatic activity.

Cation impurities seem to be mainly responsible for low citric acid yield from crude carbohydrate sources as malt and cane molasses (Shu and Johnson, 1947; and Waksman and Karow, 1946). As the organic acid formation is very susceptible to metallic ions, this mineral unbalance disturbs the metabolism of a fungus. Also, it appears that the ash present in molasses played a major role in inhibiting the production of citric acid.

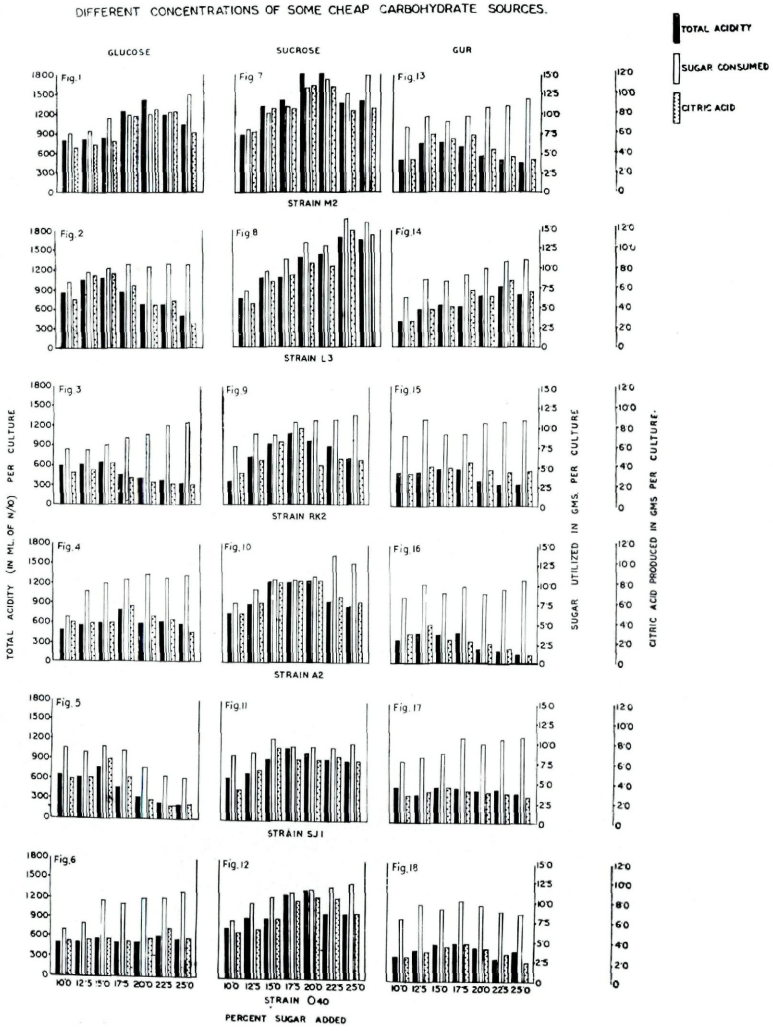
That concentration of carbohydrate in the medium is an important factor was evident from the result. The citric acid production increased with increase in the sugar concentration up to an optimum (this optimum concentration varied with the strain and sugar). This may be due to the fact that at lower concentrations the sugar present is so limited that most of its part is utilized in cell synthesis and the growth of the fungus. And at higher sugar concentrations the excess of sugar results in a faulty metabolism as indicated by only partial utilization of the sugar molecule leaving incompletely oxidized products accumulating in the medium, usually organic acids (Foster, 1949). It appears that the enzymes normally involved in complete oxidation of the substrate become saturated and the excess of sugar molecules in the medium is shunted to the subsidiary enzyme system. These subsidiary enzyme systems effect only minor changes in the substrate, which accumulates in the transferred form as citric acid.

These organisms show best evidence in support of the metabolic shunt, i. e., other factors remaining constant, the enzyme saturation can be demonstrated simply by increasing the concentration of the carbohydrates. In media with very low percentage of sugar (from 0 to 0.5—2.0%) moulds usually yield little of organic acid. But with increase in concentration of sugar from a certain percentage (2.0 and above) the accumulation of citric acid increases till about 15% of sugar concentration in the medium. This change in synthesis of citric acid is more a measure of sugar split products in excess of those required to saturate the enzyme system involved in the synthesis of protoplasm and in the oxidation of CO_2. Inhibition at high sugar concentration may be because of the fact that the osmotic pressure outside becomes higher than the osmotic pressure inside the mycelium with the result that the diffusion of substrate into the mycelium is inhibited (Foster, 1949).

Summary

Effect of the different concentrations of some cheap carbohydrates viz., glucose (dextrosol), liquid glucose, sucrose (cane sugar), sugar cane juice, 'Gur', molasses, starch and malt on citric acid producing capabi-

CITRIC ACID PRODUCTION BY SIX STRAINS OF ASPERGILLUS NIGER GROUP FROM DIFFERENT CONCENTRATIONS OF SOME CHEAP CARBOHYDRATE SOURCES.



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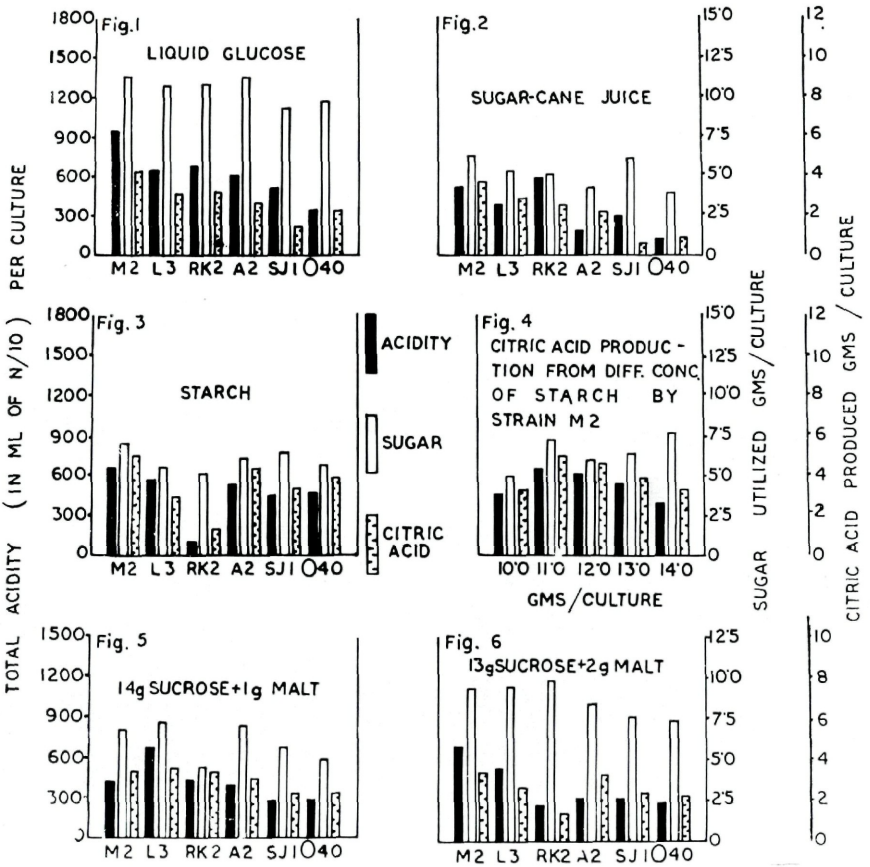

lity of six strains of the *Aspergillus niger* group (M_2, L_3, RK_2, A_2, SJ_1 and O_{40}) was observed. All the above mentioned carbohydrate sources were favourable for citric acid production except malt and molasses. Maximum yield was obtained with 15—22.5% commercial cane sugar. The strains produced fair amount of acid also on glucose, 'Gur' and starch and these sources can be used as substitutes of sugar. The optimum concentration of these sources varied with the strains tried. However, this concentration ranged between 10—20% for glucose and 'Gur'. In the case of starch the most suitable concentration range was 10—12.0%. None of the strains produced citric acid on the medium containing malt or molasses as the carbohydrate sources. Malt did not work even if it was partially replaced by cane sugar in the medium. In the case of molasses both the methods of purification viz., (i) by the treatment with potassium ferrocyanide and (ii) by precipitating phosphate impurity with lime, were ineffective. It favoured poor growth, encouraged sporulation and oxalic acid formation.

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