Pakistan J. Sci. Ind. Res., Vol. 13, No. 4, December 1970

STUDIES ON METHODS OF CITRIC ACID FERMENTATION FROM MOLASSES BY ASPERGILLUS NIGER

M.A.A. KHAN, M.M. HUSSAIN, M.A. KHALIQUE and M.A. RAHMAN

Department of Botany, University of Dacca, East Pakistan

(Received December 30, 1969; revised April 2, 1970)

A study of the effect of different concentrations of sugar and added inorganic nutrients and of different pH values of the fermentation medium on the citric acid production from cane molasses was made. The use of molasses in final concentration of 12.5–15.0% sugar was found best. The initial pH ranging from 3.5 to 6.0 in the molasses solution was found suitable for citric acid production. The concentration of added inorganic salts should not exceed 4.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.23 g $MgSO_4.7H_2O$, 0.02g $FeCl_3$, 0.0012 g $ZnSO_4$ and 0.0012 g $MnCl_2.H_2O$ in the fermentation of local cane molasses. At higher concentration of salts fungal growth increased and the citric acid production decreased. As a source of nitrogen, peptone was inferior to sodium nitrate and potassium nitrate whose effect appears to be the same in the citric acid production.

Citric acid was first isolated and crystallised from lemon juice by Scheele.[1] The acid was commercially produced from citrus fruits chiefly in Italy. Wehmer[2] found that the citric acid was a fermentation product of molds. Thom and Currie,[3] Currie,[4] and Doelger and Presscott[5] showed that the citric acid could be produced in bulk from fermentation by *Aspergillus niger*. Now-a-days the citric acid comes almost wholly from the fermentation industries of U.S.A., U.K., Germany, France and Japan. A large number of patents have been taken out in the field but the actual methods at present in use in different countries have not been made public in their entirety, mainly because of the trade secrecy.

The (1) organisms, (2) inorganic salt requirements, (3) pH of the medium, (4) temperature, (5) sugar concentration, (6) volume of the solution, (7) air supply, (8) incubation period and (9) surface or submerged culture methods are the main considerations of a method of industrial production of citric acid by fermentation.

Suitable conditions required for surface culture method have been studied from the beginning by many workers.[4,5] In general, highest yields of citric acid have been obtained in the fermentation medium adjusted to suitable pH with metal concentrations deficient for optimal growth of the fungus.

Citric acid which has many industrial uses is now wholly obtained by imports from abroad. The manufacture of citric acid can stop import and as well as solve the problem of the economic utilization of our molasses which are the chief raw materials for citric acid industries. Here the concentration of sugar and added inorganic nutrients, and initial pH value of the fermentation medium made from local cane molasses have been studied in order to find out optimum conditions for the highest yield of citric acid during fermentation. Isolation of molds has also been done in order to find out good citric acid fermenting strains of *A. niger*. Locally isolated strains were compared with 2 strains of IMI41874 and IMI75353 of *A. niger* obtained from Commonwealth Mycological Research Institute, London.

Method

The determination of citric acid followed the technique of Taylor[6] as modified by Hughes[7] and Solomos.[8] Sugars were determined according to the method of Somogyi[9] as described by Hockenhull and Herbert.[10]

Preparation of Inoculum.—The organisms were sporulated on the slanted medium of Waksman and Fred[11] in the test tube. Ten ml of sterilized distilled water was added to the tube, shaken and poured in the flask containing 90 ml sterilized water. The spore suspension was added to the fermentation medium.

The fermentation was done during this experimental work according to the surface culture technique. The flasks after inoculation of spores were left undisturbed in the laboratory at room temperature which ranged from 24.0 to 31.5°C depending on the season of the year. After the surface of the fermentation medium was covered by fungal mat, the cotton plug was removed from the flask. No extra air was supplied. Results of four experiments done under each condition were close and the average results are shown in Tables 1–5.

Results and Discussion

Isolation of Organisms.—The organism was isolated from the atmosphere in the laboratories of the Department of Botany and from the soil and

TABLE 1.—INITIAL SUGAR CONCENTRATION, TOTAL FUNGAL GROWTH, PRODUCTION OF CITRIC ACID CONSUMPTION OF SUGAR RECOVERED AS CITRIC ACID PER 100 ml SOLUTION.

Initial sugar concentration (%)	Total fungal growth (dry wt. in g)	Citric acid produced (g)	Sugar consumed (g)	Utilized sugar recovered as citric acid (%)	Total sugar recovered as citric acid (%)
5	2.00	0.50	4.97	10.00	10.00
10	2.36	3.00	7.30	40.60	30.00
12.5	2.55	3.35	7.45	45.00	27.00
15	2.70	4.10	8.15	50.30	27.00
20	2.80	4.10	8.20	50.00	20.50
25	2.67	3.80	7.70	49.00	15.00
30	2.55	3.35	7.10	46.45	11.15

TABLE 2.—INITIAL pH OF THE SOLUTION, TOTAL FUNGAL GROWTH, PRODUCTION OF CITRIC ACID, CONSUMPTION OF SUGAR AND SUGAR RECOVERED AS CITRIC ACID PER 100 ml SOLUTION.

Initial pH of the fermentation medium	Total fungal growth (dry wt in g)	Citric acid produced (g)	Sugar consumed (g)	Utilized sugar recovered as citric acid (%)	Total sugar recovered as citric acid (%)
1.00	—	—	—	—	—
2.00	2.27	2.70	6.80	41.00	18.00
2.5	2.45	3.00	7.05	42.54	20.00
3.0	2.72	3.50	7.25	48.27	33.33
3.5	2.80	4.06	8.10	50.12	27.06
4.0	2.90	4.10	8.25	50.35	27.70
4.5	2.87	4.04	8.25	49.00	26.93
5.0	2.85	4.00	8.25	48.78	27.00
5.5	2.88	4.06	8.34	48.68	27.06
6.0	2.88	4.00	8.35	47.90	27.66

TABLE 3.—DIFFERENT CONCENTRATIONS OF ADDED INORGANIC SALTS, TOTAL FUNGAL GROWTH, PRODUCTION OF CITRIC ACID, CONSUMPTION OF SUGAR AND SUGAR RECOVERED AS CITRIC ACID PER 100 ml OF SOLUTION.

Concentration of added inorganic nutrition	Total fungal growth (dry wt in g)	Citric acid produced (g)	Sugar consumed (g)	Utilized sugar recovered as citric acid (%)	Total sugar recovered as citric acid (%)
Single (control)	3.20	6.20	13.25	45.50	39.50
Double	4.10	4.50	13.70	33.00	29.co
Triple	4.50	3.20	14.10	22.00	22.20
Half	2.00	3.20	7.78	45.00	23.20
Quarter	1.00	2.75	7.30	34.00	18.40
Nil	0.60	1.74	7.00	25.00	11.50

TABLE 4.—INFLUENCE OF DIFFERENT SUBSTANCES ADDED TO FERMENTATION MEDIUM ON THE CITRIC ACID YIELD.

Added nutrition	Total fungal growth (g)	Citric acid produced (g)	Sugar consumed (g)	Utilized sugar recovered as citric acid (%)	Total sugar recovered as citric acid (%)
Control	4.62	4.20	10.50	40.40	28.10
$NaNO_3$ replaced by KNO_3	4.60	4.25	10.70	39.30	28.30
$NaNO_3$ replaced by peptone	2.00	2.36	5.80	40.60	16.73
Trace of Cu, B, Mo added to control	4.30	4.27	10.80	39.50	28.44
Humus extract added to control	4.72	4.30	10.75	40.40	28.70

TABLE 5.—CITRIC ACID PRODUCTION CAPACITIES OF 17 STRAINS OF *A. nigar*.

Serial No.	Isolates	Total fungal growth (g)	Citric acid production (g)	Sugar consumption (g)	Utilized sugar recovered as citric acid (%)	Total sugar recovered as citric acid (%)
1.	IM 141874	2.95	3.63	8.10	45.1	24.20
2.	IM 175353	3.00	3.93	8.00	49.68	26.20
3.	CA2	2.85	3.93	7.99	49.68	26.20
4.	CA3	2.92	4.05	8.07	50.18	27.00
5.	CA4	2.61	3.80	7.90	48.23	25.33
6.	CA5	3.00	4.10	8.10	50.61	27.33
7.	CA6	3.10	3.50	8.12	43.00	23.00
8.	CA7	2.96	3.50	8.09	43.50	23.83
9.	CA8	3.05	3.60	8.10	44.44	24.00
10.	CA9	3.07	3.35	8.00	41.87	22.23
11.	CA10	2.80	3.55	7.92	44.82	23.66
12.	CA11	2.75	3.70	7.91	46.77	24.66
13.	CA12	2.70	3.55	7.90	44.93	23.66
14.	CA13	2.61	2.80	7.31	38.30	18.66
15.	CA14	3.20	2.90	7.00	40.84	19.33
16.	CA15	3.10	3.10	7.20	43.00	20.60
17.	CA16	3.00	5.70	10.00	57.40	38.00

infected stale banana, lemon, jackfruit and coconut kernel collected from the different parts of the Dacca city.

The organisms were isolated from the different sources in the Czapek's medium.[12] Spores of black-headed colonies (characteristics of *A. niger*) were transferred from petri dishes to the slanted Czapek's medium in the test tube. Cultures were purified by pouring very dilute suspension of spores in petri dishes containing the medium. After a period of incubation a small number of scattered colonies when old were found to be the same. The pure cultures were maintained on agar slant.

Morphological characteristics of all the isolates of black molds were studied and 16 were identified as *A. niger*. CAI, CA2, CA3, CA4, CA5, and CA14 were obtained from the atmosphere, CA6, CA7, CA8, CA9, and CA15 from the soil, CA10 from banana, CA11 and CA16 from lemon, CA12 from jackfruit and CA13 from coconut kernal.

Effect of Initial Sugar Concentration of the Molasses Solution.—This experiment was done to find out the optimum concentration of total sugar in the fermentation medium of molasses solution for the maximum yield of citric acid. In Erlenmeyer flask of 3 litres capacity, molasses were diluted with distilled water to contain 5, 10, 12.5, 15, 20, 25 and 30% of total sugar. 4.0 g $NaNO_3$, 1.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.23 g $MgSO_4.7H_2O$, 0.02 g $FeCl_3$, 0.0012 g $ZnSO_4$ and 0.0012 g $MnCl_2.H_2O$ were added to 1 litre of solution.[13] The pH of the fermentation medium was adjusted to 4.0 by hydrochloric acid before sterilization. Five hundred ml of the solution in each flask was inoculated with 5 ml spore suspension made from 7 day old culture of CA3. The flask was left undisturbed at room temperature. After 7 days it was again sterilized, the liquid was pressed out from the fungal mycellium, the mycelium was washed in water, dried and weighed. The contents of sugar and citric acid, and pH of the solution were determined after the experiment.

It appears from Table 1 that the dry weight of fungal mat gradually increased from 2.0 g in 5% sugar to about 2.7% g in 15, 20 and 25% sugar. The citric acid production increased from 0.5 g in 5% sugar to 3.0 g in 10%, 3.35 g in 12.5%, 4.1 g in 15 and 20%, and then it slightly decreased in 25 and 30% sugar. The sugar consumption increased from 4.97 g in the molasses solution containing 5% sugar to about 7.40 g in the media containing 10 and 12.5% sugar. It further increased to about 8.20 g in sugar concentrations of both 15 and 20% and then it slightly decreased in both 25 and 30% sugar concentrations. The conversion of utilized sugar to citric acid increased from 10% in the medium containing 5% sugar to 40 and 45% in the media containing 10 and 12.5% sugar respectively. It further increased to about 50% in the medium of 15, 20 and 25% sugar and then it decreased in 30% sugar concentration. The recovery of total sugar as citric acid increased from 10% in the medium of 5% sugar to 30% in 10% sugar concentration and then it decreased to 27% in the fermentation media containing 12.5 and 15% sugar. It decreased sharply in 20, 25 and 30% sugar concentrations.

It appears from the experimental results that the use of indigenous molasses in final sugar concentration of 12.5 to 15.0% is the best for the citric acid production from the considerations of total sugar and utilized sugar recovered as citric acid and total citric acid production (Table 1). Currie[4] advocated the use of 12.5 to 15.0% of sucrose in the citric acid fermentation medium. Porges[14] obtained maximum production of citric acid in 20% sugar solution and Doelger and Prescott[5] in 14% sucrose solution. Molasses solution in final sugar concentration of 10–20% was reported as optimum for the citric acid fermentation.[15]

Effect of Initial pH of the Fermentation Medium.—This experiment was performed to find out the optimum initial pH values of the fermentation medium required for the maximum yield of citric acid. Five hundred ml molasses solution containing 15% sugar and inorganic nutrients as mentioned in the earlier experiment was fermented in each conical flask of 3 litre capacity by CA3. The initial pH of the medium was adjusted to 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0. The experiment was conducted as in the previous experiment.

There was no fungal growth at pH 1.0 From pH 2.0 to pH 4.0 the dry weight of fungal mat gradually increased from 2.27 g to 2.90 g, the citric acid production, from 2.7 g to 4.10 g and the sugar consumption, from 6.80 g to 8.25 g, recovery of utilized sugar to citric acid, from 41% to 50% and then all of them hardly changed at pH 4.5–6.0. The conversion of total sugar to citric acid gradually increased from 18% at pH 2.0 to 33% at pH 3.0 and then it decreased to about 27% at other pH values (Table 2). The pH of the solution decreased after the experiment and was found to vary between 2.32 and 2.46.

Experimental results show that initial pH 3.5–6.0 in the molasses solution may be used for the citric acid fermentation (Table 2). The pH range of 3.5 to 4.0 was found to be most satisfactory by Sarker.[16] Sheikh *et al.*[17] obtained highest yield of citric acid with initial pH value of 6.5 when molasses solution was treated with 0.36% potassium ferricyanide.

Effect of Inorganic Nutrients Added to the Fermentation Medium.—In this experiment concentrations of the inorganic nutrients in the fermentation medium were varied in order to investigate the range of concentrations of various inorganic salts which should be added to our local molasses solution to obtain maximum yield of citric acid. Similar to the previous experiments, 4.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.23 g $MgSO_4.7H_2O$, 0.02 g $FeCl_3$, and 0.0012 g $ZnSO_4$ and 0.0012 g $MnCl_2.H_2O$ were added in 1000 ml solution in the control which was compared with different concentrations of salts, double, triple, half and quarter and without addition of any inorganic nutrients (nil). Five hundred ml molasses solution containing 15% total sugar at initial pH 4.0 was fermented in each flask of 3 litre capacity by CA3. This experiment also was performed in the same way as the previous experiments.

It appears from Table 3 that from the single concentration of nutrients to the triple concentration, a gradual increase from 3.20 to 4.50 g of the dry weight of fungal mat, a gradual decrease from 6.20 to 3.20 g of citric acid production, a gradual increase from 13.25 to 14.10 g of sugar consumption, a gradual decrease from 45.50 to 22.00% and from 39.50 to 22.20% in the conversion of utilized and total sugar respectively to citric acid took place in 100 ml solution during fermentation. The dry weight of fungal mat was 2.00 g, citric acid production was 3.20 g and sugar consumption was 7.78% per 100 ml medium containing 1/2 of the concentration of inorganic salts and all of them were further reduced in medium containing 1/4 of the concentration of inorganic salts and in medium without added salts.

It is evident from the experimental results that the citric acid production is the highest when 4 g $NaNO_3$, 1 g KH_2PO_4, 0.23 g $MgSO_4.7H_2O$, 0.02 g $FeCl_3$, 0.0012 g $ZnSO_4$ and 0.0012 g $MnCl_2.H_2O$ are added to 1 litre molasses solution. It appears that the fungal growth increases and the citric acid production decreases when the concentration of salts in the medium was raised to double and triple (Table 3), and therefore, it is suggested that the use of inorganic nutrients should not exceed single concentration of salts as used in these experiments.

Various observations have been made by a number of investigators of the effects of different concentrations of inorganic nutrients added to the fermentation medium on the citric acid production. $NaNO_3$ in 4 g in 1 litre solution was found to be better than NH_4NO_3 and $(NH_4)_2SO_4$ by Porges[14] while Butkewitsch *et al.*[18] used KNO_3 in 3.5 g in order to obtain high yields of citric acid. It has also been shown that the strains of the fungus used have a very important bearing on the salt requirements. According to Quilico and Dicapua[19] the effect of iron on citric acid production depends on the strain of *A. niger* used. Aluminium, chromium, iron, manganese and molybdenum ions stimulated the acid production in some strains of *A. niger* but zinc, copper and calcium ions inhibited the acid production[20,21] Shu and Johnson[22] reported that the trace of manganese in the medium lowered the citric acid production. Doelger and Prescott[5] reported that 2.33 g NH_4NO_3, 1 g KH_2PO_4 and 0.23 g $MgSO_4.7H_2O$ in 1 litre solution were most satisfactory. Wells and Herrick[23] suggested the following limits: 0.30–1.00 g KH_2PO_4, 0.10–0.50 g $MgSO_4.7H_2O$ and 1.60–3.20 g NH_4NO_3 per litre of solution. In general, it has been reported that higher yields of citric acid were obtained when the development of mycelium was restricted and not when stimulated and the sporulation was light or nearly absent. These results were recorded when a minimum quantity of inorganic salts was used.[24] It has also been reported that for commercial purpose the source of water supply should be carefully observed.

Effect of Various Inorganic Substances Added to the Fermentation Medium.—This experiment was performed to investigate the effect of various inorganic and organic substances added to the fermentation medium on the citric acid production. The control which contained the single concentration of all the inorganic salts used in the previous experiments was compared when 4.0 g $NaNO_3$ was replaced from the medium by (1) 3.5 g KNO_3 or (2) 6.0 g peptone, and when (3) 0.0012 g/litre of copper, boron and molybdenum or (4) humus extract (8 ml/100 ml) prepared by shaking humus with water (saturated) was added in the control. The experimental procedure was similar to that of the previous experiments.

It appears from Table 4 that during fermentation there was hardly any difference in the dry weight of fungal mat, citric acid production and sugar consumption in the different fermentation media except one containing peptone. In case of peptone, the dry weight of fungal mat, citric acid production and sugar consumption were reduced and found respectively to be 2.0 2.36 and 5.8 g. The recovery of citric acid from utilized sugar varied from 39.30 to 40.60%. The conversion of total sugar to citric acid was about 28% in all of them except in case of peptone where it was 16.73%.

Peptone nitrogen is inferior to sodium nitrate and potassium nitrate nitrogen for the citric acid production. The addition of copper, boron and molybdenum to the fermentation medium did not alter any result (Table 4).

Citric Acid Production Capacities of 17 Isolates of A. niger. This experiment was performed to compare the citric acid production capacities of 2 strains obtained from Commonwealth Mycological Research Institute, London and 15 strains obtained in this laboratory. Five hundred ml molasses solution containing 15% sugar and single concentration of different salts as used in other experiments at initial pH 4 was fermented in each flask. The experimental procedure was similar to other experiments.

It appears from Table 5 that during fermentation per 100 ml of solution, the dry weight of fungal mat varied between 2.61 and 3.20 g, citric acid production, between 2.90 and 4.10g sugar

consumption, between 7.00 and 8.12 g and recovery of utilized sugar and total sugar as citric acid, between 38.30 and 50.61%, and between 18.66 and 27.33%, respectively in strains numbering from 1 to 16. In strain CA16 the dry weight of fungal mat was 3.00 g citric acid production 5.70 g, sugar consumption 10.00 g, and the conversion of utilized and total sugar 57.40 and 38.00% respectively.

The fungal growth, citric acid production, sugar consumption and conversion of utilized and total sugar to citric acid did not show any remarkable difference in different strains tested except CA16 where 57.40% of utilized sugar and 38% of total sugar were recovered as citric acid. Industrially citric acid weight yields of 50–70% of added sugar are generally obtained.[15]

Acknowledgement.—The authors are thankful to Dr. M.A. Hannan, Senior Scientific Officer, Atomic Energy Centre, Dacca, for his help in this work.

References

1. W. Scheele, Crells Ann., **2,** 1(1784).
2. C. Wehmer, Compt. rend., **117,** 332 (1893).
3. C. Thom and J.N. Currie, J. Agric. Res. **7,** 1(1916).
4. J.N. Currie., J. Biol. Chem., **31,** 15 (1917).
5. W.P. Doelger and S.C. Presscott, Ind. Eng. Chem., **26,** 1142 (1934).
6. T.G. Taylor, Biochem. J., **54,** 48 (1953).
7. C.S. Hughes, Ph. D. thesis, University of Cambridge (1960).
8. T. Solomos, Dissertation for Ph. D. Degree (University of Cambridge, 1963).
9. M. Somogyi, J. Biol. Chem., **119,** 741 (1937).
10. D. J. D. Hochenhull and D. Herbert, Biochem. J., **39,** 102 (1945).
11. S.A. Waksman and E.B. Fred, *In Methods for Studying Soil Microflora-Plant Disease Relationships* (Burgess, Minneapolis, U.S.A., 1960), p. 148.
12. Czapek's medium (Czapek–Dox), In *Methods for Studying Soil Microflora—Plant Disease Relationship* (Burgess, U.S.A., 1960), p. 141.
13. S.C. Prescott and C.G., Dunn, *Industrial Microbiology* (McGraw-Hill, New York, 1959), p. 533.
14. N. Porges, Am. J. Botany, **19,** 559 (1932).
15. *Encyclopedia of Science and Technology*, vol. 3 (McGraw, New York 1960), p. 146.
16. P.B. Sarker, *Organic Chemistry* (Sarker Trust, Calcutta, 1960), p. 260.
17. N.M. Sheikh and G.K. Joarder, Scientific Researches, **1,** 116 (1964).
18.* W.S. Butkewitsch and M.S. Gawskaya, Compt. rend. acad. Sci., U.S.S.R. (N.S.) **3,** 405 (1955).
19.* A.C. Quilico and A. Dicapua, Chemica and industria (Milan), **14,** 289 (1932).
20. Encyclopedia of Chemical Technology, 2nd. edition, vol. 5, p. 524.
21.* E.F. Bruckmann, Biochem. Z., **335,** 199 (1961).
22.* P. Shu and M. J. Johnson, J. Bact., **54**(2), 161 (1947).
23. P.A. Wells and H.T. Herrick, Ind. Eng. Chem., **30,** 255 (1938).
24. L.M. Miall, New Scientist, **438** (1966).

*Origingal papers have not been seen.