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A Comparison of the citric Acid Production by four Species of Aspergillus niger Group*).

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Introduction.

One of the most important achievements in the field of Industrial Microbiology is the production of citric acid by fermentation. Today mycological citric acid is a huge industry in several parts of the world — in United States, Europe, Russia, England and Japan. An institute devoted to the development and improvement of this process exists in Russia. Most of the citric acid produced in the United States is fermentation citric acid. While no estimates of the total consumption of citric acid in our country or of the world are available yet it is certain that it must be enormous in amount because of its varied uses in medicinal products, for foods (as flavouring extracts, soft drinks etc.) in candies, inks, silvering, dyeing, calico printing, enlarging etc. . . The production of citric acid has engaged the attention of a large number of investigators because of its apparent enormous commercial potentialities. In spite of all this there is probably less basic established fundamental knowledge regarding it than in most other industrial processes (Foster 1949).

Citric acid formation is probably one of the most widely distributed metabolic processes known in fungi. But one species of the genus, *Aspergillus niger* van Tieghem, is well known as the organism most suitable for its production. It was Currie (1917) who stated that a well selected culture of this species is far superior to any other for the production of citric acid. Experience has shown that the key to the success in citric acid manufacture lies in the proper selection of a strain of *Aspergillus niger*.

The present study deals with an investigation made to evaluate the citric acid producing power of four strains belonging to four different species of the *Aspergillus niger* group viz., *A. niger* van Tieghem, *A. awamori* Nakazawa, *A. phoenicis* (Corda) Thom, and *A. luchuensis* Inui, isolated and identified in our laboratory. This study was also made to find out if the Indian strain of the species of *A. niger* van Tieghem is more suitable or any other species of the black Aspergilli is more effective citric acid producer under similar cultural conditions. The investigation is of special interest because

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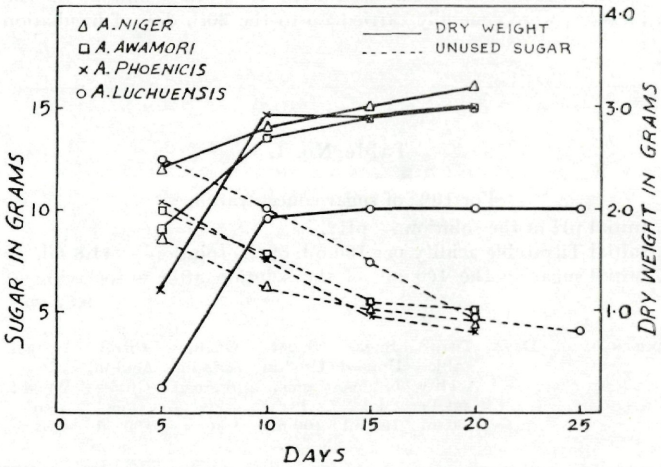
practically no work has been done to determine the potentiality of the Indian strains of Aspergilli to produce citric acid.

Material and Method.

The following basal medium, which is essentially the medium successfully used by Porges (1932), for the citric acid production in *A. niger*, was used.



1. Graph showing the changes in dry weight and utilization of sugar at different periods of incubation in the four species of *A. niger* group at 15% of sugar concentration.

$NaNO_3$	—	4.0 grams
K_2HPO_4	—	1.0 gram
$MgSO_4.7H_2O$	—	0.5 gram
KCl	—	0.5 gram
$FeCl_3$	—	0.02 gram
$ZnSO_4$	—	0.10 gram
Distilled water	—	To make it up to one litre.

To the above basal medium crystalline cane sugar was added to make the necessary percentage. The sugar used was market sugar manufactured at Ram Kola sugar mill and known by the name ,,Ram

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Kola Khand". Throughout the experiment the same sugar was used.

The pH of the culture solution was adjusted to pH 2 by adding hydrochloric acid. 100 ml. portions of the medium were poured in 300 ml. Erlenmeyer Pyrex flasks and sterilized for twenty minutes at ten pounds pressure. A heavy spore suspension was obtained by pouring 10 ml. of sterilized distilled water over a young colony (3 days old) of the fungus grown in a culture tube. The tube was shaken to ensure maximum dispersion of the spores. Half ml. of this heavy spore suspension was added to each flask of a set of 12 flasks, of one sugar concentration marked for the growth of a single species.

Concentrations of sugar used were 10%, 15% and 20%. The estimations were generally carried up to the 20th day of incubation

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Table No. 1.

For 10% of sugar concentration.

1. Initial pH of the solution — pH2.
2. Initial Titratable acidity per 100 ml. of the solution — 11.8 ml.
3. Initial sugar in the 100 ml. of the solution after autoclaving — 9.52 gms.

Species	Days	Titratable Acidity in ml Per 100 ml	Sugar Unused in gms. Per 100 ml	Sugar Used in gms. Per 100 ml	Oxalic Acid in in gms. Per 100 ml	Citric Acid in Grams Per 100 ml	Average Dry Weight in Grams
A. awamori	5	31	6.25	3.27	0.4184	0.1166	0.7553
	10	152	2.77	6.75	0.6060	0.6833	2.6226
	15	166	2.15	7.37	0.5596	0.8076	2.746
	20	187	2.04	7.48	0.1492	0.3545	2.9421
A. luchuensis	5	—	—	—	—	—	—
	10	142	3.22	6.30	0.1812	0.8705	1.8607
	15	195	2.38	7.14	0.7956	1.2456	2.2135
	20	206	1.55	7.97	0.3564	1.1511	2.2740
A. niger	5	66	6.45	3.07	0.5144	0.0691	1.2915
	10	120	3.33	6.19	0.7280	0.6022	2.5826
	15	158	2.04	7.48	0.5328	0.5501	2.8176
	20	168	1.86	7.66	0.3148	0.2676	3.0296
A. phoenicis	5	25	8.70	0.82	0.0220	0.1171	0.2362
	10	76	3.57	5.95	0.4552	0.2109	2.2602
	15	112	2.70	6.82	0.3596	0.2676	2.4394
	20	118	2.32	7.20	0.1776	0.1683	2.4836

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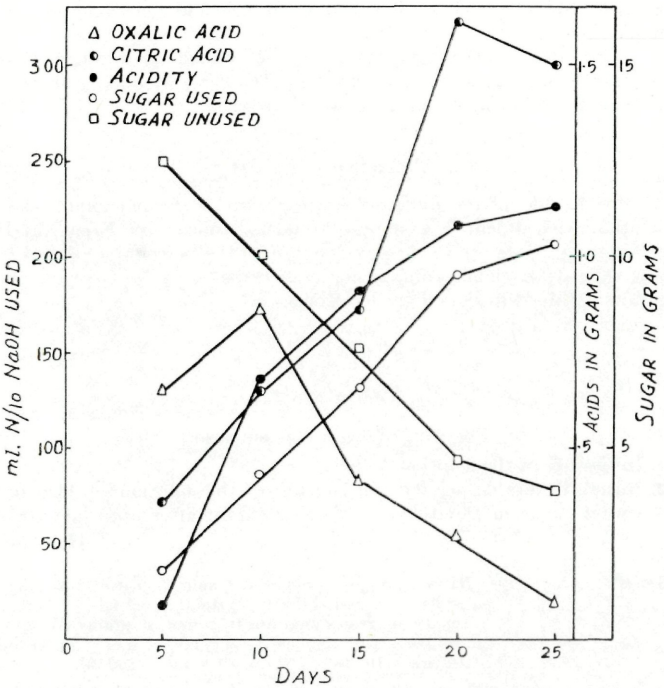

2. Graph showing the relationships of different metabolic processes in *A. luchuensis* at 15% sugar concentration.

but at times the period was extended or decreased taking in view the exigencies of the experiment.

All the four species were grown simultaneously in sets of 12 flasks of one sugar concentration. Three flasks of each sugar concentration were kept as control. The temperature of incubation varied from 25°C to 28°C.

Three flasks from each of the four different species were taken out on the 5th, 10th, 15th and 20th days of incubation. Each of the three flasks of every set were filtered through a previously dehydrated and weighed Whatman's filter paper no. 42. The fungal mat before drying was washed with distilled water and then dried at 60°C for 3 days in a hot oven. They were then transferred to a dessicator for cooling. The filter papers with the dried mat of fungus were then

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weighed quickly over an analytical balance and the difference in weight was noted.

The filtrate of the three flasks was then mixed and the volume of the filtrate was made up to 300 ml., in each case, by adding distilled water. The following estimations were made:

1. Titratable Acidity.

To 10 ml., of the filtrate a drop or two of the phenolphthalein solution was added. The solution was neutralized by N/10 NaOH solution and from the reading thus obtained the percentage of acidity was then determined. Comparision was made in each case with the results obtained in the controlled flasks.

Table No. 2.

For 15% of sugar concentration.

1. Initial pH of the solution — pH2.
2. Initial Titratable acidity per 100 ml. of the solution — 11.8 ml.
3. Initial sugar in the 100 ml. of the solution after autoclaving — 14.3 gms.

Secies	Days	Titratable Acidity in ml per 100 ml	Sugar Unused in grams per 100 ml	Sugar Used in grams per 100 ml	Oxalic Acid in grams per 100 ml	Citric Acid in grams per 100 ml	Average Dry Weight in grams
A. awamori	5	22	10.0	4.3	0.6416	0.1844	1.8732
	10	133	7.28	7.02	0.7224	0.4445	2.7572
	15	158	5.50	8.80	0.6928	0.5994	2.9246
	20	161	5.00	9.30	0.2384	0.5871	3.1104
A. luchuensis	5	19	12.5	1.80	0.6468	0.3561	0.2815
	10	137	10.0	4.30	0.8252	0.6515	1.9203
	15	182	7.69	6.61	0.4212	0.8677	2.1286
	20	216	4.76	9.54	0.2684	1.6277	2.2420
	25	228	3.95	10.35	0.0864	1.5253	2.2744
A. niger	5	23	8.80	5.50	0.2544	0.4459	2.4378
	10	141	6.45	7.85	0.4376	0.5396	2.8615
	15	173	5.40	8.90	0.7700	0.5849	3.0241
	20	198	4.87	9.43	0.4376	0.5828	3.2411
A. phoenicis	5	18	10.52	3.78	0.4272	0.4564	1.2270
	10	124	7.70	6.6	0.5968	0.5302	2.9013
	15	146	4.87	9.43	0.7472	0.8384	2.9902
	20	205	4.65	9.65	0.7260	0.9075	3.0829

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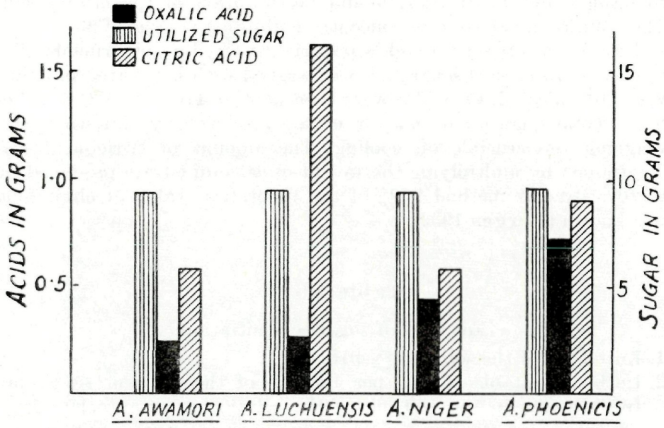

3. Histogram showing comparsions of citric acid and oxalic acid production and utilization of sugar in the four species of *A. niger* group at 15% sugar concentration and on the 20th day.

2. Total residual Sugar.

The residual sugar of the medium was determined by Bennedict's method (Haas and Hill 1928 p. 134) and comparision was made in each case with the results obtained in the controlled flasks.

3. Oxalic Acid and Citric Acid.

The oxalic and citric acids were determined gravimetrically as the calcium salts by the method employed succesfully by Bernhaur (1926). 50 ml. of the filtrate was used for this determination. To this 10 ml. of 10% $CaCl_2$ was added and neutralized with 10% ammonium hydroxide. Two ml. of a 20% Hydrochloric acid solution was then added to it and then the solution was heated for a short while. The solution was then allowed to cool. The oxalic acid present in the filtrate was thus precipitated as calcium oxalate. It was separated by filtering the solution through a tared gooch crucible, previously dehydrated and weighed to a constant weight. The calcium oxalate, thus obtained in the crucible was first washed with cold distilled water and thereafter the crucible with the calcium salt was kept in an oven at 100°C. The amount of calcium oxalate produced by the fungus was known by reweighing the cooled crucible.

The filtrate and washings after precipitation of the oxalate were then concentrated, and while hot, ammonium hydroxide was added

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to bring about neutralization and precipitation of the citrate salt. This solution was heated to concentrate it upto a volume of 25—30 ml. and was soon filtered through a preweighed tared gooch crucible. The calcium citrate, thus separated, was washed with hot water and then with 60% alcohol. Crucibles were then kept at 110 to 115°C in a hot oven. Total quantity of calcium citrate produced was known by reweighing the crucible on cooling. The amount of citric acid was determined by multiplying the weight of calcium citrate produced by 0.7709. By this method 96% of the theoretical value of citric acid was known (Porges 1932).

Table No. 3.

For 20% of sugar concentration.

1. Initial pH of the solution — pH2.
2. Initial Titratable acidity per 100 ml. of the solution — 12 ml.
3. Initial sugar in the 100 ml. of the solution after autoclaving — 19.8 gms.

Species	Days	Titratable Acidity in ml per 100 ml	Sugar Unused in grams per 100 ml	Sugar Used in grams per 100 ml	Oxalic Acid in grams per 100 ml	Citric Acid in grams per 100 ml	Average Dry Weight in grams
A. awamori	5	52	12.05	7.75	0.2320	0.2199	2.554
	10	110	8.26	11.54	0.6220	0.8135	3.4748
	15	156	6.66	13.14	0.3256	0.9674	3.5435
	20	164	4.18	15.62	0.2738	1.1360	3.6056
	25	172	3.42	16.38	0.1972	1.0764	3.6368
A. luchuensis	5	—	—	—	—	—	—
	10	13	16.52	3.28	0.2088	0.0321	1.327
	15	75	11.43	8.37	0.5084	0.4080	2.963
	20	108	10.02	9.78	0.6936	0.5353	3.2400
	25	126	8.56	11.24	0.4752	0.5686	3.4086
A. niger	5	42	13.16	6.64	0.2196	0.1854	2.7316
	10	121	8.73	11.07	0.7192	0.5779	3.2523
	15	148	4.27	13.53	0.6888	0.9016	3.5666
	20	160	5.07	14.73	0.6128	0.9765	3.6274
	25	174	4.54	15.26	0.5644	0.9595	3.6806
A. phoenicis	5	15	16.12	3.68	0.2832	0.2085	1.354
	10	101	12.74	7.06	0.4716	0.2557	3.181
	15	132	8.00	11.80	0.4864	0.5218	3.4577
	20	146	6.02	13.78	0.4048	0.7205	3.5240
	25	155	5.20	14.60	0.3752	0.6659	3.5908

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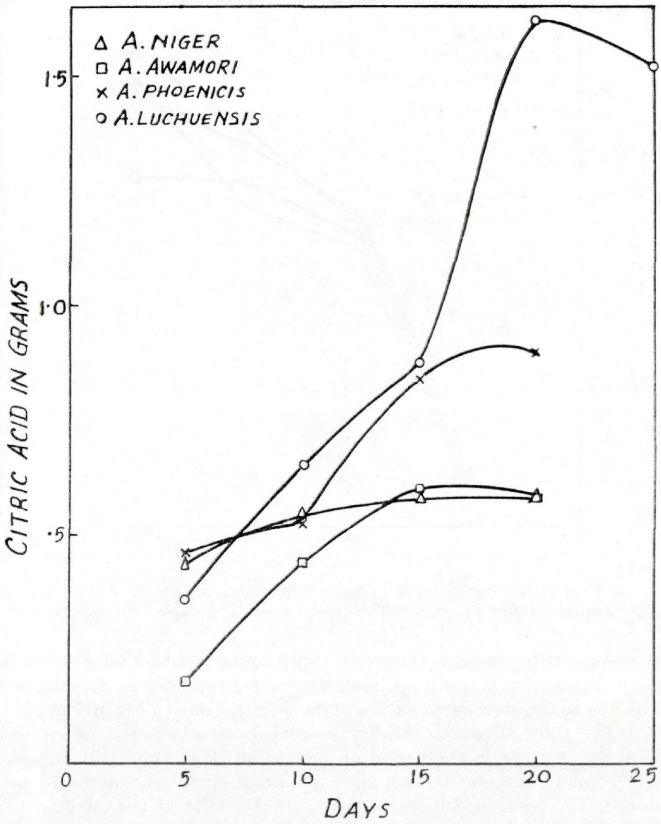

4. Graph showing the amount of citric acid produced in the four species of *A. niger* group at different periods of incubation at 15% sugar concentration.

Results and Discussion.

The results obtained at different concentration of sugar with the four species are tabulated in tables 1—3 and represented graphically in figures 1—6. From the results obtained it was apparent that the vigorous growth of all the four species was between 5th and the 10th day (figure 1). Strangely enough there was little growth of *A. luchuensis* for the first five days of the period of incubation. But in

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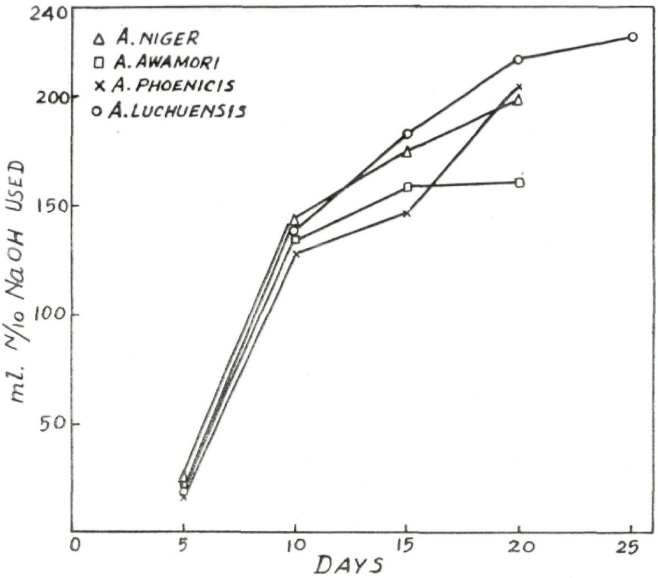

5. Graph showing changes in titratable acidity of the four species of *A. niger* gruop at different periods of incubation at 15% sugar concentration.

the other three species there was fairly good amount of growth by the fifth day. It is quite probable that this behaviour of *A. luchuensis* was due to the slow germination of its spores at such a low pH as pH 2.

The total titratable acidity in each case was found to increase with the increase in the period of incubation (Fig. 2) but this increase could not be correlated with the amount of citric or oxalic acid produced. The continued increase in the total acidity of the medium had probably been due to production of acids other than citric and oxalic.

Estimations of oxalic and citric acids produced at different periods showed that up to a certain period there was a gradual increase in the amount of oxalic acid produced by the organism with the accompanying lesser production of citric acid but after maximum oxalic acid production had been attained there was distinct and quick improvement in citric acid production (Fig. 2). Possibly the oxaloacetase enzyme produced by the fungus during the earlier period of growth was more active and was, therefore, responsible for the production of oxalic acid from oxaloacetate which is being continuously produced by Tri-carbxylic Acid Cycle. As soon as the concentration

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of oxalic acid reached the inhibitory level for the enzyme, this pathway got slackened and more of oxaloacetate was made available to condense with Acetyl coenzyme A to give rise to citric acid.

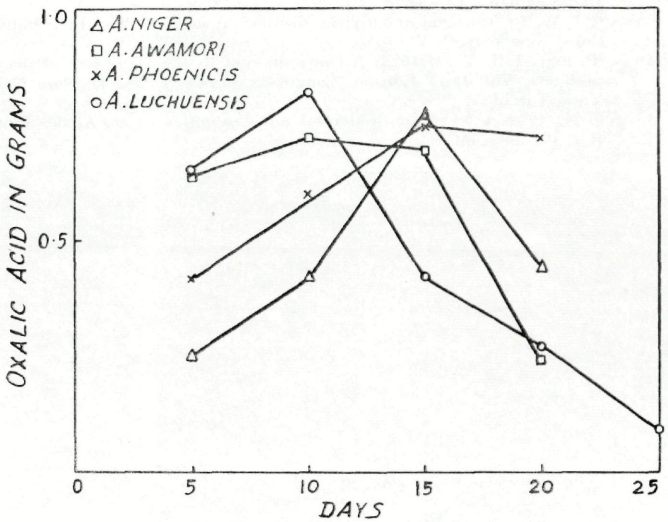
Maximum oxalic acid was found in *A. luchuensis* Inui at 15% sugar concentration on the tenth day after which it decreased (Fig. 6).

Maximum citric acid production was found in *A. luchuensis* Inui at 15% sugar concentration in 20 days (Fig. 3 & 4). Incubation after that period showed decrease in citric acid production. This is due to the utilization of the acid in metabolic processes by the organism (Fig. 2). The maximum amount of citric acid obtained at 15% sugar concentration from the different species was as follows:

A. awamori Nakazawa — — 0.5994 gram at 15th day
A. luchuensis Inui — — 1.6277 gram at 20th day
A. niger van Tieghem — — 0.5849 gram at 15th day
A. phoenicis Thom — — 0.9075 gram at 20th day.

Porges (1932), on the other hand, got a higher yield with *A. niger*, amount of acid produced in his case being 2.375 grams at 15% sugar concentration.

It is apparent from the above data that the strain of *A. luchuensis* tested was the best producer of citric acid in comparision to the strains



6. Graph showing the oxalic acid production in different species of *A. niger* group on different days of incubation at 15% sugar concentration.

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of other three species studied. But it is quite likely that better strains of *A. niger* also exist in India which might prove to be high citric acid producers. Further work on the effect of cultural conditions on the citric acid producing power of these molds is in progress in our laboratory and will be communicated later.

Summary.

An evaluation of the citric acid producing power of four Indian isolates of *A. niger* group viz. *A. awamori* Nakazawa, *A. luchuensis* Inui, *A. phoenicis* (Corda) Thom and *A. niger* van Tieghem, has been made under similar cultural conditions for the first time. It has been found that out of the four species tested the strain of *A. luchuensis* produces the maximum amount of citric acid at 15% sugar concentration. Further it has also been noticed that the germination of spores of *A. luchuensis* was slowest at pH 2 as compared with that of the other three species.

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