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MANUFACTURE OF CITRIC ACID FROM MOLASSES

Part I.—Isolation of Micro-Organisms Useful in Production of Citric Acid

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Thirty-two different black molds were isolated from various sources such as local soil, stale lemon pulp, stale pieces of bread, rotting sugar beets and contaminated agar slants; out of these seventeen organisms (apparently distinct) were found capable of producing citric acid. The organisms were isolated by crowded-plate method[1] and they were allowed to act on molasses when citric acid was produced and identified by Deniges' method.[2]

Introduction

Quite large quantities of molasses are being produced from the newly-constructed sugar mills of East Pakistan, and to solve the problem of its economic utilisation, manufacture of citric acid was considered to be one of the important ways. For this purpose a suitable micro-organism was necessary. In the present paper isolation of some citric acid-producing micro-organisms has been described. Attention has been focussed on the molds, because of the ability of some members of the group to produce citric acid.

Wehmer[3] in 1893 first described citric acid as a product of mold fermentation. Two molds, which he designated as *Citromyces pfafferianus* and *Citromyces glaber*, produced the acid from nutrient sucrose solutions containing calcium carbonate. Later Wehmer[4] reported the formation of citric acid by *Penicillium luteum* and *Mucor piriformis*. Currie[5] in 1917, as a result of his work with Thom,[6] showed that citric acid could be produced in considerable quantities by certain strains of *Aspergillus niger* when grown in acidified solutions containing sucrose and relatively small amounts of inorganic salts. Doelger and Prescott[7] in 1934, corroborated the results of Currie and made other valuable contributions concerning the fermentation. The literature of the past two decades contains many references to citric acid fermentation.

Since the historic research of Wehmer,[3] it has been shown that a large number of fungi have the ability of producing citric acid. Some of the fungi produce small yields, some produce undesirable substances, while some on account of their unstable cultural characteristics would be unsatisfactory for use on a commercial basis. Thus the choice of a strain is of great importance.

Aspergillus niger, *A. clavatus*, *Penicillium luteum*, *P. citrinum*, *Paecilomyces divaricatum*, *Mucor piriformis*, *Ustulina vulgaris*, and another species of *Mucor* have been used to produce citric acid in laboratories or on a commercial scale.

In view of the importance of molds in the citric acid industry and of the very divergent views expressed by earlier workers about the organisms, an investigation of the organisms appeared to be desirable to find out more active organisms from local sources. Preliminary efforts in this direction are described here.

Experimental

Substrates from which the Organisms were Isolated.—The organisms were isolated from soil, stale lemon pulp, stale pieces of bread, rotten sugar beets and

contaminated agar slant.

Samples of soil were collected from the Industrial Area, Tejgaon, Dacca; the lemon pulp, the stale bread and the rotting sugar beet were all collected from different local sources, and the contaminated agar slant was prepared in the laboratory by preserving the slant of medium No. 2 for some days while it was contaminated by organisms from the atmosphere.

Method of Isolation of the Organisms.—The organisms were isolated from the substrates by crowded-plate method[1] from agar plates of medium No. 2 containing 1:1000 substrate dilution. Isolated colonies of the organisms having the apparent characteristics of the *Aspergillus* group were removed asceptically to medium No. 2 slant, and then purified by streak-plate method: and then again sub-cultured on agar slant. The sub-cultures were examined under microscope to ensure their purity. In this way thirty-two black molds were isolated; out of them ten were from soil, four from lemon pulp, nine from stale bread, seven from rotting sugar beet and two from the agar slant.

Method of Choosing the Citric Acid-Producing Micro-Organisms.—For the purpose of this investigation, all the above 32 species (apparently distinct and different from one another) of black molds were used. Tests were made in order to find out the type which would ferment sucrose to the highest total acidity in 9 days of incubation at 30-32°C. For this purpose the organisms were sub-cultured in 250 ml. conical flasks containing 75 ml. of the

TABLE I.

Source	No. of organism	Titratable acidity in ml. in terms of N/100 solution, in 5 ml. of fermentation solutions after							Test for citric acid	Test for oxalic acid	Quantity of citric acid out of total acidity
		3 days	4 days	5 days	6 days	7 days	8 days	9 days			
Soil	1	16	23	32	40	45	47	48	—	—	
	2	47	81	120	125	132	136	137	+	slight	87%
	3	18	24	28	39	47	48	48	—	—	
	4	6	12	18	12	6	5	6	—	—	
	5	20	47	38	35	36	35	34	—	—	
	6	24	45	80	87	106	108	108	+	slight	90%
	7	36	52	68	89	90	95	96	+	slight	82%
	8	12	20	43	87	67	65	60	—	—	
	9	9	19	9	8	6	7	6	—	—	
	10	6	11	13	13	14	17	19	—	—	
Lemon	11	40	99	120	120	121	123	124	+	slight	90
	12	32	72	67	61	62	62	63	+	slight	91
	13	26	62	65	105	106	110	111	+	slight	83%
	14	18	29	65	85	98	109	109	+	slight	85%
Bread	15	8	15	19	23	21	27	28	—	—	
	16	20	37	32	23	24	24	23	—	—	
	17	8	16	18	22	28	36	38	—	—	
	18	6	16	20	15	11	11	12	—	—	
	19	4	17	27	29	25	24	22	—	—	
	20	8	17	20	23	22	2	21	—	—	
	21	187	275	340	350	361	381	380	+	slight	95%
	22	9	8	7	7	5	7	8	—	—	
	23	7	16	17	18	20	18	18	—	—	
Sugar beat	24	42	145	204	233	243	290	336	+	slight	93%
	25	78	190	206	212	220	240	262	+	slight	92%
	26	60	130	175	180	188	205	225	+	slight	91%
	27	71	144	197	207	219	257	284	+	slight	87%
	28	44	144	183	188	192	220	256	+	slight	88%
	29	55	114	177	197	234	297	312	+	slight	84%
	30	17	97	139	154	173	193	212	+	slight	83%
Agar slant	31	55	114	148	159	172	190	216	+	slight	89%
	32	110	212	241	239	221	220	222	+	slight	96%

—, negative ; + positive.

medium No. 4 with an initial reaction of pH 6.00 and using a 12.5% sucrose solution as a base. After the completion of the sporulation, the spores were seeded lightly over the surface of 250 ml. portion of the medium No. 5 in a glass vat of dimensions 16 × 10 × 4 cm. with the depth of the medium 13-14 mm.

Determinations were made for titratable acidity from 3rd to 9th day of the fermentation period. Qualitative tests for citric acid were made by means of Deniges' method.[2]

Titratable acidity was determined with sodium hydroxide using phenol red as the indicator and was carried out to a stable red colour. Normality in the table is expressed as ml. of N/100 solution used for the titration of 5 ml. of the fermentation solution.

Tests for oxalic acid were based on the varying solubilities of the calcium salts of oxalic and citric acids. Oxalic acid was precipitated in the cold from a neutral 10-ml. sample with calcium chloride. The filtrate remaining after the oxalic acid precipitation was reduced to a definite volume in each instance by boiling and was then autoclaved for 30 minutes at 20 lbs. steam pressure per sq. inch. The calcium citrate precipitated was washed with hot water, then dried to a constant weight at 60 C. and weighed. This method was found to be 92% of the true value as compared to quantitative determination run by a method using standard citric acid solution.

Results and Discussion

Mean of the three results are given in the table.

The organisms Nos. 2, 6, 7, 11, 12, 13, 14, 21, 24, 25, 26, 27, 28, 29, 30, 31, and 32 were found to be capable of producing citric acid with a small quantity of oxalic acid.

The work on morphological study and citric acid production under different conditions is in progress.

Compositions of the media used were as follows:—

Medium 1

Sucrose, 4%,
$MgSO_4$, H_2O, 0.025%,
NH_4NO_3, 0.225% and
KH_2PO_4, 0.030%

pH was adjusted to 2 with HCl solution.

Medium 2

Sucrose, 4%,
Magnesium sulphate, H_2O, 0.025%
Ammonium nitrate, 0.255%
Potassium dihydrogen phosphate, 0.030%
and Agar, 2.5%

The pH was adjusted to 4.5 with HCl.

Medium 3

Sucrose, 14%
NH_4NO_3, 0.223%
K_2HPO_4, 0.10%
$MgSO_4$, $7H_2O$, 0.023%

pH adjusted to 2 with HCl.

Medium 4

Molasses, 25%
$K_4Fe(CN)_6$, 0.06%
K_2HPO_4, 0.05%
pH, 6

Medium 5

Molasses, 25%
$K_3Fe(CN)_6$, 18%
NH_4NO_3, 0.223%
$MgSO_4$, 0.012% (anhydrous)
KH_2PO_4, 0.1%
pH, 6.5

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