CITRIC ACID PRODUCING MICROORGANISMS—THEIR IDENTIFICATION AND CITRIC ACID PRODUCING CAPACITY

N. M. Sheikh, G. K. Joarder and S. A. Ahmed

East Regional Laboratories, Pakistan Council of Scientific and Industrial Research, Dacca

(Received November 16, 1962)

Morphological, cultural and biochemical studies on thirteen strains of citric acid producing microorganisms have been described. All the oganisms are the different strains of the species *Aspergillus nigre*[1]. Though there is no appreciable difference in their morphological and biochemical characters, yet there are wide differences in their cultural activities towards different media and in the production of citric acid from molasses. Different media were used to determine the optimum conditions for the production of citric acid by employing the surface culture method.

Introduction

A number of organisms, [2,3] are known to produce citric acid. The literature of the past three decades contains many references to citric acid fermentation. In view of the importance of moulds 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 and a more suitable method for the production of citric acid.

In the course of this investigation, 15 moulds[4] were found to produce citric acid. Studies on their detailed morphological, cultural and biochemical characters have shown that they are different strains of *Aspergillus niger*.[1] Using these organisms for the production of citric acid, experiments were carried out in surface cultures.

Experimental

A. Identification of the Organisms

Morphological Characters.—The microscopic examination of the organisms was carried out by the slide-culture method of Henrici[5] using basic medium,[6] following incubation at 30°C. and from these studies it was observed that all these organisms belong to the group *Aspergillus niger*.[1]

Cultural Characters.—Studies on cultural characters were carried out on the media,[7] on slants at 30°C. and the readings were taken after every twenty four hours. The results obtained are given in Table 1.

Biochemical Characters.—Studies on biochemical characters were carried out on gelatin, bromocresol-purple-milk and skimmed milk,[8] and it was observed that there was no difference in their activities towards these media.

B. Fermentation

Preparation of Inoculum.—The cultures of the purified organisms were maintained on test tube slants of medium No. 20 of Table 3. The inoculum was prepared in a 250 ml. conical flask containing 100 ml. of the medium No. 21 with an initial pH 6.0 and using a 12.5% sucrose solution as a base. This solution was inoculated with spores from the slant cultures. After the completion of sporulation in 3 days, the spores were suspended in 500 ml. of water and 25 ml. portion of it was used as ino-culum for each 250 ml. portion of fermentation medium.

Surface Culture Method.—250 ml. portion of each medium (Nos. 1—11, Table 2) were taken in a glass vat of dimensions 16 × 10 × 4 cm. with the depth of the medium 13—14 mm., inoculated with 25 ml. of inoculum and incubated at 30—32°C. Qualitative tests for citric acid were made by means of Deniges' method.[9] Titratable acidity was determined with sodium hydroxide using phenol red as the indicator. Determinations were made for titratable acidity from 3rd to 12th day of fermentation period. Normality in Table 3 is expressed as ml. of N/100 solution used for the titration of 5 ml. of the fermentation solution. Tests for oxalic acid[4] were based on the varying solubilities of the calcium salts of oxalic and citric acids. After 12 days of fermentation the mycelia were separated from the broth, washed under tap until the washings became colourless and dried in an oven at 100°C. to constant weight. The results are given in Table 3.

Submerged Culture Method.—(a) Shaking Method: In the shaking method, experiments were carried out, using media Nos. 12—19. 100 ml., portion of each medium was taken in a 250 ml. conical flask, inoculated and shaken continously for eight days in microid-flask shakers. After every 24 hours the fermentation solutions were tested for citric acid by Deniges' method.[9] None of these organisms could produce citric acid under these conditions.

Table 1. (*Columns continued on next page*)

Name of the media	Growth observed after					In cases of the Sporulation		
	1 day	2 days	3 days	4 days	5 days	1 day	2 days	3 days
1. Acid-Czapek's-Agar	13	14,24,27	6,11,12 25,32	28,30,31	2		13,14,24, 25,27	6,11,12,32
2. Jensen's-Agar		12,13,14, 25	2,6,11,27	24,28,30 31,32			12,13,14, 25	2,6,11,27
3. Soil-Extract-Agar		2,6,11-14					2,6,11-14	
4. Washed-Agar		12	2,6,11,13,		14		12	2,6,11,13
5. Glucose-Asparagine-Agar	2	6,11-14,24, 25,27,28, 30-32				2,	6,11-14,24, 25,27,28, 30-32	
6. Tyrosine-Agar		2,6,11,12, 24,27,28, 30-32	25,				2,6,11,12, 24,27,28, 30-32	
7. Starch-Agar—B		12-14	2,6,11,24 30	25,27,28 31,32			12-14	2,6,11,24, 30
8. Potato-Nutrient-Agar		2,6,11,13, 14,27,28	12,24,25 30-32				2,6,11,13, 14,27,28	12,24,25, 30-32
9. Yeast-Extract-Glucose-Agar	2,6,24,25 27,28, 30-32	11-14				2,6,24,25, 27,28,30, 31,32,	11-14	
10. Calcium-Malate-Agar		2,6,12-14	24,25,27, 28,30,32	31			2,6,12-14	24,25,27, 28,30,32,
11. Potato-Glucose-Agar	2	6,11-13,24 25,27,28 30-32	14,			2,	6,11-13,24, 25,27,28, 30-32	14,
12. Basic-Medium6		2,6,11-14 24,25	27,28,30 32				2,6,11-14, 24,25	27,28,30-32
13. Potato-Plug		2,6,11-14, 27,28,30, 32	24,31				2,6,11-14, 27,28,30,32	24,31,
14. Glucose-Nutrient-Agar			2,6,11-14 24,25,27 28,30,32	31				2,6,11-14, 24,25,27, 28,30,32
15. Peptone-Gelatin-Medium			2,6,11-14, 24,25,27 28,30,32,		31			2,6,11-14, 24,25,27, 28,30,32
16. Gelatin			12,13,31,	2,6,11,14, 24,25,27, 28,30,32				12,13,31
17. Bromo-Cresol-Purple-Milk		2,13,14,25 27,30,31	6,11,24,					2,13,14,25, 27,30,31
18. Skimmed-Milk			2,6,12,24, 25,27,28 30,32	11,14	13			2,6,12,24, 25,27,28, 30,31
19. Omeliansky's-Cellulose-Medium		12,24						
20. Dubo's-Cellulose-Medium		14,28						

(These are the remaning columns of Table 1 at page 280)

following organisms

observed after		Colour of the mycelia observed			Colour of the pigment observed					No growth observed
4 days	5 days	White	Yellow	Yellowish-white	Black	Yellowish	Red	Brown	Yellowish-black	
28,30,31		2,6,11-14, 27,28,30, 31,32		24,25	2,14	13				
24,28,30, 31,32		2,6,24, 27,30,31 32	11-14 25,28	14,28					12,13	
										24,25,27,28,30-32
	14									24,25,27,28,30-32
					13					
										13,14
25,27,28, 31,32,										
						2,12			6,11,13	25
31										11
					2,6,11, 24,27,28, 30,32				13,14	
		24,28, 30-32		6,11-14,27						25
31					2,6,11,25 27,32	14			12,13	
	31	2,11-14, 24,27,	6,25,28		27		30,31	2,12, 14,24		
2,6,11,14, 24,25,27,28 30,32		6,11-14,24, 27,28,30- 32	2	25	13			28,31		
6,11,24		11,13,14		2,6,25,28,30, 31						
11,14	13	6,11		2,12,13,14,25, 27,28,30-32		12,28,30				
										2,6,11,13,24,25, 27,28,30,31,32
										2,6,11,12,13,24, 25,27,30,31,32

Table 2.—Compositions of Media.

Medium No.	Sucrose %	Molasses %	NH_4NO_3 %	KH_2PO_4 %	K_2HPO_4 %	$MgSO_4, H_2O$ %	$K_3Fe(CN)_6$ %	$K_4Fe(CN)_6$ %	$ZnSO_4$ %	CH_3OH %	KCl %	Agar %	pH %	Autoclaved % at	for
1		25	0.233	0.100		0.012	0.18						6.5		
2		25	0.233	0.100		0.012	0.18		0.00012				6.5		
3		25	0.233	0.200		0.012	0.18		0.00012				6.5		
4		25		0.100		0.012	0.18						6.5		
5		25	0.233			0.012	0.18						6.5		
6		25				0.012	0.18						6.5		
7		25	0.233	0.100		0.012			0.00012				6.5		
8		25	0.233	0.100		0.012							6.5		
9		25											6.5		
10		25	0.233	0.100		0.012	0.18			2.5			6.5		
11		25					0.18						Not adjusted		
12		25	0.110			0.012					0.15		2 with HCl		
13		25	0.25	0.25		0.012							2.0 with HCl		
14		25		0.04				0.08					6.0 with HCl	100°C.	30 min.
15		25			0.42			0.7					8.0	100°C.	30 min.
16		25	0.233	0.100									6.5	100°C.	30 min.
17		25	0.233	0.100		0.012							6.5	100°C.	30 min.
18		25	0.233	0.100		0.012			0.00012				6.5	100°C.	30 min.
19		25	0.233	0.100		0.012		0.100	0.00012				6.5	100°C.	30 min.
20	4		0.225	0.030		0.012						2.5	4.5 with HCl		
21		25			0.05			0.06					6.0		

TABLE 3.—a/b a/c/d = MAXIMUM QUANTITY OF CITRIC ACID PRODUCED IN 5 ML. OF FERMENTATION SOLUTION AND EXPRESED IN ML. IN TERMS OF N/100 NaOH SOLUTION; b = TIME IN DAYS TO PRODUCE THE MAXIMUM QUANTITY OF ACID. c = % OF CITRIC ACID IN TERMS OF WEIGHT OF MOLASSES; AND d = DRY WEIGHT OF MYCELIA IN G. PER VAT AFTER 12-DAY FERMENTATION. MOLASSES CONTAINED 50% SUGAR.

Medium No.	RGANISM No.												
	2	6	11	12	13	14	24	25	27	28	30	31	32
1	99/6 4.95/2.8	132/7 6.6/3.97	132/8 6 6/2.88	110/8 5.5/1.05	74/6 3.7/.4	139/8 6.95/3.2	219/10 10.95/4.05	200/9 10.0/4.85	198/7 9 90/4.3	160/7 8.0/5.0	117/9 5.85/4.4	106/9 5.3/3.4	123/6 6.15/3.7
2	100/6 5/2.85	153/7 7.65/3.8	144/8 7.2/3.9	110/8 5.5/1.00	91/6 4.55/0.4	139/8 6.95/2.8	211/10 10.55/4.4	206/7 10.3/5.9	189/7 9 45/4.8	162/ 9 8.10/4.0	117/9 5.85/5 3	103/10 5.15/2.9	144/6 7.2/3.13
3	99/6 4.95/3.05	123/7 6.15/3.0	137/8 6.85/4 8	120/8 6.00/0.9	76/6 3.8/0.5	142/8 7.1/3.5	190/10 9.5/4.55	197/7 9.85/5.55	188/7 9.4/4.3	206/7 10.3/4.33	117/8 5 85/4.1	106/10 5.3/2.9	186/6 9.3/4.83
4	89/6 4.45/2.13	111/7 5.55/1.32	98/8 4 9/0.5	65/5 3.25/0.85	65/6 3.25/0.4	106/8 5.3/0.5	93/8 4.65/0.9	97/6 4.85/5.45	119/6 5.95/0.6	97/7 4.85/0.48	73/8 3 65/1.05	91/5 4.95/1.5	72/5 3.6/1.6
5	117/6 5.85/5.19	128/7 6.4/3.40	136/8 6.8/4.48	114/8 5.7/1.08	77/6 3.85/0.5	140/8 7.0/1.05	186/10 9.3/4.2	217/7 10.85/5.2	201/7 10.05/4.5	191/7 9.55/4.8	123/8 6.15/1.4	103/5 5.15/3.75	168/6 8.4/5.2
6	81/6 4.05/2.4	106/7 5 3/1.40	96/5 4.8/0 9	130/8 6.5/0.70	71/7 3.55/0.4	62/4 3.1/0.7	167/8 8.35/2.75	90/6 4.5/0.5	102/6 5.1/0.65	190/9 9.5/2.6	85/8 4.25/5.9	95/5 4.7/1.9	87/6 4.35/1.9
7	70/6 3.5/3.18	122/7 6.1/2.78	95/6 4.75/2.48	60/6 3 0/4 43	86/6 4.3/5.2	106/4 5.3/4.9	110/6 5.5/6.35	145/6 7.25/4.4	116/6 5.8/4.8	145/7 7.25/9.5	80/4 4 0/8.05	68/5 3.4/3.3	120/5 6.0/9.8
8	79/6 3.95/3.61	118/7 5 9/1.35	91/6 4.55/2.55	62/5 3.1/4.00	77/ 5 3.85/5.25	127/4 6.35/5.95	113/6 5.65/6 2	103/5 5.15/5 0	115/5 5.75/5.3	133/7 6.65/10.8	84/4 4.2/4.8	89/5 4.45/4.25	125/6 6.25/9.2
9	74/6 3.7/2.17	81/8 4 05/1.31	96/5 4 8/1.13	92/7 4 6/0.95	86/6 4.3/0.7	90/4 4.5/0.75	90/6 4.5/1.2	74/5 3 7/0.6	122/7 6.1/0.80	82/7 4.1/2.75	88/8 4.4/2.55	89/10 4.45/1.35	70/5 3.5/0.65
10	97/7 4.85/3.82	117/7 5.85/4.72	113/8 5.65/2.27	117/7 5.85/1.20	76/6 3.8/0.5	128/9 6.4/3.35	175/10 8.75/3.85	90/6 4.5/2 3	180/7 9.0/4.5	195/7 9.65/5.45	68/6 3.4/0.5	108/6 5.4/3.75	125/6 6.25/3.13
11	99/7 4.95/1.98	93/7 4.65/1.5	104/5 5.2/1.00	88/8 4.4/0.5	65/6 3.25/0.25	64/4 3.2/0.7	90/6 4.5/0.85	53/7 2.65/0.3	112/6 5.6/0.55	211/7 10.55/2.55	79/8 3.95/0.7	76/10 3.3/1.4	83/6 4.15/1.05

(b) Aeration Method: The above eight media (Nos. 12—19, Table 2) were used in aeration culture method. 500 ml. portion of each medium was taken in a 2-litre measuring cylinder and sterile air was passed at the rate of 200 ml./100 ml. of the medium/minute. Aeration was continued for 6 days. After every 24 hours the fermented broth was tested for citric acid by Deniges' method.[9] It was observed that none of the organisms could produce citric acid under these conditions as well.

Discussion

Morphological and biochemical characters show that all the organisms belong to the species *Aspergillus niger*.[1] From the results of the fermentation experiments it was further observed that under the same experimental conditions, all the organisms do not produce equal quantities of citric acid, but produce different quantities in different cases. Hence it is concluded that though morphologically and biochemically they are identical to a great extent, yet there are wide variations in their cultural biological characters, which indicate that these are different strains of *Aspergillus niger*.

From the results of the fermentation experiments, it is observed that all the organisms could produce citric acid in all media. The minimum yield 2.66% (as anhydrous citric acid) was in the case of organism No. 25 in medium No. 11, and maximum yield 10.95% in the case of organism No. 24 in medium No. 1.

Inorganic salts are essential for the growth of mycelium and production of citric acid, but in most of the cases potassium ferricyanide supressed the growth of myceliumto a large extent. In all cases small quantities of oxalic acid were also formed.

Acknowledgement.—The authors express their thanks to Dr. M. Qudrat-i-Khuda and Dr S. Hedayetullah for their helpful suggestions during the progress of the work and to Dr. Salimuzzaman Siddiqui, F. R. S., for his encouragement. The authors also thank Mr. M. Rahman for his help in taking the measurements of the organisms in slide cultures.

References

1. G. Smith, *Industrial Mycology* (Edward Arnold, London, 1955), 4th Ed., p. 140.
2. H. Tauber, *The Chemistry and Technology of Enzymes* (Champan and Hall, London, 1950), 2nd Ed. p. 341.
3. S. C. Prescott and C. G. Dunn, *Industrial Microbiology* (McGraw Hill, New York, 1949), pp. 572—605.
4. M. Q. Khuda, N. M. Sheikh and G. K. Joarder, Pakistan J. Sci. Ind. Research, **5,** 59 (1962).
5. S. A. Waskman, *The Actinomycetes* (Williams and Wilkins, Baltimore, 1959), Vol. I, p. 47.
6. W. P. Doelger and S. C Prescott, Ind. Eng. Chem., **26,** 1142 (1934).
7. Salle, *Practical Bacteriology* (McGraw Hill, New York, 1943), pp. 65—148.
8. George L. Peltier, Carl. E. Georgi, F. Lawrence and Lindgren, *Laboratory Manual of General Bacteriology* (John Willy & Sons, Inc., New York, 1952), p. 53.
9. F. A. Camp, Missouri, Botan. Garden, **10,** 3 (1923).