EFFECT OF THE COMPOSITION OF THE SPORULATION MEDIUM ON CITRIC ACID PRODUCTION BY ASPERGILLUS NIGER IN SUBMERGED CULTURE[1]

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Extensive work has been done (Foster, 1939; Perlman, Dorrell, and Johnson, 1946; Porges, 1932) on the nutritional requirements of *Aspergillus niger* and on the relation between the constitution of the fermentation medium and the yield of citric acid by this organism. However, no work has been reported in which the effect of the composition of the sporulation medium has been studied. Doelger and Prescott (1934), however, have noted that successive transfers of the culture on a synthetic medium increased citric acid yields. In the course of a study of citric acid production by submerged culture (Shu and Johnson, 1947) it was noted that the composition of the sporulation medium had a great effect on citric acid yield in fermentations in which the spores were used as inoculum. The experiments reported in the present paper were designed to determine the cause of this variation in yield.

METHODS

A strain of *Aspergillus niger* from culture 72-4 (Perlman, Kita, and Peterson, 1946) was used throughout the experiments. The stock culture was carried on soil. In order to reduce the amount of soil substances carried over, cultures were carried through three successive sucrose agar slants made with medium A, shown in table 1. The second of these transfers was kept as a substock culture for the entire experiment. A water suspension of spores was made from the third transfer with 5 ml of sterile distilled water.

This suspension was used to inoculate the agar medium under investigation: 1 loopful for an agar slant and 0.5 ml for a bottle plate. All slants were made with 4 ml of agar medium in 18-by-150-mm pyrex test tubes. The slope of the slants was made approximately 15 degrees with respect to the axis of the tube. Bottle plates were made with 25 ml of agar medium in a 6-oz rectangular bottle. This amount gave a layer 0.5 cm thick with a 72 sq cm agar surface when the bottles were placed in a horizontal position. The media were sterilized at 120 C for 20 minutes. The inoculated slants or plates were incubated at 30 C until the entire agar surface was uniformly covered with spores.

Suspensions of spores grown on experimental agar media were made with 5 ml of sterile water for slants and with 50 ml for bottle plates. Approximately 1.5 ml

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of the suspension was used to inoculate 500-ml cotton-plugged Erlenmeyer flasks containing 50 ml of fermentation medium. The composition of the fermentation medium is shown as medium B in table 1. The inoculated flasks were incubated at 25 C on a shaker, rotating horizontally, describing a circle 1 inch in diameter at a speed of 270 rpm. All results reported represent the average of triplicate flasks.

At intervals of 5, 7, and 10 days, samples were taken and analyses were made for residual sugar, titratable acidity, and, in some cases, citric acid. The figures given in the tables are for samples taken at 10 days except when otherwise stated. Residual sugar was determined by the method of Shaffer and Somogyi (1933) and citric acid by the method of Perlman, Lardy, and Johnson (1944). The titratable

TABLE 1

Composition of media

CONSTITUENTS	MEDIUM A	MEDIUM B	MEDIUM C
	wt/L	*wt/L*	*wt/L*
Domino sucrose	140 g	140 g	140 g
Difco agar	20 g		
KH_2PO_4	1 g	1 g	1 g
$MgSO_4 \cdot 7H_2O$	0.25 g	0.25 g	0.25 g
NH_4NO_3	2.5 g	2.5 g	2.5 g
HCl	to pH 4.5	to pH 3.1	to pH 2.3
Trace metals			
Cu^{++}	0.14 mg	0.06 mg	0.06 mg
Zn^{++}	1.4 mg	0.25 mg	0.25 mg
Fe^{+++}	2.2 mg	1.3 mg	0.45 mg
Mn^{++}	<1 μg	<1 μg	<1 μg

The listed quantities of metals include the amounts present as impurities in other constituents of the media.

acidity was expressed in terms of anhydrous citric acid. The presence of certain trace elements was determined colorimetrically with the γ, γ' dipyridyl method for iron; dithizone for zinc; carbamate for copper; and periodate for manganese (Sandell, 1944). The yield of citric acid was expressed as the percentage of added sugar (grams anhydrous citric acid per 100 grams added sucrose).

EXPERIMENTAL RESULTS

Effect of medium on rate of sporulation. Rectangular bottle plates were used in these experiments. The composition of the media tested was the same as medium A, table 1, except for the components which were varied. The minimum time required for the spores to cover the entire plate was used as a measure of the rate of spore formation. The results are summarized in table 2. Increasing the concentration of Zn, NH_4NO_3, and KH_2PO_4 retarded the rate of spore formation, but increasing the concentration of Mn or malt extract favored spore formation. Abundant spores were formed in 48 hours if Mn or malt extract was added.

Effect of trace metals in sporulation medium on citric acid yield. Agar slants

were used in these experiments. Table 3 summarizes the results of the addition of Fe, Cu, Zn, and Mn, and their combinations, to the basal agar sporulation medium A. The presence of Mn at a level of 9.3 mg per liter lowered the acid yield to 35 per cent, which is only 50 per cent of the basal medium control. Iron added at a level of 8 mg per liter of medium also showed considerable effect.

TABLE 2

Effect of the composition of medium on rate of sporulation

CONSTITUENT VARIED IN MEDIUM A	QUANTITY PRESENT	MINIMUM TIME FOR SPORES TO COVER AGAR SURFACE
		hours
None		68
pH	6.0	68
	7.5	96
Sucrose	200 g/L	68
	50 g/L	68
KH_2PO_4	5.0 g/L	>240
	2.5 g/L	72
NH_4NO_3	5.0 g/L	>240
	0.5 g/L	52
$MgSO_4 \cdot 7H_2O$	0.5 g/L	68
	0.05 g/L	68
Mn^{++}	9.3 mg/L	48
Fe^{+++}	10.2 mg/L	60
	3.0 mg/L	68
Cu^{++}	3.5 mg/L	68
	0.5 mg/L	68
Zn^{++}	25.4 mg.L	144
	3.8 mg/L	96
Malt extract	1.5 g/L	48

Zinc alone did not exhibit any significant effect, but it exhibited some antagonistic effect against manganese. Copper showed a similar effect. Simultaneous addition of Cu, Zn, and Fe at levels of 0.34 mg, 2.4 mg, and 0.8 mg, respectively, per liter of basal medium was found to give the highest acid yield in the fermentation test. A yield of 80 per cent total acidity calculated as citric acid on added sugar was obtained in 10 days of fermentation. About 90 per cent of this total acidity was due to citric acid.

The stability of the culture in the medium (no. 18, table 3) was tested by 18 successive spore transfers. At intervals of 6 transfers, fermentation tests were

made. The results are shown in table 4. No significant changes in acid production were observed.

TABLE 3

Effect of metallic ions in sporulation media on acid production

NO.	METALLIC ION ADDED TO SPORULATION MEDIUM A				YIELD OF ACID* IN MEIUM B ON AVAILABLE SUGAR PER CENT
	Mn	Zn	Cu	Fe	
	mg/L	*mg/L*	*mg/L*	*mg/L*	
1	0	0	0	0	70
2	93	0	0	0	14
3	9.3	0	0	0	35
4	1.9	0	0	0	53
5	0	24	0	0	69
6	0	2.4	0	0	71
7	0	0	3.4	0	60
8	0	0	0.34	0	66
9	0	0	0.07	0	54
10	0	0	0	8	48
11	0	0	0	0.8	53
12	0	2.4	0.34	0	63
13	9.3	2.4	0	0	45
14	0	2.4	0	0.8	70
15	9.3	0	0.34	0	45
16	9.3	0	0	0.8	37
17	0	0	0.34	0.8	56
18	0	2.4	0.34	0.8	80
19	9.3	2.4	0.34	0	46
20	9.3	2.4	0	0.8	22
21	9.3	0	0.34	0.8	20
22	9.3	2.4	0.34	0.8	54

* Titratable acidity calculated as anhydrous citric acid.

TABLE 4

Effect of successive spore transfers of the culture on acid production

NUMBER OF TRANSFERS	YIELD OF ACID* ON AVAILABLE SUGAR PER CENT
0	80
6	80
12	87
18	75

* Titratable acidity calculated as anhydrous citric acid.

The metals might exert the effects shown in table 3 either by being carried over by the spores into the fermentation medium, or by causing some physiological changes in the spores. Since it is known (Perlman, Dorrell, and Johnson, 1946) that the presence of appreciable quantities of manganese in the fermentation medium reduces yields of citric acid in surface fermentations, it seemed desirable

to determine the quantity of Mn added to the fermentation medium by the spore inoculum. Spores were grown in bottle agar plates containing various levels of Mn. Spore suspensions from each of the bottle plates were made with 50 ml sterile distilled water containing 10 per cent ethyl alcohol. The suspensions were filtered aseptically through glass wool into previously sterilized centrifuge tubes. The tubes were then centrifuged and the supernatant was pipetted out. The spores were washed twice with 50-ml portions of distilled water and finally resuspended in 50 ml distilled water. For each of the fermentation flasks 1.5 ml of this suspension was used as inoculum. The remaining spore suspension was used for the determination of manganese.

TABLE 5

Effect on acid production of manganese carried into fermentation medium with spore inoculum

NO.	Mn ADDED TO SPORULATION MEDIUM A	Mn CARRIED TO FERMENTATION MEDIUM B	Mn ADDED TO FERMENTATION MEDIUM B	YIELD OF TITRATABLE ACID ON AVAILABLE SUGAR	YIELD OF CITRIC ACID* ON	
					Available sugar	Utilized sugar
	mg/L	*μg/L*	*μg/L*	*per cent*	*per cent*	*per cent*
1†	0	<0.02	0	66	57	64
2	0	<0.02	0.4	74	69	75
3	0	<0.02	3	50	45	56
4	0	<0.02	15	23	19	21
5†	0.93	0.4	0	68	57	66
6†	9.3	3	0	44	40	56
7†	93.0	16	0	21		

* By pentabromoacetone method.

† Results of 12 days' fermentation.

Another series of fermentations was prepared and inoculated with spores produced on the basal (Mn-free) medium. To these flasks were added amounts of manganese equal to those introduced to the first series of fermentation flasks with the spores grown on the Mn-containing media. The results are summarized in table 5. It may be seen that the amount of Mn carried over with the washed spore inoculum to the fermentation medium is sufficient to retard the acid production, and that as little as 3 μg Mn per liter of fermentation medium appreciably lowers the citric acid yield.

Addition of malt extract. As shown in table 6, the addition of Trommer's malt extract to the sporulation medium at a level of 1.5 g per liter decreased the acid yield. The organic components of the malt extract seem to be responsible for this reduction, because the addition of the equivalent amount of the malt extract ash to the agar plate medium favored acid production in the fermentation test. Furthermore, the addition of malt extract to the agar medium containing manganese at a level of 9.3 mg per liter exhibited a definite additional influence on acid production. The results are shown in table 7. This effect of Mn and malt

extract is not noticeable if the fermentation test is run by the surface culture method (table 6) with the fermentation medium C (table 1).

TABLE 6

Effect of addition of malt extract (to sporulation medium) on acid production

SUBSTANCES ADDED	QUANTITY ADDED	YIELD OF ACID PRESENT*	METHOD OF FERMENTATION
	g/L		
None		66	submerged
Trommer malt extract	1.5	55	submerged
Trommer malt extract ash	Equivalent to 1.5 g malt extract	76	submerged
Trommer malt extract	1.5	28	submerged
Mn	0.0093		
None		60	surface
Trommer malt extract	1.5	57	surface
Mn	0.0093		

* Titratable acidity calculated as anhydrous citric acid.

TABLE 7

Retardation of acid production by simultaneous presence of malt extract and manganese in sporulation medium

BASAL MEDIUM A +		YIELD OF ACID ON AVAILABLE SUGAR*
Mn	Trommer malt extract	
mg/L	*g/L*	*per cent*
9.3	0.00	33
9.3	0.01	27
9.3	0.05	12
9.3	0.10	10
9.3	1.00	11

* Titratable acid calculated as anhydrous citric acid on 7 days' fermentation.

SUMMARY

The addition of Mn and Trommer malt extract at a level of 9.3 mg and 1.5 g, respectively, to 1 liter of basal agar plate medium accelerated spore formation, whereas increasing the concentration of KH_2PO_4, NH_4NO_3, and Zn retarded spore formation.

The presence of Mn in the sporulation medium at a level of 9.3 mg per liter retarded citric acid production in submerged fermentations in which the spores were used as inoculum. The effect is shown to be attributable to the amounts of Mn carried over into the fermentation medium by the washed spore inoculum.

The presence of Mn and malt extract in the sporulation medium reduced the acid production in submerged fermentation, but not in the surface culture fermentation.

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