THE INFLUENCE OF ZINC, IRON, COPPER, AND MANGANESE ON THE PRODUCTION OF CITRIC ACID BY *ASPERGILLUS NIGER*¹

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The literature contains seemingly conflicting reports regarding the influence of zinc, iron, copper, and manganese on the production of citric acid by *Aspergillus niger* (Foster, 1939; von Loesecke, 1945); in fact, the fermentation media used by many workers has not included any of these elements other than as chance contaminants.

These reports are frequently difficult to reconcile with the known nutritional needs of the organism. It has, however, been established that the growth requirements of *A. niger* include minute quantities of the four elements under consideration, in addition to carbon, hydrogen, nitrogen, oxygen, potassium, phosphorus, magnesium, and sulfur. Raulin (1869) found zinc and iron to be essential, and this observation was confirmed by Steinberg (1919a). Bertrand and Javillier (1911) demonstrated that in addition to these two elements manganese was required. Bortels (1927) was the first to indicate the need for copper. Steinberg (1935a) confirmed the need of the organism for manganese, and Wolff and Emmerie (1930) confirmed the essential nature of copper.

If these four elements are essential for the growth of *A. niger*, then they should also be essential for the production of citric acid by any method that involves the growth of the organism. Steinberg (1935a), who was primarily concerned with the nutrition of the fungus rather than with acid production, found that zinc, iron, and copper were necessary for the development of a low pH in his medium, but that this low pH was not reached if manganese was present in amounts optimum for growth. Similarly Mulder (1939) stated that copper appeared to favor acid production as evidenced by pH changes. Currie (1917), who was concerned directly with the production of the acid, believed iron to be unnecessary for acid production, and zinc to increase the weight of the mycelium but not the yield of acid. Porges (1932) and Shu and Johnson (1948b) employed zinc and iron in their media in order to obtain high yields of citric acid. Perlman, Dorrell, and Johnson (1946) used iron in media containing very low impurities of zinc, copper, and manganese and obtained high yields of the acid, but found the addition of more zinc or copper to be harmful. Shu and Johnson (1948b) found that managanese in very low concentration caused marked decreases in yields

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of the acid. Bernhauer, Knobloch, and Iglauer (1941) observed that manganese salts had no constant effect, and that zinc, copper, or iron inhibited acid production. Porges (1932) reported that manganese and copper were inhibitory to acid production.

The present work has been directed toward determining whether or not zinc, iron, copper, and manganese are necessary for maximum citric acid production by a method involving the growth of *A. niger*.

METHODS AND MATERIALS

The method of fermentation adopted for use was that of growing the organism from conidia on the fermentation medium under stationary surface culture conditions. Changes in acid production, sugar utilization, and growth of the organism were employed as the means of determining the influence of the variable investigated.

The organism studied was *A. niger* strain 72-4 (Perlman, Kita, and Peterson, 1946) kindly supplied by Dr. M. J. Johnson, Department of Biochemistry, University of Wisconsin. It was selected for study following the testing of some 40 isolates of *A. niger* in this laboratory.

Sucrose of commercial grade was used as the carbon source. It had an ash content of 0.021 per cent, of which 47 per cent was water-soluble. By chemical analysis only sodium chloride and a slight trace of phosphate were identified in this ash. No trace of either iron or manganese was found. All other chemicals were of reagent grade. The water used was redistilled from pyrex glassware. Pyrex glassware was used throughout this work and, unless otherwise stated, was cleaned with 10 per cent nitric acid (Henry and Smith, 1946), then thoroughly rinsed with redistilled water. Stock cultures were carried in soil in accordance with the recommendations of Greene and Fred (1934).

The inoculum for each 100 ml of medium to be fermented consisted of 1 ml of a heavy suspension of conidia. This was prepared by seeding the organism from the soil stock culture to a slant of solid basal medium (see below), incubating the culture for 4 days, subculturing it to a second slant of the same composition, and incubating it for the same period. From this last culture sufficient similar slants were prepared so that one culture was available for each five flasks to be inoculated. After 4 days' incubation, conidia were collected on a sterile glass rod and transferred to sterile water; 5 ml of water were used for each slant culture. The suspension thus prepared contained approximately five million conidia per ml, as determined by means of plate counts. Glass rods were always used in inoculating slant cultures. This procedure was followed in order to obtain a uniform inoculum containing only those trace elements unavoidably present in the conidia. Conidia produced under these conditions were of a brown color.

Fermentation studies were conducted on 100-ml portions of medium contained in 250-ml Erlenmeyer flasks covered with inverted 100-ml beakers.

Following the recommendations of Doelger and Prescott (1934), the medium was adjusted to pH 2.0 after sterilization by autoclaving, and the temperature and period of incubation were set at 30 C and 10 days, respectively.

Following fermentation, all flasks were autoclaved, the mycelium from each was removed by filtration, and the filtrate was diluted to a volume of 250 ml. Aliquots of the diluted filtrate were taken for titration and analysis. The mycelia were dried at 80 C until the heavier ones showed no appreciable loss of weight over a 24-hour period; they were then weighed. Titration of acidity was done by using 0.5 N NaOH with phenolphthalein as the indicator. Citric acid was determined gravimetrically as pentabromacetone. Oxalic acid was determined by precipitating it from cold neutral solution as the calcium salt. This was removed by filtration, dissolved in H_2SO_4 , and titrated with standard $KMnO_4$ solution. Residual sugar was measured by the Lane-Eynon volumetric method (*Official and Tentative Methods of Analysis of the A.O.A.C.*, 1945), the aliquot used first being neutralized with NaOH. In the tables and text all values are based on 100 ml of medium. Thus the quantities of citric and oxalic acids given in the tables were formed from 15 grams of available sucrose. The value "Citric acid (by titration)" in the tables was derived by assuming that all the increase in titratable acidity that occurred in the fermentation was due to the formation of citric acid. This value approximates roughly the quantity of citric acid actually present, as will be observed from the data in tables 5 to 8. The values "Citric acid by gravimetric

TABLE 1
Effect of the method of cleaning glassware on the fermentation

METHOD OF CLEANING FERMENTATION FLASKS	CITRIC ACID (BY TITRATION)
	g
Washed with soap	10.1
Chemically cleaned	2.0

analysis" and "Oxalic acid" in the tables are the weights of these acids actually found to be present by analysis. The citric acid referred to is calculated as the anhydrous form.

Unless otherwise indicated in the tables, the mycelia showed no evidence of the formation of conidia.

EXPERIMENTAL RESULTS

The effect of trace elements on this fermentation was clearly indicated by results obtained early in the investigation when a study was made of the influence of the degree of cleanliness of the glassware on the yield of acid. The data of table 1 show the large variation in yield encountered when the only known variable was a difference in the degree of cleanliness of the glassware. The medium fermented in this instance was that found optimum by Doelger and Prescott (1934). It contained no added trace elements. It was also found that, if the glassware was washed with soap and water and reused, the high yield first obtained could not be duplicated; in fact, yields fell to approximately 3 g of citric acid from 15 g of sucrose. This strongly suggested the loss of some essential nutrient from the glassware through repeated use. A somewhat similar condition was met by Steinberg (1919b), who found that the apparent leaching of zinc from glassware by

its repeated use caused a reduction in the dry weight of mycelium produced by *A. niger*. Chemically cleaned glassware was used in all subsequent work, except as mentioned in connection with the preparation of the inocula.

To determine whether a high yield of acid could be obtained under our conditions by the adjustment of the concentration of certain nutrient salts without the addition of trace elements, an experiment was undertaken in which the concentration of one salt in the medium was varied over a comparatively wide range while the other two salts were held at the level recommended by Doelger and Prescott (1934). The salts used were $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and K_2HPO_4 , with either NaNO_3 , NH_4NO_3 , or NH_4Cl as the nitrogen source. None of the combinations produced a good yield of citric acid, the maximum yields being of the order of 2.5 g of citric acid from 15 g of sucrose. This experiment was repeated with two other strains of *A. niger*, isolated from soil, with similar results. It was concluded that it would be impossible to obtain large yields of the acid simply by the adjustment of the quantities of the salts in this medium. Accordingly, after careful study, a basal medium of the following composition was selected for an investigation of the influence of certain trace elements:

Basal medium

Sucrose.....	15.0 g
NaNO_3	0.6 g
K_2HPO_4	0.06 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.03 g
HCL (1N).....	1.7 ml (pH 2.0)
Water.....	to 100.0 ml total volume

For a solid medium, 1.5 per cent of agar was added to this medium, and the amount of HCl was reduced to 0.1 ml. That this basal medium is adequate, upon the addition to it of suitable quantities of trace elements, for greater growth of the organism than is needed for good acid production is shown by the data of table 8.

The four trace elements under investigation were tested singly for their effect in the basal medium in the amounts of 0.1 and 0.005 mg per cent. The salts used were $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. The results are recorded in table 2.

Increasing the quantity of zinc, in amounts up to 3.0 mg per cent, produced no further increase in acid over that obtained with 0.1 mg per cent, but rather resulted in a small decrease.

The effects of combinations of the four elements when added to the basal medium are recorded in table 3. Any combinations that did not include zinc produced very low yields of acid, and such combinations have not been given in the table.

In order to determine the approximate optimum levels of zinc and iron for acid production in the absence of added copper or manganese, experiments were conducted in which zinc and iron were separately held at 0.1 mg per cent while the other element was varied in amounts up to 0.8 mg per cent. Although the

optimum amount of zinc was found to be close to 0.1 mg per cent, the optimum for iron was indicated as being below 0.1 mg per cent. The results in table 4 show the effect of varying amounts of iron added to the basal medium to which 0.1 mg per cent of zinc had been added.

TABLE 2

Influence of copper, of iron, of manganese, and of zinc on acid production and growth of A. niger 72-4

BASAL PLUS				CITRIC ACID (BY TITRATION)	DRY WEIGHT OF MYCELIUM
Cu	Fe	Mn	Zn		
mg %	mg %	mg %	mg %	g	g
—	—	—	—	0.99	0.13
0.005	—	—	—	1.15	0.12
0.1	—	—	—	1.25	0.15
—	0.005	—	—	1.18	0.14
—	0.1	—	—	0.96	0.18
—	—	0.005	—	0.53	0.23*
—	—	0.1	—	0.36	0.20*
—	—	—	0.005	1.82	0.26
—	—	—	0.1	3.71	0.60

* Mycelia heavily covered with black conidia.

TABLE 3

Influence of combinations of copper, iron, manganese, and zinc and acid production and growth of A. niger 72-4

BASAL PLUS				CITRIC ACID (BY TITRATION)	SUGAR USED	DRY WEIGHT OF MYCELIUM
Cu	Fe	Mn	Zn			
mg %	mg %	mg %	mg %	g	g	g
—	—	—	0.1	3.68	6.0	0.58
—	0.1	—	0.1	6.50	14.4	2.06
0.005	—	—	0.1	2.50	—	0.38
—	—	0.005	0.1	0.86	3.3	0.35*
0.005	0.1	—	0.1	7.23	14.5	2.10
—	0.1	0.005	0.1	2.11	12.3	3.25†
0.005	0.1	0.005	0.1	1.95	11.8	3.86*

* Mycelia heavily covered with black conidia.

† Mycelia fairly heavily covered with light-brown conidia.

In view of the observation of Currie (1917) that under the conditions of a prolonged fermentation the organism utilized the citric acid formed, it was of interest to ascertain whether the low yields obtained in the presence of excessive iron were due to the fact that under these conditions a 10-day fermentation period was too long. The titratable acidity of a fermentation was followed when both zinc and iron in the amount of 0.1 mg per cent were added to the basal medium. It was found that on the eight day of the fermentation the acid had

attained a maximum, after which time it remained relatively constant for 3 days and then began to decrease. A shorter fermentation period would, therefore, not overcome the effects of excessive amounts of iron.

A high yield of citric acid thus had been obtained without the addition of either copper or manganese to the medium. In fact 0.005 mg per cent of copper in the presence of optimum amounts of zinc and iron was now found to cause a decrease in the quantity of acid produced, the reverse of the effect observed in the presence of excess iron (table 3). If copper or manganese, or both, are necessary for the production of a large amount of acid, then they must have been present either in the conidia forming the inoculum or as impurities in the medium, or both. That the former source was a possibility had been shown by the work of Steinberg (1935a) and Shu and Johnson (1947).

In order to minimize the carry-over of trace elements in the conidia of the inoculum, the practice of washing the agar used in the propagation medium was

TABLE 4
Influence of varying concentrations of iron in the presence of 0.1 mg per cent zinc

BASAL PLUS 0.1 MG % Zn PLUS Fe	CITRIC ACID (BY TITRATION)	SUGAR USED	DRY WEIGHT OF MYCELIUM
mg %	g	g	g
—	3.96	6.0	0.66
0.0025	5.57	9.2	0.98
0.005	7.36	11.9	1.07
0.006	8.38	12.2	1.13
0.0075	8.35	12.5	1.28
0.010	9.40	13.7	1.46
0.015	8.51	13.8	1.70
0.02	8.22	14.0	1.85
0.03	7.52	14.1	2.06
0.04	7.58	14.3	2.15
0.05	7.36	14.6	2.13
0.1	6.62	14.6	2.12

adopted. Also the test tubes used for growth of the inocula, which formerly had been washed with soap and water only, were chemically cleaned in all further work. This procedure had not been followed in earlier work as it was known that too low a concentration of trace elements in the substrate would result in the failure of the organism to form conidia.

These changes in method at once resulted in a marked reduction in the yield of citric acid when 0.1 mg per cent of zinc and 0.01 mg per cent of iron were supplied to the basal medium. It was then found that very small additions of either copper or manganese, or both, to the medium caused an increase in the production of citric acid. Tables 5 and 6 show the distinctly beneficial effect of copper or manganese, or both, under these conditions and the extremely harmful effect of either if supplied in slight excess. Table 6 shows that an increase in iron will not substitute for copper and manganese. (The optimum quantities of copper and manganese were not determined.)

TABLE 5

Influence of varying concentrations of copper and manganese in the presence of fixed amounts of zinc and iron

BASAL PLUS 0.1 MG % Zn, 0.01 MG % Fe, PLUS		CITRIC ACID		OXALIC ACID	SUGAR USED	DRY WEIGHT OF MYCELIUM
Cu	Mn	By titration	By gravimetric analysis			
$\mu\text{g } \%$	$\mu\text{g } \%$	g	g	g	g	g
—	—	8.42	7.80	0.327	11.7	1.24
0.01	—	8.35	8.37	0.445	12.3	1.47
0.1	—	8.64	8.70	0.396	13.4	1.42
1.0	—	8.80	9.03	0.408	12.8	1.47
5.0	—	6.34	5.78	0.253	10.3	1.49
—	0.01	8.96	8.92	0.438	12.5	1.30
—	0.1	9.28	9.37	0.438	14.1	1.62
—	1.0	5.28	4.91	0.445	11.5	1.90*
—	5.0	6.59	5.11	0.284	12.7	2.07†
0.01	0.01	8.06	8.00	0.426	11.8	1.33
0.1	0.1	9.15	9.07	0.432	13.8	1.58
1.0	1.0	5.02	4.63	0.414	11.3	2.03‡
5.0	5.0	5.09	4.55	0.321	11.6	2.10§

* Mycelia partially covered with light-brown conidia.

† Mycelia covered with brown conidia.

‡ Mycelia partially covered with brown conidia.

§ Mycelia heavily covered with black conidia.

TABLE 6

Influence of copper and manganese in the presence of varying concentrations of iron and a fixed concentration of zinc

BASAL PLUS 0.1 MG % ZINC PLUS Fe	NO ADDED Cu OR Mn					0.1 $\mu\text{G } \%$ EACH OF Cu AND Mn				
	Citric acid		Oxalic acid	Sugar used	Dry wt of myce- lium	Citric acid		Oxalic acid	Sugar used	Dry wt of myce- lium
	By titra- tion	By gravi- metric analysis				By titra- tion	By gravi- metric analysis			
$mg \%$	g	g	g	g	g	g	g	g	g	g
—	2.91	2.16	0.004	4.1	0.53	3.30	2.72	0.093	4.9	0.67
0.004	6.53	6.78	0.117	8.8	0.92	8.32	8.27	0.222	12.0	1.15
0.009	8.42	7.85	0.296	11.6	1.23	9.15	9.33	0.432	13.5	1.52
0.01	8.42	7.80	0.327	11.7	1.24	9.15	9.07	0.432	13.8	1.58
0.011	9.09	8.59	0.321	12.8	1.35	9.25	9.35	0.438	14.0	1.51
0.015	9.02	8.69	0.272	13.5	1.58	8.45	8.30	0.389	14.1	1.87
0.05	6.98	6.31	0.253	13.9	2.18	7.10	7.24	0.364	14.3	2.23
0.1	6.88	6.70	0.315	13.8	1.81	6.82	6.34	0.296	14.3	1.98

In order to investigate the influence that impurities of trace elements in the basal medium might have on the fermentation, the use of the organism itself as

a means of reducing those impurities was considered. The work of Bertrand and Javillier (1911) demonstrated that *A. niger* does not take up all the manganese available if it is present in appreciable quantities, but under our conditions it was hoped that the amount removed would be sufficient to emphasize the effect of a shortage of manganese, and also possibly of copper.

The organism was grown, for a period of 10 days, on the basal medium with its sucrose content increased to 17.5 grams. The mycelium was then removed by filtration, after which the filtrate was resterilized by autoclaving and used as a basal medium for this phase of the work. Table 7 records the results of one such experiment. The values given are those obtained at the end of the second 10-day fermentation period. If no trace elements were added to the depleted medium before it was used, only a very slight increase in acidity occurred during the fermentation period.

TABLE 7

Influence of copper, iron, manganese, and zinc on the fermentation of substrate previously depleted by the growth of A. niger 72-4

DEPLETED BASAL PLUS				CITRIC ACID		SUGAR USED	DRY WEIGHT OF MYCELIUM
Cu	Fe	Mn	Zn	By titration	By gravimetric analysis		
$\mu\text{g } \%$	$\text{mg } \%$	$\mu\text{g } \%$	$\text{mg } \%$	g	g	g	g
—	—	—	—	1.44*	0.76	2.9†	0.11
—	0.014	—	0.1	5.09	4.53	7.8	0.70
0.2	0.014	—	0.1	5.23	4.58	8.4	0.73
—	0.014	0.2	0.1	7.68	6.98	12.2	1.05
0.2	0.014	0.2	0.1	8.73	8.05	13.5	1.19

* Citric acid at start of second fermentation (by titration), 1.18 g.

† Sugar used at start of second fermentation, 2.0 g.

In table 8 data are presented showing that the quantities of iron, copper, and manganese desirable in the fermentation medium for high yields of citric acid are less than those needed for good growth of the organism. Comparison of the values in the left half of the table with those in the right show that, without exception under comparable conditions, 0.1 mg per cent of iron permitted greater growth of the organism than did 0.01 mg per cent, whereas, with only one exception (copper at 5 μg per cent), 0.01 mg per cent of iron permitted the accumulation of more citric acid in the medium than did 0.1 mg per cent. It will also be observed that, without exception, the lower amounts of either copper or manganese, or both, permitted less growth but greater accumulation of citric acid than did the higher amounts of those elements under comparable conditions.

To investigate the effect on the fermentation of replacing certain constituents of the basal medium with others that could be expected to be their equivalent, NH_4NO_3 and HNO_3 were substituted for the NaNO_3 and HCl of the basal medium. The substitutions were made in such amounts that the nitrogen content and the pH of the two media were identical. This medium appeared to contain lower impurities of trace elements than the basal medium previously used, as

judged by the quantity of growth it produced when used without any additions of trace elements. The weight of *A. niger* 72-4 grown on 100 ml of this medium is of the order of 0.05 grams in 10 days. Experiments of the same type as those described by the data of tables 5 and 6 were conducted using this medium, and similar but more pronounced beneficial effects of copper and manganese were observed. The requirement for manganese was found to be somewhat higher than was the case with the regular basal medium.

TABLE 8

Interrelationship of citric acid production and the growth of A. niger 72-4 as influenced by copper, iron and manganese

BASAL PLUS 0.1 MG % Zn, 0.01 MG % Fe PLUS		CITRIC ACID BY TITRATION	DRY WEIGHT OF MYCELIUM	BASAL PLUS 0.1 MG % Zn, 0.1 MG % Fe PLUS		CITRIC ACID BY TITRATION	DRY WEIGHT OF MYCELIUM
Cu	Mn			Cu	Mn		
μg %	μg %	g	g	μg %	μg %	g	g
—	—	8.42	1.24	—	—	6.5	2.06
0.1	—	8.64	1.42	0.1	—	7.3	1.94
5.0	—	6.34	1.49	5.0	—	7.23	2.10
—	0.1	9.28	1.62	—	0.1	7.10	2.11
—	5.0	6.59	2.07*	—	5.0	2.11	3.25*
0.1	0.1	9.15	1.58	0.1	0.1	6.82	1.98
5.0	5.0	5.09	2.10†	5.0	5.0	1.95	3.86†

* Mycelia covered with brown conidia.

† Mycelia covered with black conidia.

DISCUSSION

The experimental evidence presented demonstrates that zinc and iron are essential for the production of high yields of citric acid by *A. niger*. Although definite proof has not been obtained that both manganese and copper are also essential, it has been shown that, under conditions in which their inclusion as impurities in the fermentation medium and in the inoculum had been reduced to a low level, extremely minute quantities of either, or both, when added to the medium increased the production of citric acid.

The finding that excessive amounts of any one of the four elements interfere with the accumulation of citric acid is in agreement with the observations of Porges (1932), Bernhauer *et al.* (1941), Perlman, Dorrell, and Johnson (1946), and Shu and Johnson (1948b) regarding the harmful effects of one or more of these elements.

In other work (Tomlinson, 1949) the same interrelationship between zinc and iron in their effect on acid production as that reported by Shu and Johnson (1948b) was obtained. However, so far it has not been possible to obtain maximum yields of citric acid at more than one combination of these two elements if all other conditions remained the same.

The observed effect of substituting NH_4NO_3 for NaNO_3 indicates that Porges' (1932) conclusion that NaNO_3 was the preferred nitrogen source for this fer-

mentation resulted from the unrecognized effect of impurities contained in the reagents used. Likewise, it appears probable that the salt concentrations found optimum for acid production by Currie (1917) and Doelger and Prescott (1934) were dependent to a large extent on the trace elements carried as impurities by the salts employed in the medium. The observation of Szucs (1948) that the addition of a small amount of dry milk to a basal medium that had no added trace elements resulted in an increased yield of the acid may well be due, at least in part, to the same effect. It may also be possible that the relatively high phosphate concentration found desirable for citric acid production under submerged culture conditions by Shu and Johnson (1948a)—a requirement we have been unable to confirm under our conditions—resulted from a similar cause. In this connection Steinberg (1935a) has pointed out that if an essential element is omitted from the nutrient solution intentionally, then maximum growth of *A. niger* will only be obtained if that element is unintentionally added as an impurity with some other component, and this statement probably applies equally well to the production of acid by the organism.

The results obtained throughout this study have been found to be readily reproducible, provided that all conditions are maintained unchanged. However, it must be noted that our data indicate that with the basal medium employed a large proportion of the requirements of the organism for copper and manganese were satisfied by impurities in the basal medium. Also, impurities accompanying the salts probably tended to obscure some effects. For example, the manufacturer's label stated that the ferric chloride contained 0.005 per cent copper and this plus traces of manganese could well explain the apparent decrease in the influence of copper and manganese that occurred as the iron concentration was increased (table 6). Spores were observed when increased quantities of iron were added to the growth medium, thus confirming the presence of manganese in this salt.

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SUMMARY

When reagent grade chemicals and sucrose were used as components of the fermentation medium, zinc and iron were found to be essential components of the medium for maximum citric acid production by *Aspergillus niger* 72-4 when grown from conidia in stationary surface culture.

The presence of very small amounts of manganese or copper, or both, when added to the fermentation medium, permitted higher yields of citric acid to be formed than were produced in the absence of such additions.

Excessive amounts of copper, iron, manganese, or zinc in the fermentation medium interfered with the accumulation of citric acid.

The quantities of iron, copper, and manganese found most suitable for the production of high yields of citric acid were lower than the quantities of those elements required for good growth of *A. niger* 72-4.

REFERENCES

- BERNHAEUER, K., KNOBLOCH, H., AND IGLAUER, A. 1941 Formation of acid from sugar by *Aspergillus niger*. XI. Factors determining the accumulation of citric acid. *Biochem. Z.*, **309**, 151-178.
- BERTRAND, G., AND JAVILLIER, M. 1911 Influence du manganèse sur le développement de l'*Aspergillus niger*. *Compt. rend.*, **152**, 225-228.
- BORTELS, H. 1927 Über die Bedeutung von Eisen, Zink und Kupfer für Microorganismen. *Biochem. Z.*, **182**, 301-358.
- CURRIE, J. N. 1917 Citric acid fermentation. *J. Biol. Chem.*, **31**, 15-37.
- DOELGER, W. P., AND PRESCOTT, S. C. 1934 Citric acid fermentation. *Ind. Eng. Chem.*, **26**, 1142-1149.
- FOSTER, J. W. 1939 The heavy metal nutrition of fungi. *Botan. Rev.*, **5**, 207-239.
- GREENE, H. C., AND FRED, E. B. 1934 Maintenance of vigorous mold stock cultures. *Ind. Eng. Chem.*, **26**, 1297-1299.
- HENRY, R. J., AND SMITH, E. C. 1946 Use of sulfuric acid-dichromate mixture in cleaning glassware. *Science*, **104**, 426-427.
- MULDER, E. G. 1939 Über die Bedeutung des Kupfers für das Wachstum von Mikroorganismen und über eine mikrobiologische Methode zur Bestimmung des pflanzenverfügbaren Bodenkupfers. *Arch. Mikrobiol.*, **10**, 72-86.
- Official and tentative methods of analysis of the Association of Official Agricultural Chemists. 1945 6th ed. Association of Official Agricultural Chemists, Washington. *Refer to p. 570-571*.
- PERLMAN, D., DORRELL, W. W., AND JOHNSON, M. J. 1946 Effect of metallic ions on the production of citric acid by *Aspergillus niger*. *Arch. Biochem.*, **11**, 131-143.
- PERLMAN, D., KITA, D. A., AND PETERSON, W. H. 1946 Production of citric acid from cane molasses. *Arch. Biochem.*, **11**, 123-129.
- PORGES, N. 1932 Citric acid production by *Aspergillus niger*. *Am. J. Botany*, **19**, 559-567.
- RAULIN, J. 1869 Etudes chimiques sur la végétation. *Ann. sci. nat., Botan.*, **11**, 93-299.
- SHU, P., AND JOHNSON, M. J. 1947 Effect of the composition of the sporulation medium on citric acid production by *Aspergillus niger* in submerged culture. *J. Bact.*, **54**, 161-167.
- SHU, P., AND JOHNSON, M. J. 1948a Citric acid production by submerged fermentation with *Aspergillus niger*. *Ind. Eng. Chem.*, **40**, 1202-1205.
- SHU, P., AND JOHNSON, M. J. 1948b The interdependence of medium constituents in citric acid production by submerged fermentation. *J. Bact.*, **56**, 577-585.
- STEINBERG, R. A. 1919a A study of some factors in the chemical stimulation of the growth of *Aspergillus niger*. *Am. J. Botany*, **6**, 330-372.
- STEINBERG, R. A. 1919b A study of some factors in the chemical stimulation of the growth of *Aspergillus niger*. II. A comparison of two strains of the fungus. *Bull. Torrey Botan. Club*, **46**, 1-21.
- STEINBERG, R. A. 1935a The nutritional requirements of the fungus, *Aspergillus niger*. *Bull. Torrey Botan. Club*, **62**, 81-90.
- STEINBERG, R. A. 1935b Nutrient-solution purification for removal of heavy metals in deficiency investigations with *Aspergillus niger*. *J. Agr. Research*, **51**, 413-424.
- SZUCS, J. 1948 U. S. Patent 2,438,136.
- TOMLINSON, N. 1949 *Unpublished data*.
- VON LOESECKE, H. W. 1945 A review of information on mycological citric acid production. *Chem. Eng. News*, **23**, 1952-1959.
- WOLFF, L. K., AND EMMERIE, A. 1930 Über das Wachstum des *Aspergillus niger* und den Kupfergehalt des Nährbodens. *Biochem. Z.*, **228**, 441-450.