Production of Citric Acid by Mutants of *Aspergillus niger*

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SUMMARY: Mutants of *Aspergillus niger*, Wisconsin strain 72–4, were produced by multiple X-ray and ultraviolet irradiation. The mutants differed from the parent strain culturally and in citric acid production. Comparison between the yields of citric acid from the parent strain and mutants showed significant increases with the latter. In crude sugar media, such as ferrocyanide-treated brown sugar, yields of citric acid equivalent to 80 % of the sugar fermented were obtained in aerated culture, whereas only 21 % was obtained with the parent strain.

The factors which govern citric acid production by *Aspergillus niger* in submerged culture, particularly the influence of the metal constituents of the medium, were investigated in detail by Shu & Johnson (1947, 1948*a, b*), and their findings were confirmed and extended by Tomlinson, Campbell & Trussell (1950, 1951). The small amounts of iron, zinc, copper and manganese which are essential for optimal growth (Steinberg, 1935) exceed the concentrations which favour citric acid production. Thus one step towards providing suitable conditions for acid accumulation is to decrease these metal concentrations well below those required for optimal growth. Even so, it is extremely difficult to obtain constantly the highest yields of citric acid, especially from crude cane sugar, by the submerged culture method.

Three approaches to this problem are possible: (1) to strip the medium of its excess metal contaminants by ion-exchange techniques, as described in a patent issued to Miles Laboratories (1952), or by potassium ferrocyanide precipitation (Gerhardt, Dorrell & Baldwin, 1946; B.I.O.S. Final Report 220, 1946; Clement, 1952); (2) to add a growth-inhibitory agent such as methanol which was found to increase the metal concentrations permissible (Moyer, 1953); (3) to isolate metal-tolerant mutants.

In regard to this third possibility Perlman (1947), who investigated some seventy-five strains of *Aspergillus niger* for ability to produce citric acid in submerged culture, found considerable variation among them and reported that some were less affected by metal ions than others. Quilico, Panizzi & Visconti (1949) obtained mutants by X-ray irradiation which gave increased yields on Currie's defined medium (1917); they did not report on the yields of citric acid obtained from crude sugar substrates. Yuill (1951) commented briefly on citric acid production by mixed cultures of *A. niger*, some of them mutants. He stated that when two unrelated high acid-producing strains were grown together, the yield was lower than that given by either separately, but no such decrease was observed when the strains were related as mutant and parent, or as mutants from the same parent; no information was given about the ability of these mutants to produce acid from crude sugars more efficiently than the parent strain.

The present work is concerned with the study of citric acid production by mutants of *Aspergillus niger* in metal-contaminated crude cane sugar by submerged (shaken or aerated and stirred) culture. A method developed for selection of such mutants is described separately (James, Rubbo & Gardner, 1956).

METHODS

Organism

The parent culture was *Aspergillus niger* Wisconsin strain 72-4 (Perlman, Kita & Peterson, 1946). For irradiation and fermentation experiments, cultures were grown on the 14 % sucrose agar defined medium described by Shu & Johnson (1947) and on beer wort agar. On these media sporulation was prolific after 3-5 days of incubation at 28°.

Irradiation techniques

X-ray irradiation. Freshly formed spores were spread aseptically on the surface of a 4 % agar gel and exposed to X-rays in doses of 45,000 and 60,000 r. The spores were then washed off the agar with water containing a wetting agent, Soaxit (sodium dioctyl sulphosuccinate, 1/10,000), and the suspension filtered through sterile cotton-wool to remove clumps. The death-rate was estimated by comparison of the viable count (plate method) with a haemocytometer count of the total number of spores, and found to be 99 % with a dose of 45,000 r. and 99.9 % with 60,000 r. In later experiments 1 ml. of an aqueous suspension of spores, previously filtered through cotton-wool and containing 2×10^6 spores, was irradiated in a small Petri dish covered with cellophan.

Ultraviolet irradiation. Filtered suspensions of spores in water, containing 2×10^6 spores/ml., were irradiated, at room temperature (c. 20°) in a clear quartz tube of 2 cm. diameter, by a vertical 15-watt G.E. low-pressure germicidal lamp (2537 Å.). The tube was rotated at 180 r.p.m. with its axis parallel to and 12 cm. from the lamp. The irradiations were performed in a dark room and the irradiated suspensions were shielded from light until plated out in order to minimize any photoreactivation effects. Exposure for 15 min. gave a 99.9 % kill.

The plan for preparation of high-yielding mutants was similar to that outlined by Dulaney, Ruger & Hlavac (1949) for improving streptomycin yields by *Streptomyces griseus*, in that it involved exposure of the spores of successively induced mutants to mutagenic agents. All strains selected were preserved by lyophilization of spores harvested from beer wort agar.

Fermentation techniques

Submerged culture methods. Some mutants which showed superior acid-producing ability on cane molasses substrate by surface culture were tested as submerged cultures in flasks on a rotary shaker (1 in. throw, 200 r.p.m.) at 28° and finally in 5 l. fermentation flasks equipped for aeration and agitation.

The shaken flasks, of 500 ml. capacity and loosely plugged with cotton-wool, contained 50 ml. of medium. Three preparations containing sucrose were used: white sugar of the ordinary commercial grade; a brown crystalline product ('brown sugar'); cane molasses. Their content of those trace metals important in the fermentation is shown in Table 1.

Table 1. *Metal content in different batches of cane sugar*

Type of sucrose preparation	Metal content (p.p.m.)			
	Copper	Iron	Zinc	Manganese
White sugar	0.06–0.20	1.42–3.00	0.24–1.00	0.45–1.40
Brown sugar	3.3–4.7	65–88	1.1–5.7	1.6–7.0
Molasses	7.6–7.8	104–360	4.4–5.7	39–49

All media contained (g./l.): sucrose preparation, 140; KH_2PO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; NH_4NO_3 , 2.5. In the case of the white sugar medium, trace metals were added as follows (mg./l.): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0; these being the quantities given by Shu & Johnson (1947). The pH value was adjusted to 3.0 for white sugar, 5.6 for brown and 5.0 for molasses. The flasks were inoculated with a suspension of mycelial fragments prepared in shaken cultures containing plastic beads. Cultures were incubated for 9 days before being analysed for acidity and residual sugar.

For aerated submerged fermentations a battery of four 5 l. round-bottom reaction flasks of Hysil glass mounted in a water-bath at 28° was used; the equipment was similar to that described by Kelly, Miller & Hale (1952). Air inlet tubes and baffle plates were glass, stirrers were of glass or stainless steel. The rate of air flow was 4 l./min. and the speed of the stirrer was 600 r.p.m., both being the maximum obtainable with our equipment.

Fermentations were started by inoculating 1 l. of 2%, w/v, sugar medium with 100 ml. of a suspension of mycelial fragments. Incubation with aeration for 24 hr. produced an actively growing mycelial mass, to which 3 l. medium containing high sugar and low ammonium nitrate (187 g. and 0.83 g./l. respectively) were added. The final concentration of sugar in the combined media was 14%.

When potassium ferrocyanide was used in the brown sugar medium it was added to the hot medium after autoclaving, usually 0.25–0.50 g./l. for the shaken cultures and 0.75–0.90 g./l. for aerated submerged fermentation. In cane molasses a much higher concentration (3.0 g./l.) was required for high yields in shaken cultures.

Acidity was determined by titration with 0.25 N-NaOH and residual sugar by Cole's ferricyanide method (1933). At the conclusion of a fermentation the acid products were identified and their proportions estimated by partition chromatography, with butanol + water containing 2N-formic acid as solvent (Lugg & Overell, 1948).

RESULTS

Origin and description of mutant strains

The distinguishing cultural characteristics of the strains of *Aspergillus niger* investigated are shown in Table 2 and Pl. 1. It will be seen that the mutants are easily distinguished by their restricted colony development and delayed sporulation. After 3 days of incubation on Czapek-Dox medium the mutants gave raised non-sporulating compact colonies (popcorn colonies), while the parent strain 72-4 gave a flat spreading colony with spores. When grown on beer wort agar slopes strain 72-4 spored prolifically in 2-3 days whereas, after 7 days, the felt of mutant X 20 was unevenly covered with spores. It is interesting to note that mutant V 7-1 spored freely only on the upper portion of the slope after 9 days of incubation (Pl. 1), a character shared by all the other mutants except X 20.

Table 2. *Origin and distinguishing features of Aspergillus niger strains*

<i>Aspergillus niger</i> strain	Origin	Time and degree of sporulation on beer wort agar (days/degree*)	Colony size on Czapek-Dox agar after 8 days (central point inoculation) (diameter, mm.)
72-4 (Wild type)	University of Wisconsin	3/+ + + +	35
X 20 (mutant)	X-ray irradiation of 72-4	7/+ + +	10
V 4 (mutant)	UV irradiation of X 20	9/+ +	8
V 4-1 (mutant)	UV irradiation of V 4	14/+	8
V 4-2 (mutant)	UV irradiation of V 4-1	14/+	8
V 7 (mutant)	UV irradiation of X 20	9/+ + +	8
V 7-1 (mutant)	UV irradiation of V 7	9/+ +	8
V 7-2 (mutant)	UV irradiation of V 7-1	9/+ +	8

* + + + + = entire felt covered with black spores; + + + = 3/4 felt covered with black spores; + + = 1/3 felt covered with black spores; + = < 1/3 felt covered with black spores.

When grown on the surface of liquid media the mutants in general developed felts which were lighter and more friable than those of the parent strain 72-4. On cane molasses mutant V 7-1 grew as a light, non-sporing felt covering the entire surface of the medium, mutant X 20 grew as an incomplete moderately sporing felt, while the parent strain 72-4 produced a tough, heavily sporing band of felt restricted to the edge of the medium. Submerged cultures in shaken flasks reflected the restricted mycelial development of the mutants which was seen on solid media. These characteristics, like the fermentative ability, have remained unchanged through many subcultures over the past 2 years.

Fermentative activity in submerged culture

Fermentations in shaken flask culture with parent strain 72-4 and mutants X 20 and V 7-1, using various sugar substrates were studied. The results are summarized in Fig. 1. The average yield from all substrates was higher with the mutants than with the parent culture. With crude sugars the superiority

of the new strains was most evident. On treated and untreated molasses the yields of acid with mutant V7-1 were about twice those obtained with mutant X20, a similar ratio having been obtained in surface fermentation (James *et al.* 1956).

Fig. 1. Average yields of citric acid (columns) and range of variation (arrows with number of fermentations analysed) in 9-day shaken flask cultures at 28°.

The results recorded in Table 3, based on those shown in Fig. 1, indicate that the yields of acid progressively decreased as the metal content of the medium (contributed by the sugar) increased. The loss of efficiency was most pronounced with the parent strain and least with mutant V7-1. However,

Table 3. Influence of substrate on yields of citric acid

Nine-day shaken flask fermentation at 28°.

Strain	Yields of citric acid*				
	White sugar	Brown sugar		Molasses	
	(%)	$K_4Fe(CN)_6 \cdot 3H_2O$ -treated	Brown sugar untreated	$K_4Fe(CN)_6 \cdot 3H_2O$ -treated	Molasses untreated
	(%)	(%)	(%)	(%)	(%)
72-4	100	61	33	23	0
X20	100	74	32	34	11
V7-1	100	98	52	71	32

* For purposes of comparison the yields of citric acid from white sugar are calculated as 100% and the yields from other sugars adjusted accordingly.

from an industrial point of view, it is apparent that untreated cane molasses is unsuitable as a substrate for citric acid production with the mutants available, although mutant V7-1 gave outstandingly good yields in ferrocyanide-treated sugar or cane molasses.

In the fermenters, where aeration was combined with mechanical agitation, the results (Fig. 2) generally confirmed the findings with surface and shaken fermentations, mutant V7-1 being superior to mutant X20 and this in turn superior to parent strain 72-4. It is worth noting that mutant V7-1 in ferrocyanide-treated brown sugar was better in aerated fermenters than in shaken flasks, whereas with mutant X20 the difference was negligible.

Fig. 2. Average yields of citric acid (columns) and range of variation (arrows with number of fermentations analysed) in 11-day aerated and stirred cultures at 28°.

A closer analysis of the aerated fermentation is given (Fig. 3) for brown sugar media, with and without ferrocyanide treatment. It will be seen that the production of citric acid was most rapid with mutant V7-1, reaching a yield of 64 % in 9-11 days in the ferrocyanide-treated medium. At the conclusion of these fermentations, when the acidity had reached its maximum, the culture fluid usually contained 1-2 % sugar. Therefore, when the results are calculated on sugar consumed the yields are higher than those shown in Figs. 2 and 3, reaching 21 % for the parent strain 72-4, 48 % for mutant X20 and 80 % for mutant V7-1 in 9 days in the best runs. Such yields compare favourably with those regarded as satisfactory in an industrial process.

Utilization of citric acid as a substrate

It was thought that the difference in citric acid production between the parent strain and mutants of *Aspergillus niger* might be due to differences in their ability to utilize citric acid as a substrate, the parent being more active than the mutants. In order to test this point surface felts and submerged pellets of *A. niger* mycelium were harvested from sucrose medium, washed and

transferred to a medium containing 6% citric acid as sole source of carbon. In addition, parallel tests were done in 6% citric acid medium containing 4% sucrose. These replacement cultures were incubated at 28° in static and shaken flasks and the utilization of citric acid determined by titration with 0.25N-NaOH and also colorimetrically (Saffran & Denstedt, 1948). The results

Fig. 3. Rate of citric acid production (—) and sugar utilization (---) in aerated and stirred cultures at 28°.

A, B, parent strain 72-4 on untreated and on $K_4Fe(CN)_6 \cdot 3H_2O$ -treated brown sugar.

C, D, mutant X20 on untreated and on $K_4Fe(CN)_6 \cdot 3H_2O$ -treated brown sugar.

E, F, mutant V7-1 on untreated and on $K_4Fe(CN)_6 \cdot 3H_2O$ -treated brown sugar.

(Table 4) showed that the rate of disappearance of citric acid in both static and shaken cultures was similar for the parent strain and mutants. The discrepancy between the total titratable acid and citric acid estimations, which developed towards the end of the fermentation, indicated that acids other than citric were being produced. When sucrose was present (Fig. 4) there was no apparent utilization of the citric acid by any of the cultures until the sugar concentration had fallen to zero. These findings clearly showed that the high yields of citric acid in the mutant cultures (and their restricted growth rate)

were not due to their inability to metabolize the citrate formed. In any case it appears unlikely that any of the cultures will attack citrate while sucrose is present in the medium. It is obviously important to take advantage of the citrate-sparing effect of sucrose if highest yields of citric acid are to be obtained commercially.

Table 4. Utilization of citric acid by strains of *Aspergillus niger*

Strain	Method of cultivation	Citric acid yield in culture fluid				
		Initial		After 4 days; by titration	After 7 days	
		Titration	Colorimetric		By titration	Colorimetrically
Citric acid (mg./ml.)						
Parent 72-4	Surface	59	59	41	13	2
	Shaken	63	68	12	5	N.D.
Mutant X20	Surface	57	59	26	9	0
	Shaken	65	70	8.5	3.5	N.D.
Mutant V7-1	Surface	58	59	25	7	0
	Shaken	66	N.D.	13.5	2	N.D.

N.D. = not determined.

Fig. 4. Utilization of citric acid by strains of *Aspergillus niger*.

DISCUSSION

The aim of this investigation was to study the fermentative activity of mutants of *Aspergillus niger*. These mutants were induced by X-ray and ultraviolet irradiation of spores and selected by paper culture technique

(James *et al.* 1956). They were culturally distinct from the parent strain and stable in all characteristics studied.

The results show that all the mutants were markedly more active in producing citric acid than the parent strain under all conditions of fermentation, namely in static, shaken and aerated submerged cultures with commercially pure sucrose or crude sucrose such as cane molasses. In aerated fermentations these differences were most pronounced in that one mutant, V7-1, gave fourfold to sixfold higher yields of citric acid than the parent strain 72-4. Mutant V7-1 appears to be suitable for large-scale production of citric acid since it is capable of giving yields of citric acid equivalent to 80 % of the sugar fermented in ferrocyanide-treated crude brown sugar media. With beet molasses, a substrate similar in metal content to brown cane sugar, comparable results might confidently be expected. Unfortunately, beet molasses was not available to us in the present study.

Several points of interest emerge from this work. In the first place it was apparent that the best strains are multiple-step mutants produced by exposure to mutagenic agents. These differed from the parent strain in requiring up to 9 days for sporulation, in growing as small compact colonies (Table 2) and in producing lighter and more friable felts on liquid media as well as smaller amounts of mycelium in submerged culture. The constant correlation between high yields of citric acid and restricted development suggests that the mutants may be deficient in certain metal-dependent enzymes associated with growth, in particular those concerned with the utilization of citrate. Experiments reported here (Table 4, Fig. 4), indicate, however, that the restriction of growth and the enhanced production of citric acid were not concerned with the inability of the mutants to utilize citric acid as a substrate.

The yields quoted for *Aspergillus niger* 72-4 on white sugar medium are considerably lower than those reported by Shu & Johnson (1947, 1948*a, b*); this is probably due to a higher concentration of metals in our media. For example, the concentration of manganese (60-196 $\mu\text{g./l.}$) in the white sugar medium was considerably in excess of the optimal level of 1 $\mu\text{g./l.}$ determined by Shu & Johnson. Little attention was given to the purification of media in this work since our aim was to produce organisms capable of giving high yields of citric acid from impure substrates.

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EXPLANATION OF PLATE

Comparison of colonial characteristics (6 days, 28°, Czapek-Dox plates) and sporulation (9 days, 28°, on beer wort agar slopes) of the parent strain 72-4 and mutants X20 and V7-1.

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