Effect of Trace Elements on Citric Acid Fermentation by *Aspergillus niger*

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Citric acid yields of 98.7% (sugar consumption basis) were reached in shaker flasks with mutant UV-ET-71-15 of *Aspergillus niger* in a resin-treated sucrose medium of the following composition (g/100 ml): sucrose, 14.0; NH₄NO₃, 0.20; KH₂PO₄, 0.10; MgSO₄·7H₂O, 0.025; and (mg/liter): FeSO₄, 0.15 to 0.75; ZnSO₄, 0.10; and CuSO₄, 0.01. Yields of 75% were obtained in medium with resin-treated clarified syrup and 68% with ferrocyanide-treated blackstrap molasses. Optimal conditions included selection of appropriate pellets as inoculum at 3%, pH of 4.5, temperature at 30°C, agitation at 250 rev/min, and fermentation time of 8 days. The mutant tolerated high concentrations of trace elements.

Among the problems associated with citric acid production by strains of *Aspergillus niger* under submerged growth conditions, those related to the control of the trace-element concentration play an important role. Recent contributors to this subject include Noguchi and Johnson (6), Millis et al. (5), and Clark (1).

The optimal concentrations of such ions as Fe²⁺, Cu²⁺, Zn²⁺, and Mn²⁺ vary so widely with the different strains under study that it is necessary to adjust the composition of the medium to avoid the inhibitory effects caused when these cations are present in toxic concentrations.

Some of the methods we have previously used to solve this problem include (i) selection of tolerant strains, (ii) addition of substances to decrease the trace-element content of the fermentation media, (iii) treatment of the carbohydrate raw material to minimize inhibition effects, and (iv) development of potentially high-yielding mutants with a higher tolerance to trace elements.

The present paper deals with the utilization of a high-yielding mutant of *A. niger*, previously obtained (10) by a combination of ultraviolet (UV) and ethylenediamine treatment, to study the effect of specific trace elements and phosphate on the citric acid fermentation of treated and untreated sugar sources.

MATERIALS AND METHODS

Raw materials. Refined commercial sugar from two types of sugar mills (producing sugars with relatively high- or low-trace-element content), blackstrap molasses, and sugar cane liquor ("clarified syrup," International Society of Sugar Cane Technologists terminology) were studied.

The blackstrap molasses was used either as received or after treatment with K₃Fe(CN)₆ by the method of Horitsu and Clark (3). The sucrose and clarified syrup, at a 14% concentration (sucrose basis), were passed through a glass column of Amberlite IR-120 prepared as follows. Appropriate amounts of the resin were washed in a glass column and were then washed successively with 2 N HCl, redistilled water, 1 N redistilled NH₄OH, and redistilled water.

Strains. *Aspergillus niger* ATCC-233; the parent strain M-172, which was isolated from a Columbian soil (7); and two mutants, UV-6 and UV-ET-71-15, obtained from the latter (10) were transferred from the soil stock cultures through several successive potato dextrose agar slants to sucrose-salts agar slants. Mutant UV-ET-71-15 does not sporulate well in the usual media but does produce abundant spores in the following (g/100 ml): sucrose, 0.25; meat extract, 1.0; NaCl, 0.5; and agar, 1.5. After 4 to 6 days of incubation at 28°C, the spores were suspended in 10 ml of sterile distilled water to prepare the inoculum.

Preparation of inoculum. The spores were harvested. Suspensions were prepared and standardized at a concentration of 10⁷ to 2 × 10⁸ and were then used to inoculate 50 ml of sterile seed media in 250-ml Erlenmeyer flasks. These were incubated at 28°C on a rotary shaker at 250 rev/min for 17 to 24 hr. After that time, those flasks showing good formation of small, firm, spherical pellets were selected as inoculum for the fermentation media (3%, v/v).

Seed media. Different seed media were tested and the following was finally selected (g/100 ml): sucrose, 6; NH₄NO₃, 0.25; KH₂PO₄, 0.1; MgSO₄·7H₂O, 0.025; agar, 0.2; and distilled water, 100 ml (pH 4.5).

Fermentation media. Two previously reported media [A-1 and G (10)] and three basic media, as shown in Table 2, were first tested in a screening procedure by using the UV-6 mutant as a standard...
TABLE 1. Trace element content of raw materials

| Raw material       | Mn** | Fe** | Zn** | Cu** |
|--------------------|------|------|------|------|
| White sugar        | 0.3-1.2 | 1.1-3.1 | 0.18-0.98 | 0.04-0.31 |
| Blackstrap molasses| Trace-0.8 | 0.5-2.0 | 0.1-0.3 | 0.03-0.15 |
| Clarified syrup    | 8.1-12.7 | 78.0-190.5 | 3.9-24.7 | 6.6-37.5 |
|                    | 0.0-0.3 | 0.3-13.0 | 0.1-1.5 | 0.01-10.0 |

a High content of trace elements.
b Low content of trace elements.

c For citric acid production in 250-ml shaker Erlenmeyer flasks at 250 rev/min with 50 ml of medium.

When sucrose was not resin treated, either no trace elements or small quantities were added depending on the amounts initially present. All fermentation media were adjusted to pH 4.5 (except molasses at pH 6.8) and incubated at 30 C for 8 to 10 days.

Estimation of growth and chemical determinations. Dry mycelial weight, pH, residual reducing sugars, citric acid production, and the presence of other acids were estimated by published methods (7, 8, 9). Citric acid yields were calculated as the anhydrous acid on the basis of sugar consumed. Oxalic and gluconic acids were detected by paper chromatography. The cations in the raw materials were determined by the methods of Noguchi and Johnson (6). The effect of resin treatment on the ion content of raw materials was estimated by means of absorption spectrophotometry. All cation values of the raw materials are expressed in ug/ml; those of culture media are in mg/liter.

RESULTS

Table 1 gives the trace element content (µg/ml) of the raw materials used: commercial white sugar with relatively high- or low-trace-element content, blackstrap molasses, and clarified syrup. Blackstrap molasses showed the highest content of trace elements. The concentration of the cations Fe**, Zn**, and Cu** tolerated by the UV-ET-71-15 mutant were 2.2, 3.8, and 0.5 mg/liter, respectively, giving yields of citric acid from 3.0 to 5.0 g/100 ml in 10 days. It is apparent that this culture is highly tolerant of trace elements.

The UV-6 mutant was tested in the five different media given in Table 2. Untreated white sugar with a high content of trace metals was used in medium A-1; methanol and corn steep liquor were present in medium A-1; and small amounts of trace elements were in medium G. The highest yield was obtained in medium G.

In the three other media, untreated white sugar with a low content of trace elements was added. These media differed in concentrations of NH₄NO₃, KH₄PO₄, and MgSO₄. Results were inferior to those obtained with media A-1 and G, indicating that the addition of trace elements (either as such or present in corn steep liquor) resulted in better citric acid production. Similar results were obtained with mutant UV-ET-71-15.

The trace elements of medium G were added to medium 3, and the citric acid yields of the parent strains (233 and M-172) and the two mutants (UV-6 and UV-ET-71-15) were determined. Data, as shown in Table 3, indicate the UV-ET-71-15 mutant to be superior with an average citric acid yield of 98%.

Instead of sugar, untreated molasses and mo-
Table 3. Culture selection in medium 3 with trace elements of medium G

| Culture          | Citric acid (g/100 ml) | Final pH | Yield of citric acid (%) |
|------------------|------------------------|----------|--------------------------|
|                  | 4 days  | 8 days  | 10 days                  | 62.1  | 68  | 3.32  | 6.5 |
| 233              | 0.6a    | 1.2     | 2.5                       | 3.1   | 58  | 58.4  | 61  |
| M-172            | 1.3     | 3.0     | 3.4                       | 3.7   | 48  | 58.4  | 61  |
| UV-ET-71-15      | 4.7     | 7.4     | 8.1                       | 2.2   | 98  | 68.2  | 61  |
| UV-6             | 3.2     | 5.4     | 7.0                       | 2.6   | 65  | 68.2  | 61  |

a Values represent mean value in three tests.

Table 4. Effect of K₄Fe(CN)₆ treatment of molasses on citric acid production in medium 3

| Culture             | Yield of citric acid (%) |
|---------------------|--------------------------|
|                     | Not treated with K₄Fe(CN)₆ | Treated with K₄Fe(CN)₆ |
|                     | With trace elements 8a | Without trace elements | With trace elements | Without trace elements |
| UV-6                | 60                       | 55                      | 63                   | 58                   |
| UV-ET-71-15         | 62                       | 58                      | 68                   | 62                   |

a Values represent mean value at 10 days in three tests.

Table 5. Effect of resin treatment of sugar on citric acid production by mutant UV-ET-71-15

| Medium | Citric acid (g/100 ml) | Final pH | Yield of citric acid (%) |
|--------|------------------------|----------|--------------------------|
|        | 8 days                 | 10 days  | 62                       | 68                         |
| 1      | 2.3                    | 2.9      | 2.5                      | 23                         |
| 1-R    | 4.1                    | 5.5      | 3.4                      | 58                         |
| 3      | 4.3                    | 5.8      | 2.2                      | 51                         |
| 3-R    | 5.1                    | 6.8      | 2.1                      | 65                         |

a Figures represent mean value in five tests.
b R = resin treatment, no cations added.

Table 6. Effect of resin treatment on the trace element content of sucrose and clarified syrup

| Determination     | Cation (µg/ml) |
|-------------------|----------------|
|                   | Fe⁺  | Cu⁺  | Zn²⁺ | Mn²⁺ |
| Sucrose untreated | 1.73 | 0.27 | 0.56  | 0.05  |
| Sucrose resin-treated | 0.50 | 0.14 | 0.06  | 0.0  |
| Clarified syrup untreated       | 12.90 | 2.35 | 1.41  | 0.25  |
| Clarified syrup resin-treated    | 0.94 | 0.26 | 0.11  | 0.0  |

Table 7. Effect of trace element concentration on citric acid yield and growth of mutant UV-ET-71-15

| Medium | Optimal concn (µg/liter) | Mycelial wt (mg/ml) | Yield of citric acid (%) |
|--------|--------------------------|---------------------|--------------------------|
|        | Fe⁺  | Zn⁺⁺ | Cu⁺⁺ | | |
| 1      | 0.15a | 0.1 | 0.01 | 17.5 | 58  |
| 2      | 2.2  | 3.8  | 0.48 | 21.2 | 57  |
| 3      | 0.75a | 0.1 | 0.01 | 12.1 | 62  |

a Values represent mean at 8 days in five tests.

Table 8. Influence of resin treatment and the addition of trace elements with mutant UV-ET-71-15

| Medium | Optimal concn (µg/liter) | Final pH | Mycelial wt (mg/ml) | Yield of citric acid (%) |
|--------|--------------------------|----------|---------------------|--------------------------|
|        | Fe⁺  | Zn⁺⁺ | Cu⁺⁺ | | |
| 1-R    | 0.15a | 0.1 | 0.01 | 2.2  | 12.1 | 98.0  |
| 2-R    | 2.2  | 3.8  | 0.48 | 2.4  | 16.2 | 63.6  |
| 3-R    | 0.75a | 0.1 | 0.01 | 2.5  | 13.4 | 98.7  |

a R = resin treatment.
b Values represent mean at 8 days in five tests.
TABLE 9. Influence of phosphate concentration on citric acid yields of mutant UV-ET-71-15 in medium 3

| Conc of KH₂PO₄ (g/liter) | Yield of citric acid (%) | Final pH | Presence of gluconic or oxalic acid |
|-------------------------|--------------------------|----------|-----------------------------------|
|                         | Medium 3 | Medium 3-R | Medium 3 | Medium 3-R | Medium 3 | Medium 3-R |
| 0.25                    | 48.1b    | 66.6      | 2.5      | 2.4        | -        | -        |
| 0.50                    | 48.5     | 66.7      | 2.5      | 2.2        | -        | -        |
| 1.00                    | 48.7     | 65.0      | 2.5      | 2.2        | -        | -        |
| 1.50                    | 53.4     | 64.4      | 2.7      | 2.3        | -        | -        |
| 2.00                    | 58.1     | 61.3      | 2.6      | 2.3        | +        | +        |
| 2.50                    | 57.6     | 63.0      | 2.6      | 2.2        | +        | +        |
| 3.00                    | 58.5     | 62.5      | 2.6      | 2.5        | +        | +        |
| 3.50                    | 58.5     | 62.5      | 2.5      | 2.3        | +        | +        |
| 4.00                    | 58.8     | 61.0      | 2.5      | 2.3        | +        | +        |

a R = resin treatment, no cations added.
b Values represent mean at 8 days in three tests.

TABLE 10. Influence of sugar substrate on citric acid yields of mutant UV-ET-71-15

| Substrate (14% sucrose) | Selected mediuma | Yield of citric acid (%) |
|-------------------------|------------------|-------------------------|
| White sugarb            | 3-R              | 98.7c                   |
| Blackstrap molasses     | 1-K              | 68.0                    |
| Clarified syrup         | 3-R              | 75.0                    |

a Cations added as in medium 3, Table 7. R = resin treatment; K = treatment with K₄Fe(CN)₆.
b Low content of trace metals.
c Values represent mean at 8 days in five tests.

were detected by paper chromatography of the fermentation broths with the usual concentration of KH₂PO₄ (1.0 g/liter). However, when higher amounts of KH₂PO₄ (2.5 g/liter) were used, these other acids were present (Table 9). The yields of citric acid were between 48.5 and 66.7% with untreated and resin-treated sucrose, respectively, at a KH₂PO₄ level of 0.5 g/liter. No cations were added in these cases.

In other studies, blackstrap molasses and clarified syrup behaved in a similar manner as regards the response to prefermentation treatment. Several tests showed that the yields of citric acid in media with clarified syrup were better than molasses but not as good as white commercial grade cane sugar under the conditions of the experiments. The best yields obtained with these three substrates and the mutant UV-ET-71-15 are shown in Table 10.

DISCUSSION

Trace elements. The importance of trace elements in the submerged citric acid fermentation of raw commercial sugar substrates has been repeatedly pointed out (1, 2, 5, 6, 7, 9). In the present investigation, great variability was found in the Fe²⁺, Zn²⁺, and Cu²⁺ content of the raw materials used. Reproducibility of the citric acid yields was not accomplished until the trace-element concentration of the medium was properly controlled.

Although very small amounts of these trace elements were apparently necessary for good citric acid synthesis by the mutants under study, these cultures were capable of resisting unusually high cation concentrations (2.2, 3.8, and 0.5 mg of Fe²⁺, Zn²⁺, and Cu²⁺ per liter, respectively) in addition to those already present in the sugar substrate itself. The largest amounts of citric acid were, however, repeatedly obtained when only low concentrations of these three cations were added to the medium. Control of the trace element concentration has ensured reasonable reproducibility of the fermentation yields.

Sugar treatment. The removal of excess trace elements by either ferrocyanide or resin treatment of sugar followed by the proper addition of trace elements was associated with good and reproducible citric acid yields. Our results confirm previous observations (3, 4, 6, 7).

Mutants. The development of mutants of A. niger allowed a higher average yield from all the crude substrates tested than the parent culture. Mutant UV-ET-71-15, obtained by a combined treatment of UV and ethyleneimine (10), was superior to the UV-6 mutant as well as to the parent strain. This culture has resisted higher concentrations of trace elements, thus resembling the mutants obtained by Gardner et al. (2) and Millis et al. (5), and has maintained its properties and characteristics after a storage period of 2 years in sterile soil. In the fermentation media selected, this mutant developed small pellets with a morphology similar to that described by Martin and Waters (4).

Inoculum. The type and concentration of the inoculum was critical, with best results depending on the selection and number of pellets.

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