ULTRAVIOLET-INDUCED MUTATIONAL CHANGES IN ENZYME ACTIVITY OF *ASPERGILLUS TERREUS*

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Received for publication October 7, 1958

In previous publications Arpai (1957) and Arpai and Valentin (1957) described the production of ultraviolet-induced mutants of *Aspergillus terreus*, using nondifferentiated spore material and conidia differentiated by sedimentation rate. A limited number of the irradiated strains were morphologically altered and produced more itaconic acid than did the parent strain. The products of these strains and mutants have been studied with the view to determining the pathways of biosynthesis of unsaturated acids (Arpai and Valentin, 1957) which were recognized by the metabolic patterns of the mutants.

Only one acid (itatartaric acid) related to itaconic acid has been found to be produced exclusively by ultraviolet-induced mutants (Stodola *et al.*, 1945). The biosynthesis of itatartaric acid probably proceeds by enzymatic oxidation. Direct evidence for this was obtained by using fungal itaconic oxidase in the metabolic patterns of the itatartaric acid producing mutants (Arpai, 1958*a*, 1959). Some properties of the itaconic oxidase have been described (Arpai, 1958*b*). Since no correlation of morphological characters with the physiological properties of *A. terreus* are known, it seemed desirable to test both the morphological variant and the parent culture. In this way, the isolated enzyme may provide useful contributing evidence to the biochemical hereditary characters in the genetic analysis of the ultraviolet-induced variants.

MATERIALS AND METHODS

Organism. The microorganisms employed were a mutant of *A. terreus* producing itatartaric acid, and the parent strain producing only itaconic acid (Arpai 1957, 1959).

The irradiation techniques for production of the mutations, using the fraction of spore suspension with the highest sedimentation rate and the single-spore isolations of survivors, are also described in the studies cited.

Morphological analysis. The growth and cultural characteristics of the single-spore colonies of the parent strain as well as the survivors were followed on standard Czapek-solution agar. The microscopical analysis was performed using the usual techniques on native and strained preparations. The dimensions were determined micrometrically.

Investigation of the metabolites. The production of itaconic and itatartaric acids was established in the fermentation liquors of surface cultures. Each culture was used in five parallel experiments. Itaconic acid was established qualitatively, using the modified procedure developed by Halliwell (1952). The standard itaconic acid used was obtained from the firm Lachema, Brno. The itaconic acid produced was determined quantitatively by the method of Friedkin (1945). To determine itatartaric acid, a standard with a melting point of 103 C was prepared using low-temperature oxidation of itaconic acid as described in detail by Arpai (1958*a*).

Enzyme preparation. Cell-free extracts were obtained from the mycelial pads of surface cultures of the organisms. Details of this operation have been described (Arpai, 1958*b*). Dependence of the enzyme activity on the hydrogen ion concentration (pH) of the environment was followed in buffers at pH 2.7, 3.2, 4.16, 4.8, 5.5, 6.2, and 6.9.

Respiratory studies. Measurement of the oxidation reaction of itaconic acid in itatartaric acid was made manometrically by standard Warburg techniques in air at 37 C. The details of the manometric methods have been given in previous publications (Arpai and Valentin, 1957; Arpai, 1958*a*).

RESULTS

The morphological analysis has revealed that the characteristics of the mutant differs markedly from the parent strain in the appearence of the colony and in structural details. Colonies of the

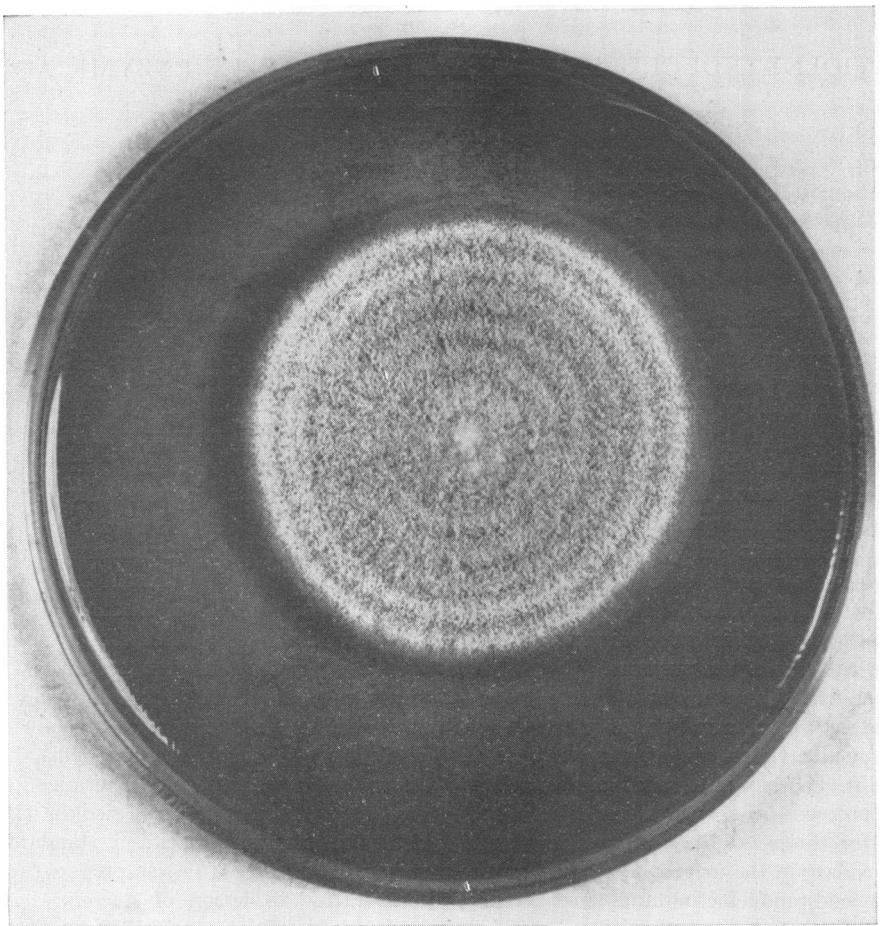

Figure 1. Parent strain of *Aspergillus terreus*

parent strain (figure 1) growing rapidly at 27 C, attain a diameter of 6 to 8 cm and a depth of 1 to 2 mm in 4 days. The colony has an entire margin, concentric furrows, and the central area is somewhat lighter in color. Heavy sporulation occurs throughout, with massed conidial heads giving colonies a cinnamon to light brown color at maturity. The conidial heads are columnar and compact, with the uppermost portion slightly expanded. They vary from 30 to 40 μ in diameter and from 200 to 300 μ in length. Conidiophores are sinuous, smooth walled, colorless, and arise from well defined foot cells. The conidiophores vary from 150 to 200 μ in length and from 4 to 5 μ in diameter; the walls are comparatively thick, ranging from 0.3 to 0.6 μ at the upper portion, to 0.5 to 0.8 μ at the basal area. The vesicles are domelike with closely crowded sterigmata in two series. The primary sterigmata are closely crowded, parallel, and range from 5.0 to 6.0 μ in length by 1.5 to 2.5 μ in width, each bearing 2 or 3 secondary sterigmata. The secondary sterigmata are closely crowded and parallel (5.0 to 6.0 μ by 1.0 to 1.5 μ). Conidia appear adherent in long chains, are light golden in color when viewed under high magnifications, smooth walled, and slightly elliptical (1.5 to 2.0 μ by 2.0 to 2.5 μ). The vegetative mycelium hyalin is thin walled (diameter from 1.0 to 3.0 μ) and irregularly and frequently branched.

Colonies of the mutant (figure 2) are spreading, thin, and light tan in color; they consist almost exclusively of coarse, thick walled, submerged hyphae. Conidial heads are produced sparingly and are small and atypical in pattern. Conidiophores may fail to develop vesicles. Heads are

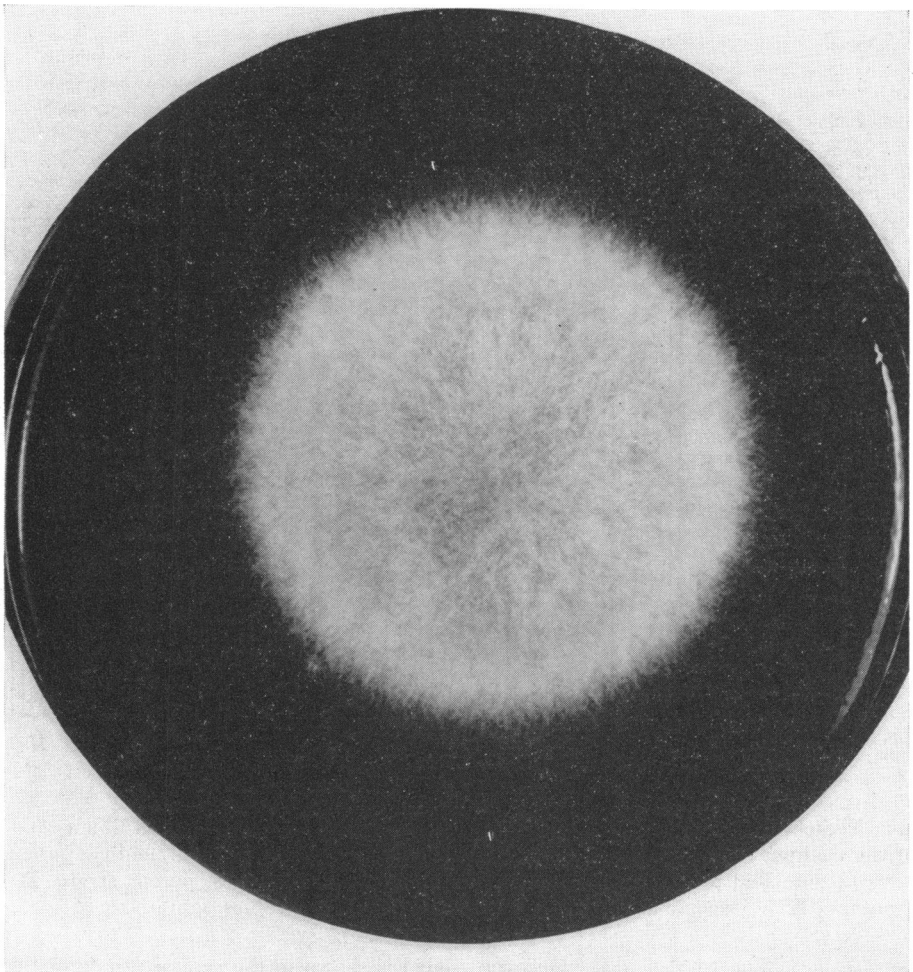

Figure 2. Mutant strain of *Aspergillus terreus*

more loosely columnar than compact and comparatively short. Sterigmata are normal in size, shape, and arrangement, but conidium formation is incomplete and individual spores are not properly differentiated and form chains. Septation in the chain is incomplete and individual conidia appear somewhat vacuolate. This mutant is stable in culture.

The biochemical analysis showed metabolical differences between the two cultures. Fermentation tests established that the parent strain belongs to the medial active itaconic-acid producers and, under standard fermentation conditions, gives yields of this acid of 37.5 per cent after 8 days, based on glucose consumed. The total acidity of the fermented liquor under these conditions reached a value of about 500 ml of 0.1 N alkali per 10 g of consumed glucose, whereas the acidity corresponding to the itaconic acid quantity produced in the substrate amounted to a value of about 470 ml of 0.1 N alkali. Hence it follows that the parent strain produced, almost exclusively, itaconic acid. Itatartaric acid could not be determined among the acid-forming components present in the fermentation liquor.

The mutant produced maximum itaconic acid in 6-day cultures, when the production, in a typical case, reached about 9.5 per cent, based on the glucose consumed. In the course of the following days of cultivation, a decrease of itaconic acid content in the substrate took place, reaching a relative minimal value of about 3 per

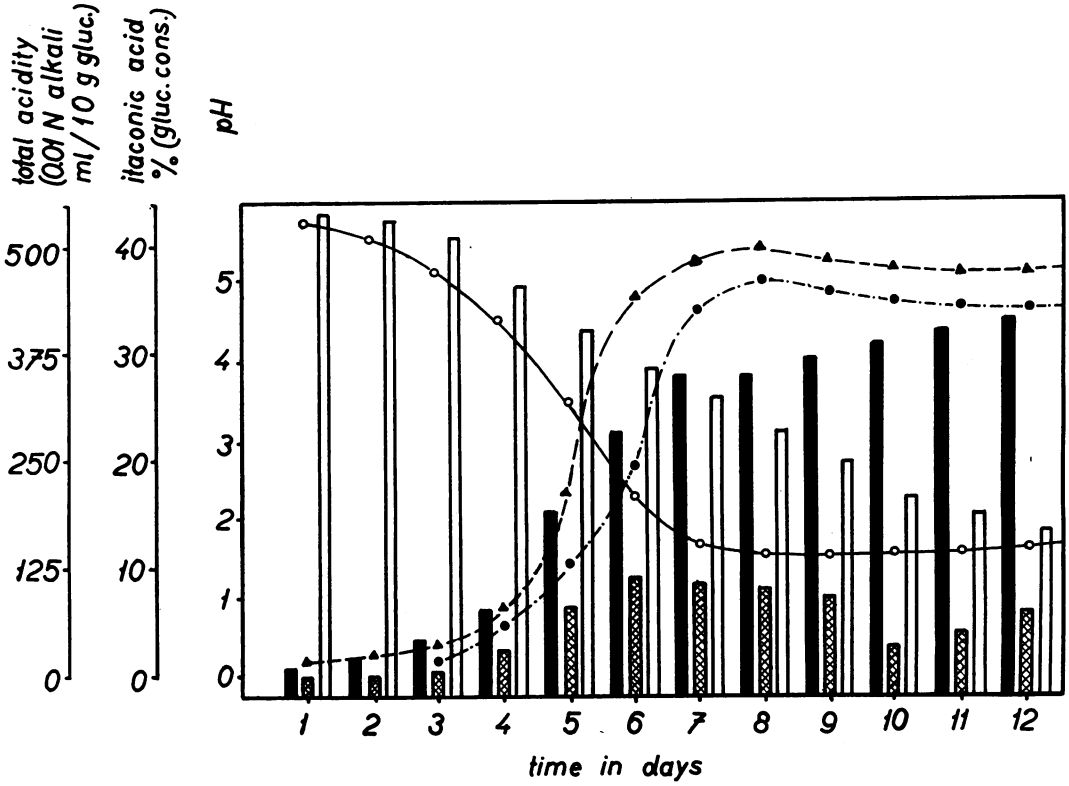

Figure 3. Typical itaconic acid fermentations, total acidity in the fermentation liquor, and the pH values of the medium for the parent and the mutant strains. ▲——▲ = total acidity, parent strain; ●——● = itaconic acid production, parent strain; ○——○ = pH values, parent strain; ■ = total acidity, mutant; ▩ = itaconic acid production, mutant; □ = pH values, mutant.

cent after 10 days. The total acidity, however, further increased and reached a value of 410 ml of 0.1 N alkali in this interval. Simultaneously, the occurrence of saturated acids including itatartaric acid could be detected in quantities ranging from 5 to 7 per cent of the base mentioned. In the further course of fermentation, a rise of the itaconic acid concentration in the medium was observed, as can be seen from the graph of a typical experiment (figure 3). This graph compares the course of the fermentation of itaconic acid production, of the total acidity, and the pH values for the parent strain and the mutant.

Essential changes were detected not only in the biochemistry of the fermenting cultures, but also in the activity of the cell-free extracts prepared from the parent strain and the mutant. The ability of cell-free extracts prepared from the mycelial pads of the mutant to oxidize itaconic acid is shown in the typical results of the manometric studies (figure 4). The rate of oxygen uptake by the enzyme obtained from the mutant was 32 μL per hr per mg, at pH 4.2. However, the oxygen uptake by the enzyme from the parent strain was only about 5 μL per hr per mg and was not significantly greater than the endogenous respiration. An inspection of the results suggests interesting relationships between pH and endogenous respiration and oxidation activity. The enzyme obtained from the mutant was most active within the range pH 4.2, forming approximately stoichiometric amounts of itatartaric acid. The activity of the itaconic oxidase decreased with a further decrease of pH, so that oxygen uptake at a pH of 2.7 was similar to values for the endogenous respiration (figure 5). On the other hand, the cell-free extracts prepared from the nonirradiated parent strain did not adapt to the

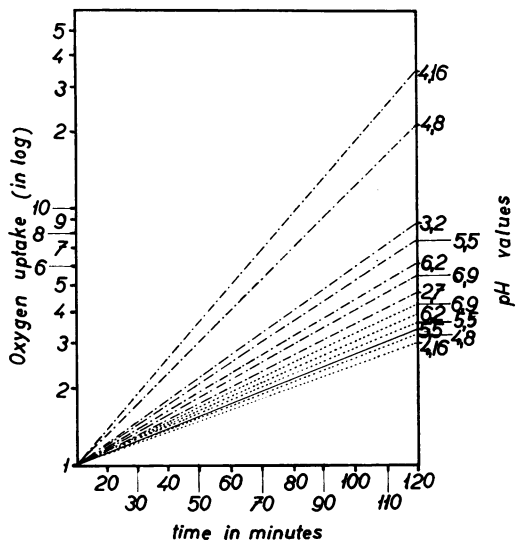

Figure 4. Typical results of the manometric studies of the oxidation of itaconic acid by enzymes prepared from the mycelium of the parent strain and of the mutant at various pH values. —— = parent strain; ·—·— = mutant; and ···· = mutant endogenous respiration.

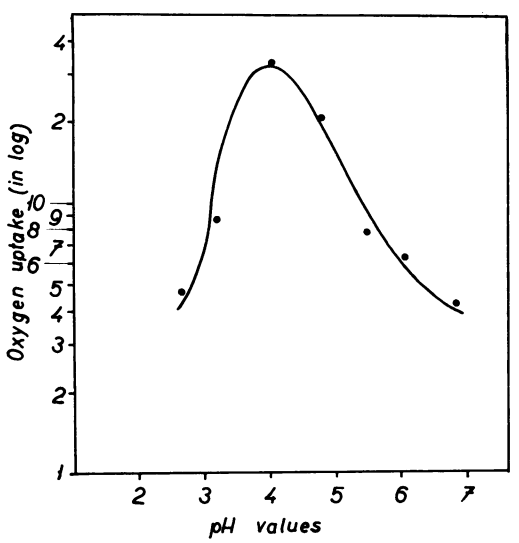

Figure 5. Activity of the itaconic oxidase and its dependence on the pH value of the medium.

oxidation of itaconate. The values in figure 6 show that a difference exists between the enzymes of the mutant and parent strains in the destruction of itaconic acid.

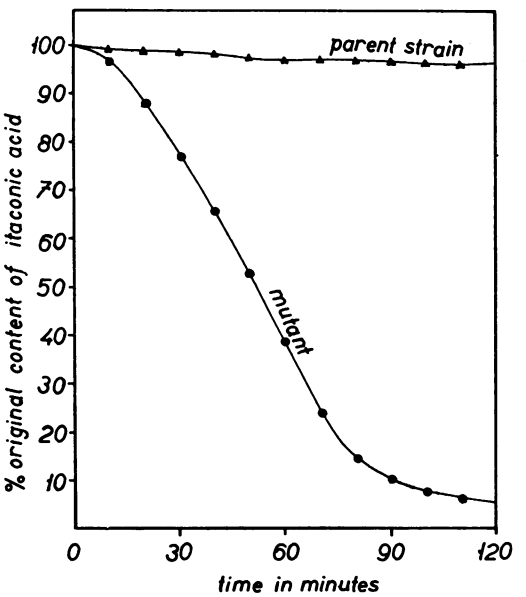

Figure 6. Percentage change of itaconic acid with respect to time by enzymes of the parent and mutant strains at optimal conditions.

DISCUSSION

It must be pointed out that the optimum pH, established for the enzymatic oxidation of itaconic acid agrees approximately with the actual acidity of the fermentation substrate of the mutant in the neighborhood of the inflection point on the production curve of itaconic acid. At this point, an intensification of the function of the itaconoxidase system leading to an increased production of itatartaric acid became evident. Some decrease in concentration of the itaconic acid in the fermented liquor took place. This may be explained by the conversion due to itaconic oxidase excreted into the medium. The kinetics of this conversion are determined not only by factors limiting the activity of the enzyme, but also by the conditions of the excretion. With decreased pH, the itatartaric acid production and also the enzymatic conversion slows down or stops, which explains the phenomenon that the itaconic acid concentration in the medium increased and a mixture of itaconic and itatartaric acid was found.

The genetic constitution of an organism has a decisive influence on the chemical composition and reactions of its parts and also on the met-

abolic patterns of the organisms (Wagner and Mitchell, 1955). In practice, this approach has been applied successfully to studies and to detection of mutants of microorganisms. Itaconic oxidase, as well as the corresponding itatartaric acid, appears to be a biochemical characteristic of induced variants. Also the recognized essential changes in the enzymatic mechanisms of the oxidation system of *A. terreus* can be explained as the result of an induced genetic mutation.

SUMMARY

Where comparing the morphological characteristics and the prevailing fermentative abilities of *Aspergillus terreus*, a mutant was detected. This mutant was specifically distinguished by the fact that it produced itatartaric acid. A soluble cell-free enzymatic extract can be prepared from the mycelium which will oxidize itaconic acid to itatartaric acid. Similar enzymatic activity does not occur in extracts prepared from the mycelial pad of the parent strain. The difference between the irradiated and nonexposed cultures is evaluated as a genetic change in the biochemical hereditary character of the fungus. The occurrence of a mixture of itaconic acid and itatartaric acid in the fermented medium can be explained by the fact that the activity of the enzymatic system producing itatartaric acid appears only in a certain phase of the fermentation, and is evidently limited by the pH of the medium. The partial conversion of itaconic acid in the fermentation liquor is determined by the kinetics of excretion of the itaconic oxidase. A further decrease of pH in the medium inhibits activity of itaconic oxidase, and brings about additional production of itaconic acid.

REFERENCES

ARPAI, J. 1957 *Frakciovanie spór pri selekcii producentov itakónovej kyseliny*. Ed. Biologické práce (SAV), Bratislava.

ARPAI, J. AND VALENTIN, F. 1957 Štúdium kinetiky biosyntézy kyseliny itakònovej. Chem. zvesti, **11**, 669–680.

ARPAI, J. 1958*a* Enzymatická oxydácia kyseliny itakónovej na kyselinu itavinnú. Chem. zvesti, **12**, 532–439.

ARPAI, J. 1958b Itaconic oxidase: an enzyme from an ultraviolet-induced mutant of *Aspergillus terreus*. Nature, **182**, 661–662.

ARPAI, J. 1959 Morfologické a biochemické sledovanie genetických zmien u mutanta *Aspergillus terreus* vyvolaného UV-svetlom. Biológia, **14**, 52–58.

FRIEDKIN, M. 1945 Determination of itaconic acid in fermentations liquors. Ind. Eng. Chem. Anal. Ed., **17**, 637–639.

HALLIWELL, G. 1952 Chromatographic detection of acids in cultures of *Aspergillus niger* on various substrates. Nature, **196**, 1063–1064.

STODOLA, F. H., FRIEDKIN, M., MOYER, A. J., AND COGHILL, R. D. 1945 Itatartaric acid, a metabolic product of an ultraviolet-induced mutant of *Aspergillus terreus*. J. Biol. Chem., **161**, 739–742.

WAGNER, R. P. AND MITCHELL, H. K. 1955 *Genetics and metabolism*. J. Wiley & Sons, Inc., New York.