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STRAIN SELECTION IN CITRIC ACID FERMENTATION*—A REVIEW

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A NUMBER of organic acids are produced by moulds and bacteria through the process of fermentation, and citric acid is one of the most important metabolic products now produced commercially by fermentation with specific moulds, mostly strains of *Aspergillus niger*.

Wehmer[29] was the first to report that citric acid was produced from sugar through fermentation by moulds named by him as *Citromyces pfefferians* and *C. glaber*. Later, many other fungi have been found to ferment sugar to produce citric acid, but today some strains of *Aspergillus niger* are used for commercial production of citric acid in many countries and the problem has been attacked from diverse angles by various investigators. The present paper is intended to report the several techniques adopted by various investigators to improve the yield of citric acid in the laboratory.

When one is to improve the yield of a product elaborated by a microorganism. two general pathways are open: (*a*) improvement of the parent strain and (*b*) improvement of the environmental condition. That means, an organism capable of synthesizing more of the compound in question is of prime importance, which should then be studied

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to find out the optimal fermentation condition for better yield. Herein lies much of the essence of industrial microbiology.

Now the question arises as to how to improve the existing strain? From the mycological standpoint the answer is to search for new strains in nature or to search for strains which may arise as natural variants among the single spore colonies of the parent strain. But to depend only on this technique is not encouraging, as spontaneous mutation is a very slow process and as such, this process of selection can never be the sole applicable technique in the field of microbial industry to improve the yield.

The alternative method is, evidently, to search for mutants which may be induced amongst the progeny of the parent strain, after teratment with mutagens. This idea of treating microorganisms with various mutagens and to search for improved mutants among the surviving progeny has now been recognised as the best means to secure strains of improved potency. Kresling and Stern[16] first reported mutants of*Aspergillus niger* producing considerably more citric acid than the parent strain. They used ultraviolet light and radium as mutagens. But the feasibility of these observations were not realized till 1945, when Demerec[7] reported a new mutant $x - 162$ of *Penicillium notatum* showing improved yield of penicillin in comparison with the parent strain. Since this observation the process of induced mutation and strain selection has been used in improving the yield of various metabolic products.

However, in the search for new strains for improved citric acid production, selection was made by Gerhardt *et al.*[10] amongst 20 monosporous isolates of a citric acid producing strain of *Aspergillus niger*, but no improved strain was obtained. In some instances an apparently superior strain was isolated but on continued cultivation 'the property was rapidly lost'. The author[1] also searched for improved variants among about a thousand monosporous colonies of a citric acid producing wild parent, but failed to secure any strain of improved potency. It may be that, search should be made among thousands of colonies. For Savage[23] found only 3 superior streptomycin producing strains by studying 3,700 colonies of *Streptomyces griseus*. Raper[22] also reported that out of more than 60,000 isolates of *Penicillium notatum* a few strains were found to produce somewhat better yields than the parent stock.

Investigators are generally inclined to search for improved mutants among the surviving colonies formed after treatment with various mutagens. Kresling and Stern's findings have been reported earlier. Nakazawa and Simo[19] treated *A. niger* with radium and secured some mutants in which acid production was greatly increased. Detailed data are not reported in the available abstract. Imshenetskii *et al.*[14], through the use of ultraviolet radiation, obtained an *A. niger* mutant producing 16–22% more citric acid than the parent strain. The yield of citric acid from the sugar consumed varied from 57–74%. Shcherbakova[27] exposed two strains of *Aspergillus niger* to uv irradiation and ethylenimine treatment, and combined treatment with uv irradiation and ethylenimine. The experiments resulted in some mutants which appeared to be superior to the initial parent strains in respect of citric acid yield. From a study of 40,000 colonies surviving uv irradiation, Millis and her associates[18] obtained a mutant of *A. niger* yielding about four times increased production of citric acid than the wild parent. Exposing 10 strains of *A. niger* to uv rays for 1·5 to 24 hours, Ilczuk and Gruszczynska[12] observed 9 strains to produce 39 mutants of high citric acid yielding capacity, compared with the parent. They reported that weak strains required lower uv doses of 1·5 to 2·0 hours to produce mutants of higher yielding capacity, while active parent strains required higher uv doses of 24 hours for securing mutants which showed smaller gains in citric acid production. The author carried on a series of mutagenic treatments with a parent strain of *A. niger* with a view to improving the yield of citric acid. She used nitrogen mustard[2], gamma radiation from Co^{60}, uv rays[3] and colchicine[4]. In all these experiments she studied 2,500 colonies and save colchicine she obtained mutants showing significant improvement over the immediate parent in respect of acid production. Figure 1 shows her experimental results where starting with a parent strain producing 28 mg of citric acid per ml, she finally secured through second step mutation programme a mutant CGU 163 producing as much as 72 mg of citric acid per ml, *i.e.*, the mutant was more than two and a half times superior to the original parent. Colchicine proved unsuccessful in securing high yielding mutants.

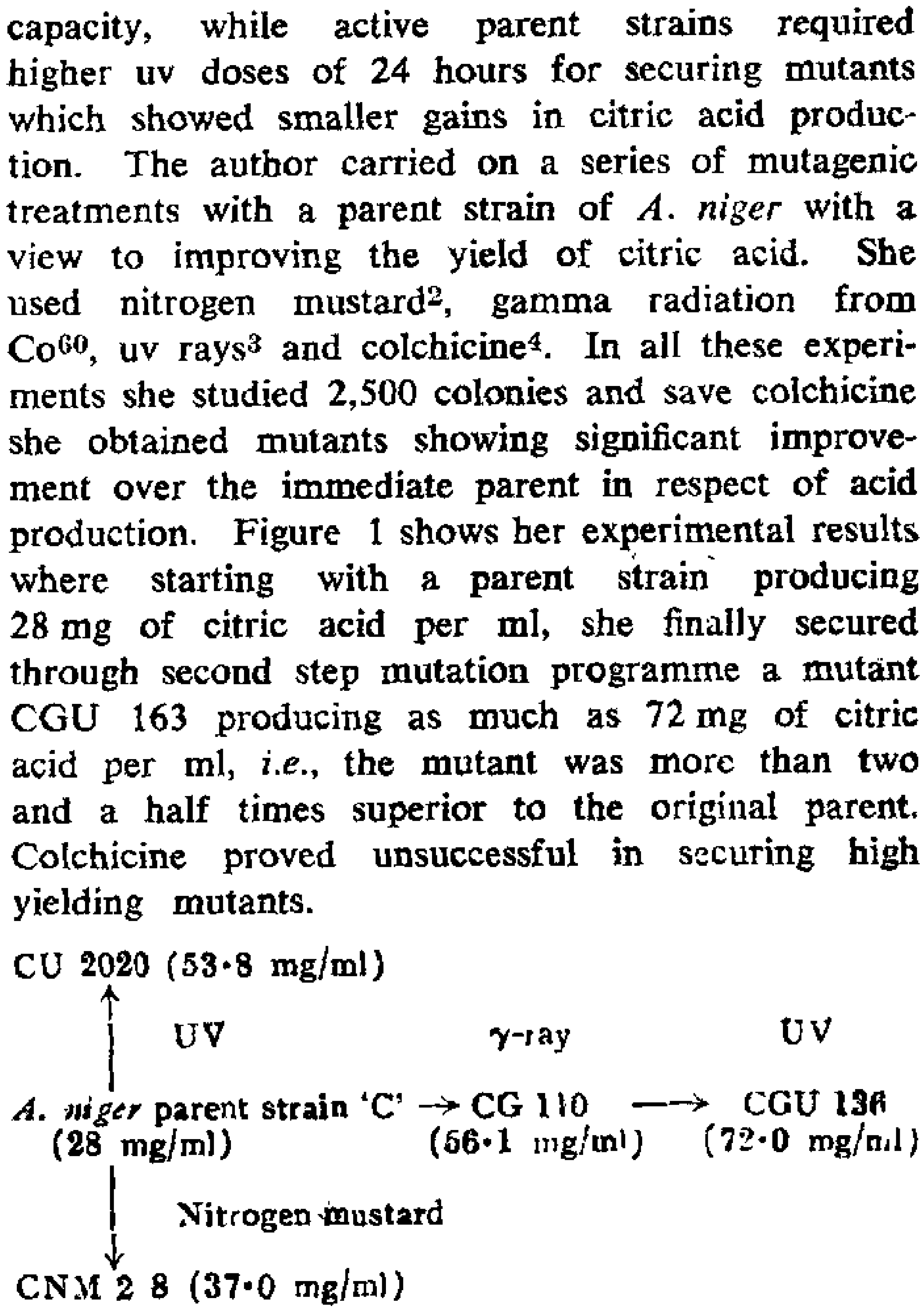

FIG. 1

By successive uv irradiation of 4 strains of *A. niger* a series of mutants was obtained by Seichertova and Lopold[24] and compared with the wild parent 11% of the mutants showed increase in citric acid yield. Pashev *et al.*[20] obtained cultures through anastomosis of various *A. niger* strains and irradiated these with 7,000 R of X-rays. Some of the surviving mutants showed 10–20% increased production of citric acid.

All these observations strongly suggest that with intensive strain selection *plus* irradiation or chemical treatments of the organisms to induce mutant races, yields of citric acid may be greatly increased.

Besides induction of mutation, the problem of strain selection has also been carried out through somatic recombination and polyploidization. In Japan breeding experiment has been undertaken to improve the citric acid producing strains of *A. niger*. Dr. Abe and his coworkers (Ikeda[11]) have successfully selected a few diploids capable of producing more citric acid than either parent. Polyploids were also synthesized between a haploid and a diploid and between two diploids of *A. niger* by Abe and his associates. It was reported that one tetraploid strain produced citric acid in a yield that was about 85% of the total sugar ; in contrast with the wild parent producing citric acid with a yield of 53%. Seichertova and Leopold[25] synthesized heterokaryons between mycelia of normal strains of *A. niger* and their uv-induced mutants. Among the heterokaryons isolated, most active strains were obtained from parents producing large amounts of citric acid in addition to oxalic acid, or in cases in which one of the parents produced a medium amount of citric acid and no oxalic acid. Takami[28] made several mixed cultures with three citric acid producing *A. niger* strains, but in no case significant effect was observed in respect of productivity. On the other hand, in one case yield value of citric acid was considerably decreased.

Once a high yielding strain is obtained it is advisable that this mutant should next be studied carefully to determine the conditions optimum for its productivity. For example, the mutant CGU 163, obtained by the author (Fig. 1) generally produced about 72 mg of citric acid per ml showing about 2·5 times superiority over the original parent[5]. Next, slight changes in the composition of the medium together with little alterations in physical factors like incubation temperature, pH of the medium, etc., resulted in the mutant producing about 119 mg of citric acid per ml[6], *i.e.*, the improvement achieved was more than 4 times compared with the parent.

Attempts were often made to correlate productivity with morphological characters. According to Protod'yakonov and Kresling[21] strains of *A. niger* giving a high yield of citric acid do not form distinctly notched, darkly pigmented conidia as do those giving a low acid yield. The latter strains were also reported to form irregular or star-shaped heads while those giving a high yield, form smoother heads. Later, Kresling[17] again reported that weak acid producing strains of *A. niger* show more fat in the mycelium than stronger acid producing strains. Shcherbakova[26] reported that cultures of *A. niger* yielding higher than the parent are morphologically different from the latter. Later, Imshenetskii and Shcherbakova[15] observed that morphological mutants of *A. niger* were characterised by both high and low yielding capacity. According to them, most high yielding cultures occur among morphological mutants. Ilczuk[13] studied 131 morphological mutants of *A. niger* derived from uv treatment and reported that 55% of these were high yielders of citric acid compared with the wild parent. Detailed investigations in this regard were made by the author[2,3]. While cultures in Czapek Dox Agar (CDA) medium did not reveal any correlation, fermentation medium, on the other hand, provided criteria so as to differentiate between the strong and weak acid producers. Non-spored or sparsely sporulating cultures were generally associated with higher potency than the heavy-spored ones. More precisely, colonies of compact growth with wrinkled surface producing very few spores suggest the possibility of possessing yield capacity worth considering in citric fermentation. Similar morphological characteristics of high yielding strains of *A. niger*, as seen in fermentation medium, have been reported by Doelger and Prescott[8] and Gardner and his associates[9].

A point worth mentioning is that, treatment with various mutagens[1] produced substrains in CDA medium, a majority of which resembled the parent in morphological characters. Substrains of both altered and unaltered morphology showed wide variation in the degree of acid production. But while these were mostly associated with decreased potency, occasionally substrains were secured showing increased potency. Once all these substrains are grown in fermentation, there is the chance to differentiate the high yielding strains from low yielding ones on the basis of characters described earlier. Hence, it may not seem unjustifiable to infer that whenever a mutation programme is to be undertaken all the substrains, irrespective of their morphological characters in CDA or in any other solid medium, should be critically tested with regard to their capacity for citric acid production, otherwise there will remain every chance of missing the highly potent strains.

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