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# Studies on the Metabolism of *Aspergillus niger*

## I. The Effect of Aeration on the Formation of Citric and Oxalic Acids in Surface Mould Cultures

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As early as 1927 Rippel and Bortels[1] advanced the assumption that carbon dioxide is essential for the metabolism of moulds and accordingly, not only an unnecessary end product of metabolism. This assumption was later confirmed by the experiments of Foster *et al.*[2] who, using $C^{11}$ as a tracer in carbon dioxide, found *Aspergillus niger* to yield citric acid from sugar containing the tracer carbon in the carboxyl groups. They used a submerged culture according to Kluyver and Perquin[3].

The fact that carbon dioxide participates in the formation of citric acid in *Aspergillus* prompted the present investigation which deals with the effect of aeration on the citric and oxalic acid formation in surface mould cultures. It is not improbable that a decrease in the percentage of carbon dioxide developed in respiration affects the amounts of fermentation products formed.

### EXPERIMENTAL

*Aspergillus niger* (strain HC I of the laboratory) was grown as a surface culture in 1000 ml Erlenmeyer flasks containing 100 ml nutrient solution. The composition of the nutrient solution was the following: 2.25 g ammonium nitrate, 0.6 g potassium dihydrogenphosphate, 0.4 g potassium monohydrogenphosphate, 0.25 g magnesium sulphate, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.003 g $MnSO_4 \cdot 4H_2O$, 0.50 g $FeCl_3 \cdot 6H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 130 g sucrose, and glassdistilled water to one litre. Prior to autoclaving the media were adjusted with HCl to pH 2.0.

The flasks were plugged with cotton, and into some of them sterile air was admitted near the mould pellicle. The fermentations were allowed to continue for 13 to 16 days. The nutrient solution gave a 2 cm layer of medium when the flasks were placed in a horizontal position.

Acid formation was followed by taking samples from the flasks with a sterile pipette the lower end of which was passed through the mould pellicle into the nutrient solution. Care was taken not to mix the nutrient solution while moving the flasks. Citric acid was determined according to Pucher *et al.*[4] by oxidizing at room temperature with $KMnO_4$ in a KBr-Br solution to pentabromacetone, by de-halogenizing, and by titrating the bromine ion argentometrically. Oxalic acid was estimated by precipitation as the calcium salt at pH 5 and by subsequent permanganate oxidation. The results recorded here are mean values of two parallel experiments.

In aerated cultures a uniform pellicle of mycleium developed about 2 to 3 days later than in unaerated cultures. Correspondingly, in the latter ones fermentation started earlier. In the aerated cultures the total amount of sugar consumed was greater but the titratable acidity did not markedly differ from that of unaerated cultures (Table 1).

*Table 1. Consumption of sugar and increase in titratable acidity of aerated and unaerated cultures.*

| Duration of fermentation days | Sugar left in medium % | | 0.125 *N* NaOH per ml fermented medium ml | |
|---|---|---|---|---|
| | Aerated | Unaerated | Aerated | Unaerated |
| 4 | 98.0 | 68.5 | 0.3 | 0.4 |
| 6 | 90.0 | 52.5 | 0.3 | 2.0 |
| 8 | 50.0 | 45.0 | 1.5 | 2.5 |
| 10 | 33.0 | 36.0 | 2.6 | 2.9 |
| 12 | 26.5 | 33.5 | 3.4 | 3.1 |
| 14 | 24.9 | 32.5 | 3.8 | 3.2 |
| 16 | 23.5 | — | 3.8 | — |

In comparing the aerated cultures and unaerated cultures with each other quite noteworthy differences were observed in the relationships between the citric and oxalic acids produced (Fig. 1).

The relation between the maximum yields of citric and oxalic acids is given below:

| | Citric acid m*M* | Oxalic acid m*M* |
|---|---|---|
| Aerated | 0.0145 | 0.170 |
| Unaerated | 0.0645 | 0.060 |

Thus the aeration nearly trebles oxalic acid formation at the same time decreasing citric acid production to one-fourth.

## DISCUSSION

Some of the hypotheses which have been proposed for the explanation of the mechanism of citric acid fermentation involve *inter alia* the breakdown of the sugar molecule to simpler compounds, such as acetaldehyde or acetic acid,

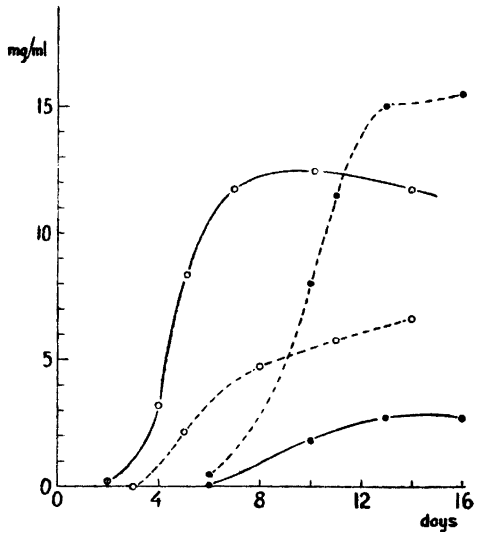
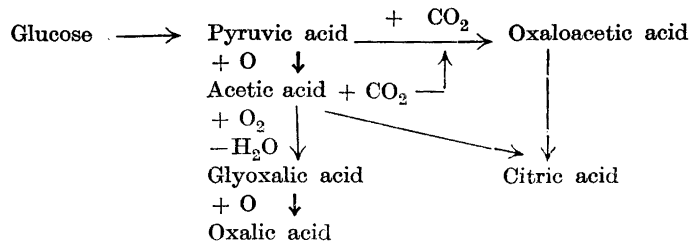



***Fig. 1.*** *The effect of aeration on the production of citric and oxalic acids.*

—— *Citric acid*
····· *Oxalic acid*
● *Aerated*
○ *Nonaerated*

which are then used for the synthesis of citric acid (*cf. e. g.* [5,6,11]). This assumption has been critisized because carbon dioxide is not regularly formed in the citric acid fermentation to the extent presumed on decarboxylation of pyruvic acid. Wells *et al.*[7] showed that such a hypothesis is incompatible with the high yields of citric acid from sugar under some conditions. High yields of citric acid were observed also by Raistrick and Clutterbuck [8] and by Butkewitsch and Gajewskaja [9]. On the contrary, it has been noted that *Aspergillus niger* forms from sugar small amounts of acetaldehyde which proves at least a partial decomposition of sugar through the $C_3$-compounds [10,11]. In fact, as early as 1928 Virtanen [11] advanced the idea that citric acid is formed as a condensation product of oxaloacetic acid and acetic acid.

The observation made by Foster *et al.*[2] that carbon dioxide takes part in the formation of citric acid *via* the Wood-Werkman reaction (pyruvic acid + $CO_2$ → oxaloacetic acid) throws new light on the mechanism of citric and oxalic acid formation. The mechanism may now be interpreted as follows:



According to the above mechanism it is possible to understand the great quantities of citric acid present in such reaction series where an incomplete alcohol fermentation forms the initial stage of the reaction chain. The carbon dioxide liberated in incomplete alcohol fermentation is re-fixed by the Wood-Werkman reaction wherefore the quantitative conversion of sugar to citric acid is theoretically possible. The formation of citric acid, on the other hand, is restricted in the above scheme by the removal of carbon dioxide from the reaction system. When the pH of the nutrient solution is 2 it is incapable of fixing $CO_2$ as a bicarbonate. In unaerated cultures the $CO_2$ arising as a respiration product accumulates as gas in the very near vicinity of the mycelium, whereas in aerated cultures it follows the outflowing current of air. Foster and Davis [12] could not achieve a $CO_2$-deficiency in the mycelium of vigorously fermenting *Rhizopus nigricans* in a high vacuum. In the aerobic experiments described above the fermentation is, however, so much slower that it is possible to remove by aeration significant amounts of $CO_2$ from the interior of the fungus cells. Now, the $CO_2$ fixation is suppressed and with that also the citric acid formation. The acetic acid, consequently, is converted into oxalic acid in greater quantities than in unaerated cultures. It may be possible that the abundant formation of oxalic acid in neutral reaction can also be explained by the fixation of carbon dioxide as a carbonate.

## SUMMARY

Aeration of the surface culture of *Aspergillus niger* nearly trebles oxalic acid formation while decreasing citric acid production to one-fourth.

On the basis of this result the significance of the Wood-Werkman reaction in citric acid fermentation has been discussed.

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