Reduction of Incubation Time in Citric Acid Fermentation by Vermiculite

Citric acid is still widely produced by surface culture methods. The long time of 8–10 days to complete the fermentation is a major disadvantage of the process. The present article describes means for reducing this incubation time to about half the usual. The method involves the use of synthetic substrates adsorbed on vermiculite granules, similar to the procedure described for amylase production[1].

Materials and methods. The strain of *Aspergillus niger* used here is that originally used by SHU and JOHNSON[2] (No. 72-4) and by MARTIN et al.[3] (No. A-1-233). Stock cultures of this organism were maintained on a modified Czapek-Dox agar[4]. Conidia for inoculation of the fermentation substrate were produced on substrate B[2] (see below), solidified with 2% agar, and adjusted to pH 5–5.5.

The inoculum was obtained by adding some sterile water to the sporulated cultures, shaking vigorously and filtering the suspension of conidia thus obtained through sterile cotton wool in a glass funnel under aseptic conditions. The conidia were washed 3 times in sterile distilled water and an aliquot was counted in a hemocytometer. The fermentation substrates were inoculated by adding a small volume (about 2 ml/250 ml) of this suspension to the fermentation substrate in bulk, so that an inoculum density of $5 \cdot 10^7$ conidia/100 ml resulted.

The following fermentation substrates were used[2]. Substrate A which contained: sucrose (purified), 140 g/l; KH_2PO_4, 1 g/l; $MgSO_4 \cdot 7H_2O$, 0.25 g/l; N, 0.87 g/l (0.055 as $(NH_4)_2SO_4$–N, and the rest as NH_4NO_3–N); and HCl (distilled), added after autoclaving (120 °C, 15 min) to adjust the pH to 3.1. Sucrose was purified according to the method of SHU and JOHNSON[5], using $Al_2(SO_4)_3$ and NH_4OH to obtain a precipitate of $Al(OH)_3$, which, together with adsorbed trace elements, was removed by centrifuging. In substrate B the sugar is not purified and the ammonium nitrate concentration is 2.5 g/l (0.87 gN/l). This substrate contains no ammonium sulphate, since no purification of the sugar with aluminium sulphate and ammonium hydroxide was carried out. Both substrates were used with and without the trace element supplementation according to SHU and JOHNSON[5], i.e. Zn, 0.5 mg/l (as $ZnSO_4 \cdot 7H_2O$), and Fe, 1.3 mg/l (as $FeCl_3 \cdot 6H_2O$). All chemicals used were of Analar (British Drug Houses Ltd.) grade.

For stationary cultures without vermiculite, 20 ml portions of inoculated substrate were aseptically dispensed in 100 ml sterile 'Davisil' conical flasks. The vermiculite cultures were obtained by autoclaving 4 g vermiculite (commercially available 'Micafil' of South African origin) in the above flasks and adding aseptically 15 ml of inoculated substrate. Incubation temperature was 25 °C.

Total titratable acidity, citric acid, and residual sugar were determined at various stages of culture development. The culture filtrates were obtained by alternated washing with water, and pressing with a rubber bung, of the mouldy vermiculite or the mycelium mat on a piece of fine-mesh fabric in a Büchner funnel.

Citric acid was determined by the method of GODET et al.[6], and residual sucrose was quantitatively estimated according to STILES, PETERSON and FRED[7] after hydrolysis of the sucrose by boiling for 5 min in 0.1 *N* HCl followed by neutralization with 0.1 *N* NaOH; the results were obtained from glucose calibration curves and calculated as sucrose equivalents.

Results and discussion. As can be seen from Figure 1, the incubation time to obtain the maximum yield of citric acid is reduced from 10 to 5 days if vermiculite is

[1] J. MEYRATH, J. Sci. Fd Agric. *16*, 14 (1965).
[2] P. SHU and M. J. JOHNSON, J. Bact. *54*, 161 (1947).
[3] R. STEEL, C. P. LENTZ, and S. M. MARTIN, Can. J. Microbiol. *1*, 299 (1955).
[4] *The Oxoid Manual* (The Oxoid Division, Oxo Ltd., London 1961), 2nd ed., p. 83.
[5] P. SHU and M. J. JOHNSON, J. Bact. *56*, 577 (1948).
[6] TH. GODET and R. CHARRIÈRE, Mitt. Lebensm. Unters Hyg. *37*, 317 (1946).
[7] H. R. STILES, W. H. PETERSON, and E. B. FRED, J. Bact. *12*, 427 (1926).

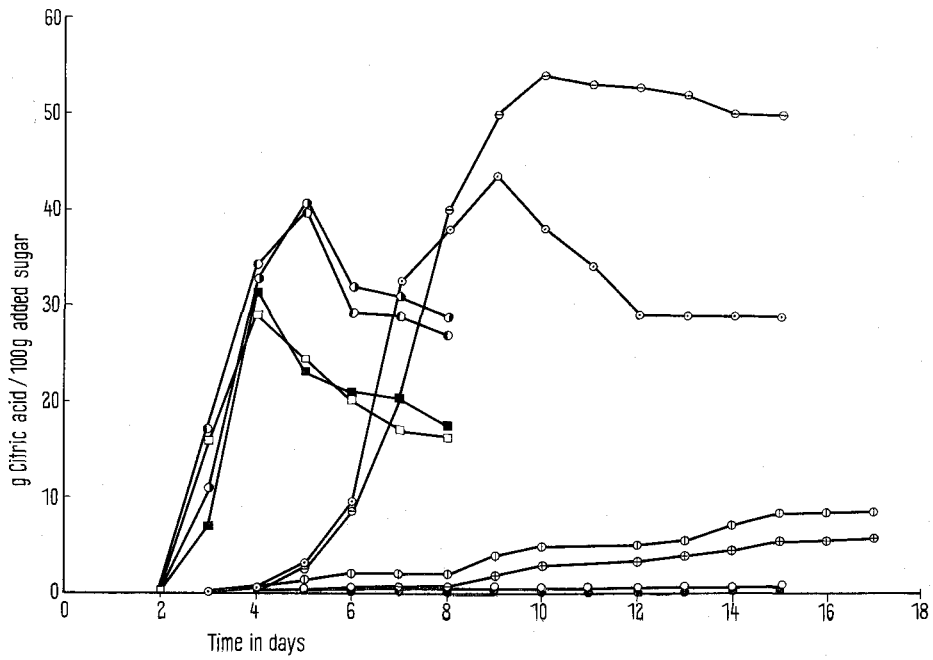

Fig. 1. Progress curves of citric acid production (g citric acid/100 g sugar added) in various substrates with and without vermiculite. Presence of vermiculite (solid stationary cultures); ■ = substrate A + purified sucrose; □ = substrate A + trace elements; ◑ = substrate B + unpurified sucrose; ◐ = substrate B + trace elements. Absence of vermiculite (liquid stationary cultures): ● = substrate A without trace elements; ⊙ = substrate A + trace elements; ○ = substrate B without trace elements; ⊖ = substrate B + trace elements. Absence of vermiculite (liquid shaken cultures): ⊕ = substrate A without trace elements; ⊙ = substrate A + trace elements.

added to the substrate. This reduction in incubation time is due partly to a shortening of the time elapsing until measurable citric acid formation takes place, and partly to a higher total rate of production in the measurable phase of the fermentation. Figure 1 shows also that this phenomenon is similar in substrates A and B, provided they are supplemented with trace elements. Addition of vermiculite results also in a somewhat reduced yield of citric acid, as can be seen from Figure 1. This has now been shown to be mainly due to the formation of insoluble Mg-citrate, since the amount of exchangeable Mg present in this type of vermiculite is approximately 14% weight/weight. Strictly speaking, the insoluble portion of citrate should be taken into account also for the evaluation of the rates of production. This, however, has been omitted for the present discussion.

The shortened incubation time is obviously of industrial importance, and while the presently used strain, under the conditions described, does not give yields as high as are often expected in industry there is great likelihood that other citric acid strains would react similarly towards addition of vermiculite. This assumption is supported by the fact that vermiculite increases the overall rate of metabolism rather than shifting it qualitatively; this increase in metabolic rate is correct at least as far as catabolism is concerned, since the rate of sugar utilization in the presence of vermiculite is almost proportionately increased with that of citric acid formation (Figure 2). Furthermore, as can be seen in Figure 2, the ratio of citric acid to total titratable acids (calculated as citric acid) follows the same trend over various stages of the citric acid fermentation in the presence as in the absence of vermiculite. No attempt has as yet been made to assess how far the assimilatory activity of the mould is affected by vermiculite.

It is also of interest that the rate of amylase production in *A. oryzae* is strongly stimulated by the presence of vermiculite[1]. There is some indication that in this mould too the overall rate of metabolism is increased[8]. Furthermore, evidence has been presented that the stimulation of amylase production is also partly due to the establishment of a favourable trace element balance[9], due to vermiculite functioning as a cation exchanger with particularly high affinity for heavy metal ions. The trace element requirement in vermiculite substrate of our *A. niger* strain has not yet been studied in detail, but from Figure 1 it is apparent that the trace elements Fe and Zn, which are required in the liquid substrates A and B, are supplied with the natural vermiculite.

From earlier work the conclusion has already been reached that the stimulation of the rate of metabolism by vermiculite is unlikely to be due to better exchange of gases, since neither the rate of amylase production nor the rate of growth in early stages of the culture development is increased if very shallow layers of substrate, shaken cultures or deep cultures are used. Similarly, with the presently used strain, the mere improvement of the rate of exchange of gases does not stimulate growth of citric acid production if shaken cultures are used, as can be seen from Figure 1; in fact both the rate of growth and the rate of citric acid production are under these circumstances smaller than in surface cultures. The inhibition of citric acid production in shaken cultures is a reminder of the possible importance of carbon dioxide in citric acid synthesis, as originally observed by FOSTER[10] and by MARTIN et al.[11,12], since it is possible that an insufficiently high partial pressure of carbon dioxide is established in shaken cultures as compared with surface cultures. It might be considered that vermiculite improves access of oxygen and yet does not decrease the carbon dioxide partial pressure too strongly, so that a most favourable balance between the 2 gases is established in the culture. There is, however, no great likelihood of this being the main reason for the stimulatory function of vermiculite, since, as is shown in Figure 1, the very early phase of citric acid production is favourably influenced by vermiculite. At this stage, the enhancement of the rate of exchange of gases should play a minor role, in view of the absolutely smaller rates of uptake of oxygen and production of carbon dioxide per unit volume of the culture. Further experiments on this subject are in progress[13].

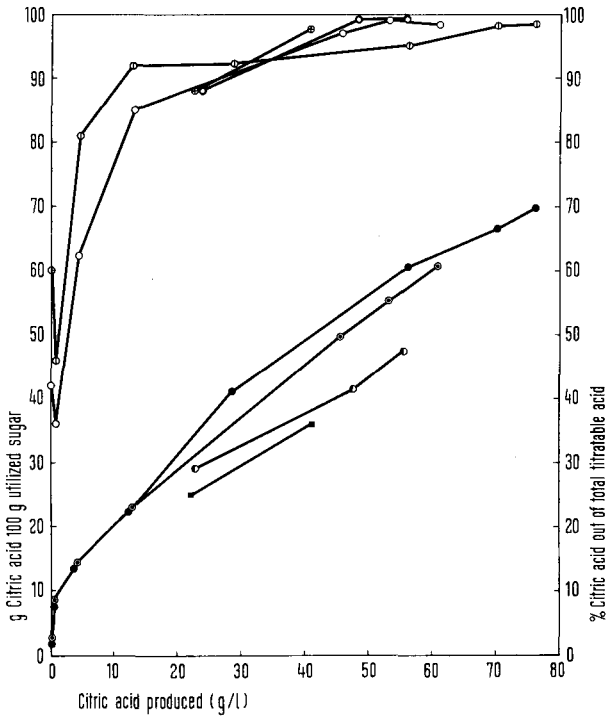

Fig. 2. Yield of citric acid (per unit weight of sugar utilized) and proportion of citric acid of total titratable acid at various stages of the fermentation (indicated by the citric acid content) in trace element supplemented substrates A and B. Presence of vermiculite (solid stationary cultures): ■ = substrate A, ● = substrate B (g citric acid/100 g sugar utilized); ⊕ = substrate A, ◉ = substrate B (% citric acid out of total titratable acid). Absence of vermiculite (liquid stationary cultures): ○ = substrate A, ● = substrate B (g citric acid/100 g sugar utilized); ○ = substrate A, ⓘ = substrate B (% citric adid out of total titratable acid).

[8] J. MEYRATH and M. H. IKRAM, to be published.
[9] J. MEYRATH and M. H. IKRAM, Proc. Soc. gen. Microbiol., Jan. 1966.
[10] J. W. FOSTER and S. F. CARSON, J. Am. chem. Soc. *72*, 1865 (1950).
[11] S. M. MARTIN, P. W. WILSON, and R. H. BURRIS, Archs Biochem. *26*, 103 (1950).
[12] S. M. MARTIN and P. W. WILSON, Archs Biochem. *32*, 150 (1951).
[13] Thanks are due to Dr. S. M. MARTIN, National Research Council, Ottawa, who kindly supplied the strain of *A. niger* used in these experiments, to Miss DIANA BRABSON and Miss JUNE MCKINLAY who provided technical assistance, and to Professor E. O. MORRIS for his support and stimulating interest.

Zusammenfassung. In Gegenwart des exfolierten Tonminerals Vermikulit wird die Zitronensäurebildung durch *Aspergillus niger* besonders stimuliert. Dabei wird die Induktionsphase verkürzt sowie die gesamte Gärgeschwindigkeit im messbaren Bereich der Zitronensäurebildungsphase erhöht. Die Stimulierung scheint einzig auf verbesserten Haushalt der Spurenelemente zurückzuführen zu sein.

J. MEYRATH and S. A. AHMED

Department of Applied Microbiology and Biology, The University of Strathclyde, Glasgow (Scotland), May 6, 1966.

A Spindle Analogue in *Saccharomyces cerevisiae* Hansen

In a previous communication[1], an illustration was presented of a tubular spindle-like structure around which the chromatin was disposed as a coiled element in vegetatively dividing cells of *Saccharomyces cerevisiae* (cf. Figure 5 of this report). The explanation was offered of the alignment of the daughter complements of chromosomes on this structure as a prelude to the subsequent distribution between the mother cell and the bud, with the aid of centrosomes. In the present report evidence is presented for what appears to us as stages in the formation of such a spindle-like element.

S. cerevisiae, Hansen (CBS 1171), obtained from the yeast division of the Centraalbureau voor Schimmelcultures at Delft, in Holland, was the yeast used in these investigations. Cells were from a 24 h growth on barley-wort-agar medium which was in turn obtained by inoculating cells from a 16-hour-old synchronized liquid culture, as detailed in the earlier communication[1]. 1 batch of smears was fixed in Carnoy's fluid for 2 h (Figures 1–7) and the other in Helly's (Figure 8) for 10 min[2]. The fixed cells were stained with Giemsa with prior extraction in NaCl at 60 °C for $1^1/_2$ h and hydrolysed in NHCl at 60 °C for $8^1/_2$ min[1,3].

In Figure 1 can be seen the centrally-located deeply-stained chromatin through which has emerged a fine unstained tubular structure with a stained knob, giving the appearance of a germinating seed. The cell at (a) in Figure 2 reveals the gradual elongation of the tube at one pole of the chromatin mass. A stained spherical dot is also seen. The chromatin in the cell in Figure 3 looks expanded. In Figure 4, the central unstained tube terminates in 2 divided stained entities at 1 pole, and the chromatin is wrapped round the central tube in 2 densely stained coils. The cell in Figure 5 reveals the spindle-like element in a better perspective with the chromatin tightly coiled round. Figure 6 is suggestive of loosely coiled chromatin, and at close examination reveals 3 dumbbell-shaped and 2 discrete chromosomes at the top (arrow). 2 parallel complements of chromosomes are seen in Figure 7. Each of these could be seen terminating in a thin drawn-out thread, converging at the pole in a stained dot. A second stained dot is seen at the other pole. Figure 8 is presented to show that comparable pictures were obtained with Helly-fixed cells also. Here again, at (a) a very fine unstained tube is seen piercing the deeply-stained chromatin. The anchoring of the ends could also be made out (arrow). In cell (b) a similar thin unstained element is seen attached to the chromatin (arrow). It may be of interest here to recall the centriolar structures connected to an unstained furrow, with a centrally-situated stained mass, demonstrated by the hematoxylin technique of SUBRAMANIAM[4].

The structure, as seen in these photographs, is very suggestive of a spindle element. The individual and divided stained entities at the poles, as seen in these cells, confirm the earlier demonstration of centrosomes

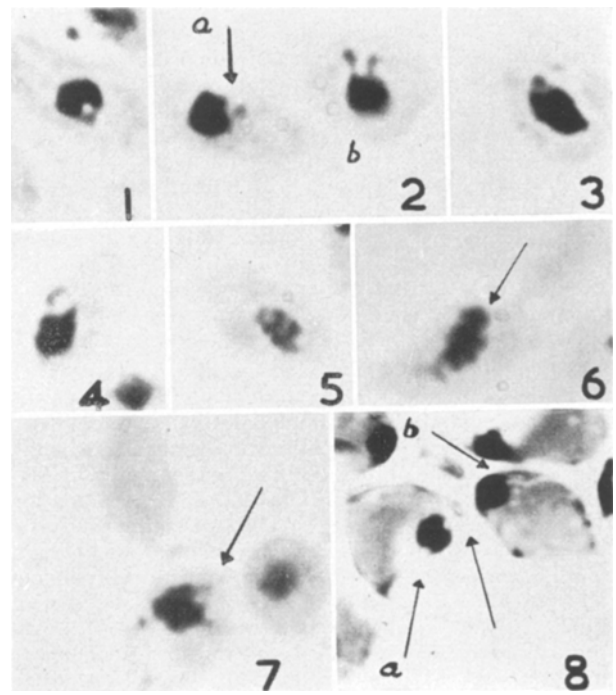

Fig. 1. Early stage in the appearance of the central tube. Ca. × 2800.

Fig. 2. Elongation of the tube with a centrosome in cell (a). Dividing centrosomes are seen in cell (b). Ca. × 2800.

Fig. 3. Chromatin expanded on the tube. Centrosome seen. Ca. × 2800.

Fig. 4. Tube more clear. Dividing centrosomes at one pole. Ca. × 2800.

Fig. 5. Chromatin coiled round the tube. Ca. × 2800.

Fig. 6. Coiled appearance of chromatin. Chromosomes arranged in tiers. Ca. × 3000.

Fig. 7. Parallel arrangement of chromosomes. Note the 2 thin threads converging in a centrosome. Ca. × 3000.

Fig. 8. Slender tube piercing through the chromatin (Helly-Giemsa preparation). Ca. × 3000.

1 L. S. PRAHLADA RAO and ALIE KOOPMAES, in press (1966).
2 C. F. ROBINOW and J. MARAK, J. Cell Biol. *29*, 129 (1966).
3 A. T. GANESAN, C. r. Trav. Lab. Carlsberg *31*, 149 (1959).
4 M. K. SUBRAMANIAM, Nature, Lond. *168*, 427 (1951).