A PAPYROGRAPHIC MICRO-METHOD FOR A DETERMINATION OF THE ORGANIC ACID MAKE-UP OF FERMENTED BEERS

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IN the course of our studies on the fermentative production of organic acids by various types and strains of fungi, maintained in the National Collection of Type Cultures, India, we were confronted with the problem not only of screening them with respect to their overall acid-producing capacity, but also of determining the organic acid make-up of the beer obtained by fermenting a standard sugar medium with each one of the fungal cultures.

The encouraging success which attended our studies on the papyrographic separation and characterisation of amino acids in micro quantities of protein hydrolysates[1] suggested the possibility of adapting this technique for a study of organic acids in fermented beers.

Preliminary experiments revealed that unlike amino acids, organic acids when run on the phase-pairs like water/*n*-butanol, were found to leave long diffuse tails behind the heads and the excursions were found to decrease with a fall in the concentration of the acids in the original solution. Lugg and Overell[2,3] attribute these effects to the ionisation of the acids in the aqueous phase and their adsorption by the filter paper. They have worked out the conditions under which discrete separations of organic acids could be secured, by incorporating a volatile acid in the mobile phase, which suppresses the ionisation of the other acids and displaces them from the filter paper by competitive adsorption. Acetic and formic acids have been employed as the volatile constituent of the mobile phase. In our studies we have adopted this modification to a micro-scale with excellent results.

Experimental

For single samples, the test tube microtechnique described earlier[1] was followed. When, however, a number of samples had to be analysed, as for instance, when a comparative study of the organic acid make-up of beers produced by different types of fungi, had to be made, the samples were simultaneously run on the same sheet of paper under identical conditions. This was accomplished by spotting the different samples on the filter paper, which, after drying and rolling into a cylinder, could be made to stand without any support. The vessel used consists of an ordinary cylindrical diet jar (10 cm. dia × 20 cm. ht.) provided with a ground-glass cover, which could be sealed airtight by means of an adhesive tape. The solvent was prepared by shaking up equal volumes of distilled water, *n*-butanol and an adequate amount of acetic acid to yield initially a 2-3 moles solution in the aqueous phase. The phases separate within a short time.

The aqueous phase is placed at the bottom of the jar while the butanol-acetic phase is kept in a petri-dish cover placed at the bottom. The filter paper (19 cm. × 18 cm.) was spotted with the test samples at points 2 cm. apart and in a line about a centimetre high from the lower edge of the paper Quantities of the test beer varying from 0·001 to 0·005 ml. are delivered on to the spot by means of capillary pipettes. Care is taken to see that the diameter of the spot does not exceed 3-4 mm. After drying, the paper is rolled into a cylinder, "bosstitched" and hung for a couple of hours in the cylinder without dipping into the solvent; during this period, the filter paper gets itself saturated with the aqueous and volatile solvent vapours. The cylinder is then lowered into the centre of the petri dish containing the mobile phase. The run usually takes about five hours at the room temperature (24-25° C.) with the *n*-butanol-acetic as the mobile solvent.

After the run, the filter paper cylinder is air-dried over night and then passed through an oven at 60° C. for 5-10 minutes. The cylinder is then unrolled and treated with an alcoholic solution of brom-cresol green (40 mgm. per cent. in 95 per cent. alcohol) either by spraying or by dipping. The positions occupied by the acids are revealed as yellow spots against a greenish blue back-ground. (See Figs. 1 to 4 which represent faithful reproductions of the papyrograms.)

Discussion

Fig. 1 shows that a mixture of the six acids, each of them being present at the level of 10 γ in the test spot, can be separated into discrete spots on the papyrogram. The excursion of a given acid is definitely influenced by the presence of another acid. It is, therefore, clear that the R_F values have little significance so far as the reading of the papyrograms of mixtures

are concerned. The relative positions occupied by the different acids from a mixture remain fortunately constant for any given phase pair. If, therefore, a known mixture of pure organic acids (reference mixture) is simultaneously papyrogramed along with the test mixture (*e.g.*, fermented beer) the spots of the reference mixture will serve to interpret the spots of the test fluid.

Fig. 1.

Papyrogram of Individual and Mixture of Organic Acids. Abbreviations: *Ox.* (Oxalic); *Ci.* (Citric); *M.* (Malic); *L.* (Lactic); *S.* (Succinic), *Fu.* (Fumaric).

Figs. 1 and 2 show that except for oxalic and citric acids which yield elongated spots, the other acids appear as circular spots. By keeping the size of the intitial test spots nearly constant, it is easy to obtain a semiquantitative idea of the relative concentration of a given acid in the mixture, by comparing the size of the acid spot with a series of standard reference spots (see Fig. 2) obtained by papyrograming known quantities of the acid under

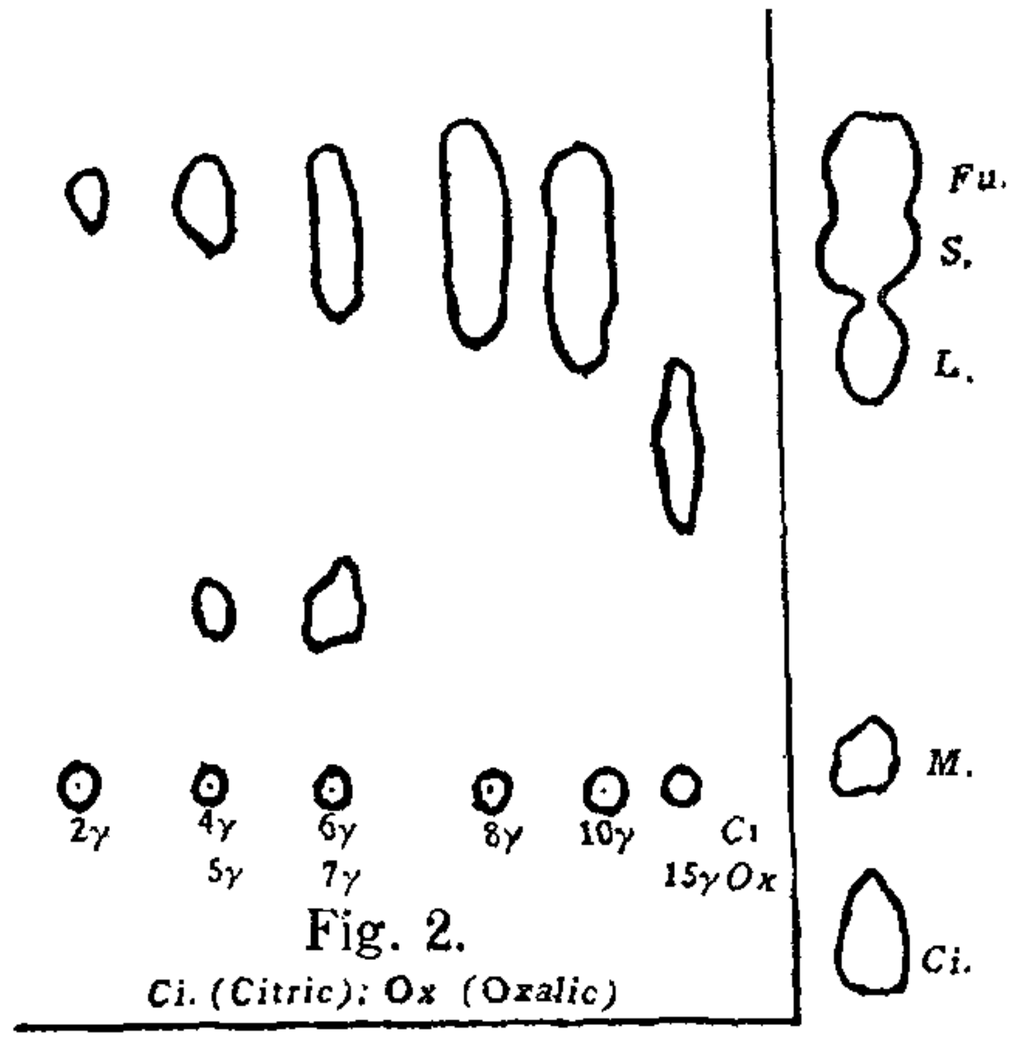

Fig. 2.

Ci. (Citric); Ox (Oxalic)

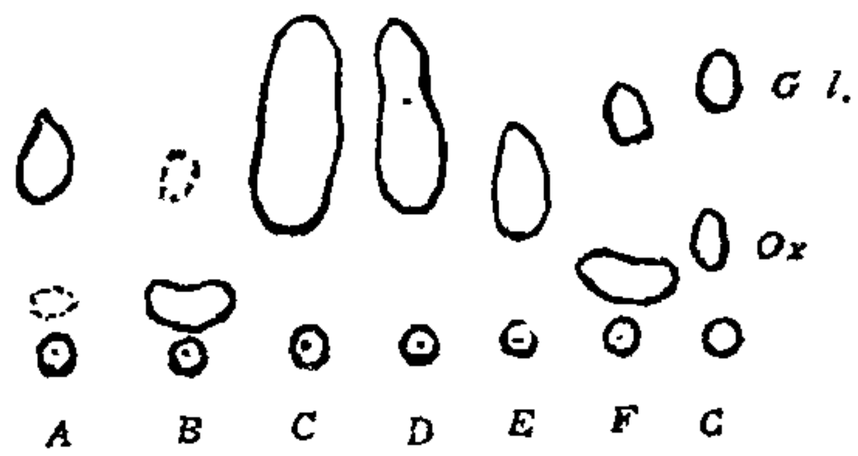

Fig. 3.

Papyrogram of Beers Fermented by Fungi.

A. oryzae—*A*: at initial pH 2 and B: at initial pH 6·8.

A. mucer—C: at initial pH 2 and D: at initial pH 6·8.

A. penicillium—E: at initial pH 2 and F: at initial pH 6·8. *F*: Reference Mixture of Organic Acids; *Gl.* (Gluconic).

the same conditions. Such estimations are obviously permissible and limited only to a certain well-defined range of acid concentrations. Quantities higher than 10-15 γ of each acid in the starting spot introduce complications in this micro-method. Lactic, succinic and fumaric acids, whose spots are closely situated, tend to merge into a continuous spot but remain distinctly discernible by the bulbular form of the spot.

Overloading the test spot with a heavy concentration of the mixture, will result in a continuous patch; the presence of a relatively high concentration of a single acid in the test mixture will give an elongated spot enveloping the spots produced by the acids which occupy

the nearby positions. This is illustrated in Fig. 4 where the beer fermented by a strain of *Aspergillus niger*, a high citric acid yielder, is papyrogramed. The sample contains about 70 mgm. of citric acid per ml. of the beer, and 0·001 ml. of the beer was spotted. The citric acid spot is seen to cover the malic acid position also.

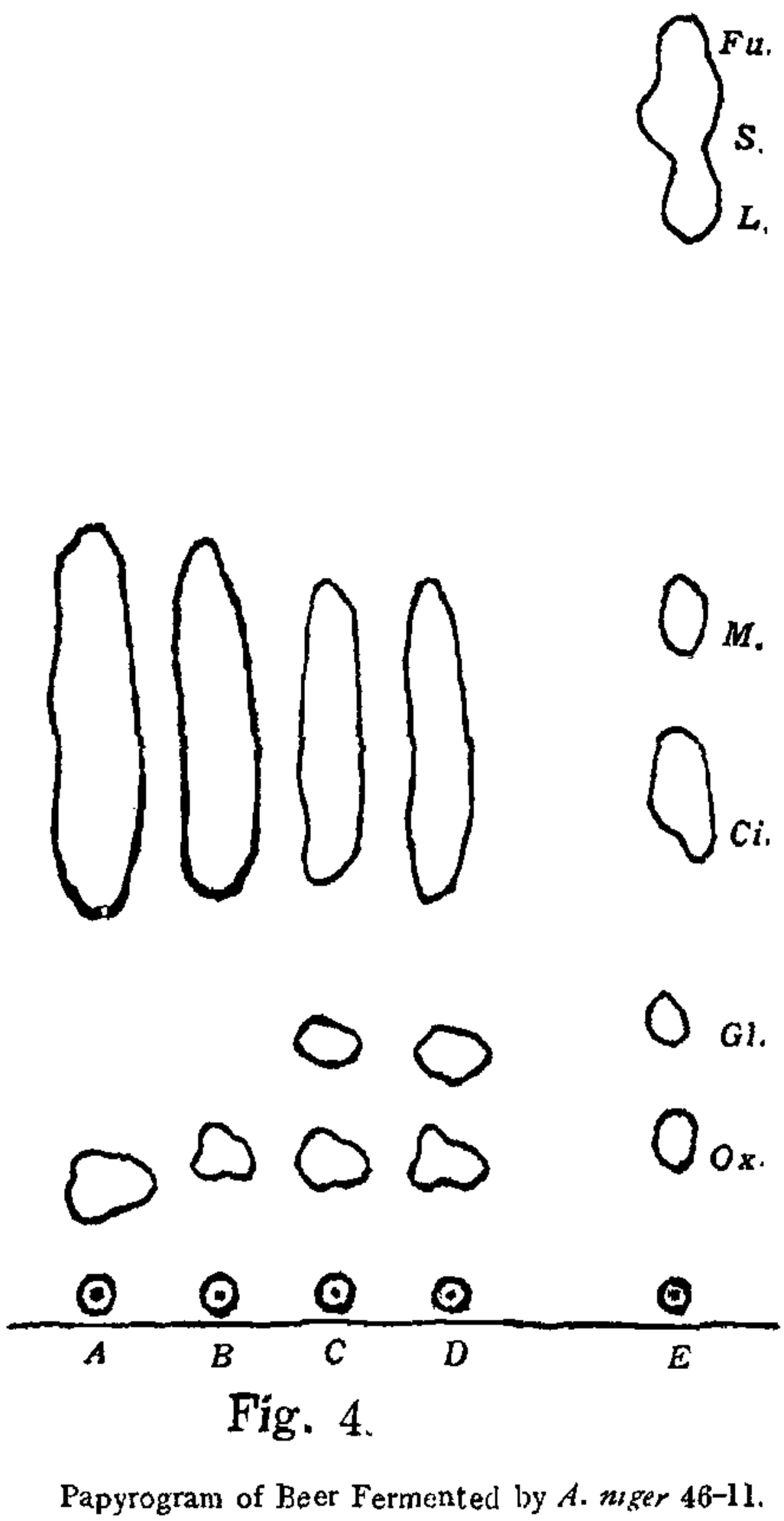

Fig. 4.

Papyrogram of Beer Fermented by *A. niger* 46-11.
A and B : with an initial pH 2.
C and D : with an initial pH 6·8.
E : Reference Mixture.

At the moment, the taxonomic classification of fungi is largely based on morphology while in the case of bacteria and yeasts, the classification takes into account the bio-chemical activities of the organisms. The fermentability of different types of sugars, for instance, has been extensively employed for the characterisation of bacteria and particularly yeasts. The bio-chemical performance of fungi as a factor in their characterisation has not been extensively recognised probably because of the lack of suitable, reproducible and rapid methods of estimation. It is believed that the acid-producing capacity both in its qualitative and quantitative aspects might serve to characterise certain groups of fungi and distinguish certain strains of a particular type of organism. The papyrographic method which we have developed for characterising the acids, might be one such method which has the merit of (*a*) rapidity, (*b*) reproducibility, (*c*) simplicity and elegance, (*d*) ease of manipulation and (*e*) adaptability to micro quantities.

We have explored this possibility, by taking a few types and strains of fungi, growing them in a standard medium and subjecting the resulting beers to a papyrographic analysis. Two different hydrogen-ion concentrations, pH 2·0 and pH 6·8 have been chosen for these studies. The results are presented in Figs. 3 and 4

From a study of the position and intensity of the spots it was found that the strain of *A. niger* produces large qualities of citric acid and traces of oxalic and gluconic acids. Beers fermented at pH 6·8 contain a smaller quantity of citric acid while a definite increase in the quantity of oxalic acid and gluconic acids is indicated.

The penicillium yields appreciable amount of oxalic and traces of gluconic acids at the neutral pH while with initial acid pH only gluconic acid is obtained.

A. oryzæ give appreciable quantities of gluconic acid with initial acid pH and appreciable quantity of oxalic acid and traces of gluconic acid with the initial pH at 6·8, while the mucor has been found to form gluconic acid in appreciable amounts at both the pH. These results have clearly demonstrated that a considerable amount of data with respect to the acid-producing capacity of fungi could be obtained by this simple and elegant method of analysis. As suggested by Lugg and Overell,[2,3] the spots could be excised, the acid extracted by steeping them in water and titrated. We can thus obtain a quantitative data.

The applications of this method are many; it offers a convenient method for making a comparative study of the acid-producing efficiency of the various mutants resulting by the irradiation or chemical treatment of a given fungus. The variation in the organic acid make-up of beers fermented under different experimental conditions—effect of temperatures, pH,

trace elements, atmospheres, concentration of various nutrients and forms of carbon and nitrogen—can be determined.

SUMMARY

A papyrographic micro-method for the separation, characterisation and semi-quantitative determination of the non-volatile organic acids in a mixture of them, is described.

The method is characterised by its simplicity, elegance, rapidity and ease of manipulation and has been shown to be adaptable to micro quantities of test samples.

The applicability of this method for a determination of the organic acid make-up of beers fermented by fungi has been demonstrated. The employment of this method as a helpful routine for a taxonomic characterisation of fungi, for evaluating the comparative acid-producing efficiency of different types, strains and mutants of fungi, and for a study of the optimum conditions favouring the production of a given acid, is suggested.

Further, the method offers possibilities in the detection of intermediates[4] and new acids formed during fermentation of carbohydrates just as the papyrographic method helped in the detection of new amino acids.

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1. Govindarajan, V. S., and Sreenivasaya, M., *Curr. Sci.*, 1950, **19**, 39. 2. Lugg, J. W. H., and Overell, B. T., *Nature*, 1947, **160**, 87. 3. —, *Aust. J. Sci. Res.*, 1948, **1A**, 98. 4. *Added in proof:* Use of buffered solvent and application to study of path of carbon in photosynthesis in given by Benson, A A., *et. al. J. Amer. Chem. Soc.*, 1950, **72**, 1710.