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On the Presence of *Acetobacter oxydans* in Apple Juice

BY C. R. MARSHALL AND V. T. WALKLEY

Seager, Evans and Co. Ltd., 14 Deptford Bridge, London, S.E. 8

SUMMARY: *Acetobacter oxydans* has been identified in pure natural apple juice. A new technique which possesses some advantages over existing methods has been developed for demonstrating capsule formation by this organism and by other species of *Acetobacter*.

Grove (1915) isolated five different types of acetic acid bacteria from thirty to forty ciders and described their cultural characters when grown in malt extract solution containing 2 % ethanol; the most commonly occurring was *Bacterium xylinum* but the others were not identified. Vandecaveye (1927) reported two strains of vinegar bacteria which did not form a pellicle, observed during an examination of cider vinegar. These organisms produced large quantities of acetic acid in fermented ciders and were catalase positive. Using their oxidative powers as a basis of differentiation between the species, he identified his strains as *Acetobacter aceticum* and *A. rancens*.

During the course of a microbiological investigation carried out at a commercial apple juice factory in Gloucestershire, we isolated and identified a large number of yeasts, moulds, and bacteria present on the fruit that were capable of inducing spoilage in apple juice. Their ability to develop in apple juice and their resistance to pasteurization were observed and will be reported elsewhere. Among the bacteria encountered were two species of *Acetobacter*, of which one was identified as *A. xylinum* Brown, similar to *Bact. xylinum* found in cider by Grove. The other species appeared to be a variety of *A. oxydans* (Henneberg) Bergey *et al.* Since the presence of this latter organism in apple juice has not hitherto been recorded we describe its characters in the present paper.

It was first isolated from a mixed plate culture which had been seeded with a sample of apple juice taken after several months' bulk storage at 37° of a heavily carbonated juice. The incidence of the organism increased from the third to the tenth month of storage to a maximum of 900 organisms/ml. juice. Thereafter, a decrease was observed, and 6 months later the organism was detected with difficulty. High carbonation and low temperature sufficiently arrested its development and prevented any impairment of the organoleptic properties of the juice. Subsequent investigations showed that it was frequently present on the apples used for processing and its presence in the juice was ascribed to this source.

METHODS

Bergey's Manual (1948) has reclassified the entire group of vinegar bacteria under the genus *Acetobacter*, all species possessing the common characteristic of forming at some period of their development either a pellicle or a surface film on the surface of suitable liquid media. It describes seven principal

species, together with two species considered by Beijerinck to be varieties of *A. rancens*, and also seventeen ancillary species which it is declared require further study before any change in nomenclature is advisable.

As a group the acetic acid bacteria are all Gram-negative, non-spore forming, and give a negative response to the Voges-Proskauer reaction. They oxidize ethanol to acetic acid, and various organic compounds to organic acids. Walker & Tošić (1948) have stated that, with the exception of *A. peroxydans*, all known *Acetobacter* species are catalase positive. Kulka & Walker (1946) established that capsule formation also is probably a characteristic feature of the genus. The keys to the identification of certain species given by *Bergey's Manual* (1948), Shimwell (1948), and Walker & Tošić (1946*a, b*), were found helpful for identifying our isolates.

Media. Apple juice and Blickfeldt's solution were used as liquid media. These solutions were used for preparation of solid media with 2 % agar, but for this purpose apple juice was usually diluted to decrease the sugar content to 1–2 % and 0.2 % w/v $(\text{NH}_4)_2\text{HPO}_4$ added.

Gram reaction. The reliability of the staining technique was checked by mixing together on the slide containing the unknown organism a Gram-positive organism (*Micrococcus candidus*) and a Gram-negative organism (*Bacterium coli*).

Catalase test, was carried out according to the method used by Walker & Tošić (1948) using sterile malt extract (sp.gr. 1036) buffered with CaCO_3 and solidified with 1.5 % agar.

Biochemical reactions were conducted in yeast water with addition of 1% (w/v) of the appropriate sugar or organic substrate. The tests were incubated at 25° and the tubes examined at intervals up to 2 weeks.

Capsule staining. To a 5 % (w/v) solution of gelatin in water at 60° sufficient Cotton Blue powder is added to give an intense dark blue colour forming the contrast medium, powdered eosin (1–2%) is then added, and the whole thoroughly mixed. Films are prepared on a warm stage at 30–37°. A little of the liquid gelatin stain is placed on a wet film on a warm slide of the organism under study. Observation is carried out on the warm stage after mixing and covering with a warm cover-glass. The bacterial cells appear pink against a blue background, the capsule remaining colourless. Thin films are easily prepared by this method. To assist differentiation and to prevent coalescence of capsules, the culture may previously be diluted with very dilute sodium hydroxide. This method does not involve drying or undue manipulation of the material and damage and distortion to the capsule are minimized. Observations of the capsules of both *A. xylinum* and *A. oxydans* by this method revealed a characteristically uniform appearance although slight differences in measurement, as compared with Indian Ink methods, were sometimes detected.

RESULTS

Cultures were obtained from apples and from juice. In the former case epidermal swabs were made and teased out and suspended in sterile water. After making suitable dilutions these were plated on suitable solid media.

When examining juice for bacteria we found it convenient to centrifuge the samples and to wash the deposit with distilled water before making a film, as this avoided the juice charring or becoming a sticky mass.

The following morphological and cultural characters and biochemical reactions were found.

Solid media. On apple-juice agar and Blickfeldt's solution agar at 25° the organism appeared barrel-shaped, with a tendency to be coccoid. After 24 hr. the colony appeared clear, colourless and circular with a mucoid tendency. After 48 hr. it appeared greyish and opaque and the mucoid tendency had increased. After 60 hr. it became grey-white, circular, refractile, entire edge, mucoid in texture, the centre uniform and slightly globose. After 70 hr. the colony was 2.5 mm. in diameter, round, globose, grey-white in colour and opaque, thickly mucoid, entire edge, raised and less refractile than at 48 hr.

Slope cultures. At 24 hr. on apple-juice agar slopes, the colony was similar in appearance to that on agar plates. After 72 hr. the colony had begun to run along the surface towards the bottom of the slope. This was observed at 20° and at 25°.

Apple-juice agar stab. Stab cultures at 25° showed very slight growth along the line of inoculation for a distance of 5–6 mm. Good growth developed on the surface. After 3 weeks the growth was more luxuriant than that obtained by any other cultural method. After 6 weeks the surface had become stiff in texture. The colony was still viable up to 12 weeks at 20° and 25°.

Gelatin media. No liquefaction in 3 weeks.

Liquid media. Under anaerobic conditions there was no growth in apple juice. Under aerobic conditions at 25° the organism exhibited formation of diplococci with a tendency to short chain formation. After 48 hr. a slight surface scum appeared as a greyish white haze, which became uniform through the medium by 72 hr. and ultimately disappeared. After 7 days a thin greyish white film had spread over the entire surface, with a slight tendency to climb the walls of the glass. This film sank when disturbed.

Optimum temperature. The organism grew well at 20–25°, between 25° and 30° growth was less good, and above 30° restriction became even more pronounced.

Motility. Neither cultures in sloppy gelatin nor liquid medium showed motile organisms.

Gas formation. Gas formation was not evident in aerobic culture in glucose-yeast water, ethanol-yeast water or acetic acid-yeast water at 25°.

Biochemical reactions. Acid was produced from glucose and sucrose accompanied by heavy growth, and from maltose, lactose, galactose, and dextrin with slight growth. There was very little growth and only a slight acid formation in the presence of raffinose. Acid was produced in the presence of 2 % ethanol accompanied by fair growth.

Other characters. The organism was Gram-negative, catalase positive, non-spore forming, and gave a negative response to the Voges-Proskauer reaction. The presence of a capsule was demonstrated, by our own technique

and by the methods of Gin and Baker. The following measurements were obtained:

Gin's method: $1.2 \times 1 \mu$. to $1.4 \times 1.2 \mu$.

Baker's method: $1.2 \times 1.2 \mu$. to $1.4 \times 1.5 \mu$.

Our method: $1.3 \times 1.3 \mu$. to $1.5 \times 1.5 \mu$.

DISCUSSION

Presence of a capsule, negative responses to Gram staining and the Voges-Proskauer reaction, absence of spore formation, production of catalase, together with ability to produce acetic acid from sugars and ethanol establish the organism as a species of *Acetobacter*. Non-oxidation of acetic acid, absence of pigment formation, and optimum growth at $20-25^{\circ}$ eliminates all the principal species in *Bergey's Manual* with the exception of *A. oxydans*. *Bergey's Manual* mentions, however, that motile forms of the latter organism occur and that the organism has been encountered in the form of rods, $0.8-1.2 \times 2.4-2.7 \mu$., either singly or in chains. Another difference from our own organism is that according to *Bergey's Manual*, *A. oxydans* does not produce acid from lactose.

These differences may be reconciled by study of other publications. It is possible that our organism was cultivated at too high a temperature for motility to be observed, for Jorgensen (1939) remarked that *Bact. oxydans* [syn. *A. oxydans* (Henneberg) Bergey *et al.*] can be induced to produce motile cells by cultivation at 20° on wort agar followed by transplantation into diluted beer. Shimwell (1948) confirmed that in many species of acetobacter motility sometimes occurs only at certain stages in the development of the culture. He also stated that although all members of the genus are rods, in some the cells are so short and so ellipsoidal and even coccoid in form that they are frequently mistaken for true cocci. Jorgensen also asserted that the cells of *Bact. oxydans* may appear in pairs or as fairly long chains.

The ability of our organism to produce acid from lactose and from other sugars is a characteristic shared by *A. industrium* (Henneberg), Bergey *et al.* Walker & Tošić (1946*b*), however, claimed that *A. oxydans* can also produce acid from lactose, but Shimwell regarded *A. oxydans* and *A. industrium* as identical. Jorgensen makes a distinction between the two species for he remarked that although both organisms form a thin film on liquid media, that of *A. oxydans* tends to climb the glass (a characteristic exhibited by our organism), whereas that formed by *A. industrium* falls to the bottom. On these grounds, therefore, we feel justified in identifying our organism as *A. oxydans*.

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