STUDIES ON ACETOBACTER

I. Isolation and Characterization of the Species

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Received February 8, 1955

(Communicated by Dr. P. Maheshwari, F.A.SC.)

The importance of acidity development in the alcoholic liquors has been realized from the very early times but it is only during this century that the exact role of acetic acid bacteria in the acetification process is being understood. The recent recrudescence of interest in these bacteria is another strong pointer to their increasing importance and many attempts have been made to utilize them even in industries other than in the manufacture of vinegar (Prescott and Dunn, 1949). But in our country even the quality of vinegar produced and marketed lack many of the attributes associated with a good product and leave much room for improvement. Furthermore the raw materials suitable for vinegar manufacture are available in abundance and invite exploitation for the economic development of our country. As a first step towards this, it was considered worthwhile to examine varieties of materials for the presence of these bacteria and indicate the sources which would help bring about the desirable type of fermentation for the industrial production of vinegar. In this paper are presented the details of the methods employed for isolation, characterization and identification of Acetobacter, a genus in which may be found species neither well defined nor fully described. It would also be clear from the comparative descriptions presented that some of the species placed by Bergey, et al. (1948) in the appendix to the genus Acetobacter (p. 185–89) deserve as much recognition as the better-characterized species mentioned under the key to the genus. In this paper are also presented the characteristics of some isolates which fit in with the descriptions given for Acetobacter but which differ from it by forming gas in the carbohydrate media.

MATERIALS AND METHODS

The thirty odd species mentioned in this paper were isolated by us from such diverse sources as grapes (Vitis vinifera L.), mangoes (Mangifera

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indica L.), pine-apples (Ananas sativa L.), sapotas (Achras sapota L.), oranges (Citrus aurantium L.), plums (Prunus domestica L.), strawberries (Fragaria indica Andr.), bananas (Musa sapientium L.), beets (Beta vulgaris L.), tomatoes (Lycopersicum esculentum Mill), raisins (Vitis vinifera L.), dates (Phantx dactylifera L.), jaggery (unclarified sugar), alcoholic liquors (neera, drakshasava and beer), soils, human and cow excreta and as aerial contaminants. Where extraction of the juice was possible from the cleansed material, the extracted juice served as the inoculum; where this was not possible, a portion of the material was directly introduced into the medium utilized for isolation purposes.

Though various complex media have been recommended for the isolation of acetic bacteria, there have not been many attempts directed at culturing them exclusively on simple, chemically defined media such as the one designed by Beijerinck (1898). This medium stands out as the simplest and offers a satisfactory approach to the isolation of the acetic acid bacteria by resort to the enrichment culture methodology. The presence of alcohol in this medium (see composition below), besides inhibiting the growth of several bacteria, serves as a 'selective' carbon and energy source for the acetic acid bacteria. Furthermore, the medium does not appear to be favourable for the growth of several other bacteria with the result that they get easily eliminated during the enrichments. The composition of this medium is as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Tap water</td>
<td>100 ml</td>
</tr>
<tr>
<td>(NH₄)₂HPO₄</td>
<td>0.05 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Ethyl alcohol†</td>
<td>3 ml</td>
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<tr>
<td>pH adjusted to</td>
<td>6.5</td>
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This medium dispensed in 50 ml. aliquots in 250 ml. Erlenmeyer flasks was inoculated and incubated at room temperature (27 to 29°C.) until growth appeared (room temperature was observed to be better than incubation at 37°C.). The growth was examined microscopically and passed through two successive transfers in the same medium, the transfer being effected each time after a critical microscopical examination. Two transfers usually resulted in the elimination of the extraneous flora so much so that the third enrichment on streaking on the same medium solidified with agar (and containing a few drops of Andrade's indicator) was invariably found to give rise to colonies mostly made up of the acetic acid bacteria which could be

† Alcohol was incorporated after sterilization of the salt solution by autoclaving.
detected by their pink colony formation. The colonies fished out and examined were repurified and tested for their power of acetifying alcohol by titration methods using in these tests various concentration of alcohol ranging from 2 to 8 per cent.

In some instances, the contents from the third enrichments failed to give any growth on the corresponding solidified medium or gave only poor growth after a long incubation period. In these cases, apple juice agar of the following composition was made use of for isolation purposes.

*Apple Juice Agar*

- Tap water .......... 100 ml.
- \((\text{NH}_4)_{2}\text{HPO}_4\) .......... 0.2 g.
- Apple juice .......... 2% (v/v)
- Agar .................. 3 g.

The pure cultures thus obtained were tested for the production of acid from alcohol. Though Beijerinck's solution was found unsuitable for this purpose, the substitution of 0.5 per cent. peptone in some instances and 0.2 per cent. yeast extract in others for the ammonium phosphate, yielded satisfactory results. The various isolates were then carefully examined for their morphological characteristics such as shape, size, motility, capsule formation, swollen cell and thread formation as well as for various cultural and physiological peculiarities such as pigment production, giant colony formation, nature of growth on solid and liquid media, gelatine liquefaction, changes in litmus milk, growth in Hoyer's medium, behaviour in presence of ethyl alcohol, oxidation of acetic acid to carbon dioxide, production of acid and gas from carbohydrates and alcohols, production of dihydroxyacetone from glycerol and the formation of sorbose from sorbitol. Though a large number of media and their modification were utilised for each test, only those which yielded consistent and reliable results were adopted for interpretation. It may be mentioned in this connection that in general, proteose peptone (Difco) was found to be a better source of nitrogen than other peptones, and yeast extracts as a rule hastened growth and development of results.

**RESULTS AND DISCUSSION**

The identification of acetic acid bacteria has been rendered difficult because the genus has been encumbered by a large number of poorly described species. Of the thirty odd species known and described in the literature, only a few are well known and the rest may be regarded as unrecognizable. As the purpose of this paper will be better served by a detailed
description of the less-known species with only indication of differences observed from the well-known ones, species differentiations are not presented here beyond a reference to variability of behaviour, a factor to be reckoned with in all species. For instance, some acetic acid bacteria acquire the power to produce acid from a sugar or alcohol which it normally cannot utilise whereas others may lose their ability to form acid from the same substrates. Therefore, neither fresh criteria to differentiate species nor the creation of new species has been attempted. By a critical study and utilisation of the criteria previously employed, a comparative study of new isolates is presented, which we hope, will help remove some of the existing confusion and render the classification of the genus Acetobacter more clear. The thirty species described in this paper have been grouped arbitrarily on the basis of cultural and gross physiological characteristics.

**Group I. Oxidise ethyl alcohol to acetic acid and further to carbon dioxide; utilise ammonium salts as the sole source of nitrogen.**

Eight isolates from our collection fall in this group and in conformity with the opinion of Hoyer (1898) and Beijerinck (1898) we recognize these isolates as _A. aceti_ without referring them to any particular variety (strain) of the parent species. We however wish to point out here that a typical _A. aceti_ is said to produce acid from glucose, ethyl alcohol, propyl alcohol and glycol and no acid from methyl, isopropyl and butyl alcohols, glycerol, fructose, lactose, maltose, or sucrose to mention only some common laboratory tests. However, some of our strains fermented lactose, fructose, maltose and sucrose as well as butyl and isobutyl alcohols. One strain even fermented glycerol. None of the strains however produced acid from methyl or propyl alcohols. One strain also showed such a strong tendency to ascend the sides of the glass containers that we are constrained to regard it as Acetobacter ascendens, a species placed in the appendix to the genus in Bergey's Manual. We give below data which would supplement the description in Bergey's Manual and make identification of this species less difficult.

**Morphology.**—Cylindrical to ellipsoidal rods (average size 1 μ/0.7 μ) occurring singly, in pairs and in irregular clumps. Thread and swollen cell formation (at 40° C.) absent, capsulated, non-motile, non-sporulating; Gram negative and do not give the cellulose reaction with iodine solution.

**Growth on Beijerinck's solidified medium.**—Round, smooth, glistening, convex, entire colonies less than 1 mm. in diameter.

**Growth on Apple juice agar.**—Round, smooth, glistening, convex colonies with an undulating margin, opaque in the centre and transparent on the borders, size 1–2 mm.
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Giant colonies on Beijerinck’s semi-solid agar.—Smooth, opaque, white glistening colonies, 2.1 cm. in diameter.

Growth on yeast extract-glucose—CaCO₃ agar.—Smooth, flat, glistening transparent colonies.

Growth in yeast extract broth.—White thin film with a strong tendency to ascend the glass wall.

Growth in yeast extract—glucose broth.—Same as above with added turbidity of the liquid medium.

Physiological characteristics.—Catalase positive; gelatine not liquefied; produce acid and soft curd in litmus milk; litmus reduced; grows luxuriantly in Hoyer’s mineral solution. Tolerates alcohol upto 10 per cent. (v/v) and produces acid after a week. Forms acid from glucose, lactose, sucrose, ethyl alcohol, propyl alcohol, butyl alcohol and isobutyl alcohol; does not ferment maltose, fructose, methyl alcohol, isopropyl alcohol, amyl alcohol or glycerol; does not form dihydroxy acetone from glycerol medium.

Source.—Soil.

Group 2. Oxidised ethyl alcohol to acetic acid and further to CO₂; do not utilise ammonium salts as the sole source of nitrogen.

Eight other isolates could be placed in this group which include species such as A. xylinum, A. acetigenum and A. xylinoides characterised by their ability to produce a cellulosic pellicle. Only one isolate in our stock could be demonstrated to form a cellulosic pellicle and this could be referred to as a variant of A. xylinum inasmuch as it failed to form dihydroxy acetone and formed acid from isopropyl and isobutyl alcohols. Another species could be labelled as A. pasteurianum despite its ability to produce slight acid in lactose, butyl alcohol and glycerol. A third strain has been referred to as A. rancens despite the fact that it differed from a typical strain by its failure to produce acid from butyl alcohol. Another strain has been referred to by us as A. viniaceti inasmuch as it agreed in general with this species as described in Bergey’s appendix to the genus. However, some additional data on the species compiled by us are briefly presented below:—

The organism is encapsulated and non-motile.

On Beijerinck’s solidified medium.—Smooth, white glistening colonies: similar but larger colonies are formed on apple juice agar. No giant colony formation on semi-solid apple juice agar.

On yeast extract-glucose—CaCO₃ agar.—Pale yellow, abundant, glistening moist, opaque growth.
Catalase present—Gelatine not liquefied. Acid and soft curd in milk; litmus reduced. Very slight growth in Hoyer's mineral solution after 2 weeks. Tolerate 8 per cent. (v/v) alcohol in which acid is formed after 10 days. Oxidation of alcohol and acid slow but complete. Slight acid from lactose. Produces dihydroxy acetone from glycerol medium.

Source.—Pine-apple.

Two other strains isolated one each from human intestine and jaggery are referable to as *A. plicatum* even though some minor differences are indicated in their physiological behaviour. Both were Gram negative, non-motile, capsulated, occurring singly, in pairs or short chains; the one from human intestine showed few swollen cells. Both give smooth, moist, glistening, transparent to transluscent, cream coloured, round, entire colonies on apple juice agar, but do not form giant colonies on the semi-solid medium of the same base. In yeast extract glucose medium viscous growth on top with a tendency to ascend the wall. In yeast extract ethyl alcohol medium, sticky growth at the bottom and on yeast extract-glucose-CaCO₃ agar cream coloured moist, watery, transluscent, raised, butyrous growth. Catalase present; gelatine not liquefied; litmus reduced and acid formed in milk; no growth in Hoyer's mineral solution; tolerates 5 per cent. alcohol in which growth appears after 24–36 hours and acid formation after 2 weeks. Oxidation of alcohol and subsequently of acid complete. Acid from glucose, maltose, fructose, ethyl alcohol; human strain also from sucrose and isobutyl alcohol; jaggery strain from propyl alcohol; no acid from methyl, isopropyl, butyl, amyl alcohol or glycerol.

The remaining two isolates presented similarities to *A. turbidans* by forming transparent to transluscent colonies with oil-like consistency on apple juice agar and wort agar: Giant colony formation was absent; on yeast extract glucose-CaCO₃, white, moist, watery, opaque, butyrous growth. In yeast extract medium, growth along the sides of the tubes with a scanty deposit. In the same medium with glucose, ring, and liquid very turbid. In ethyl alcohol yeast extract medium the growth was good with a yellowish powdery deposit; catalase positive; no liquefaction of gelatine; reduction of litmus; acid and soft curd in milk. No growth in Hoyer's medium; slight growth in 8 per cent. alcohol, acid being detectable after 8 days; oxidation of alcohol to acid and to CO₂ complete; glucose, ethyl alcohol, isobutyl alcohol and glycerol only fermented to form acid. No acid in lactose, maltose, sucrose, fructose, propyl and isopropyl alcohol, butanol, methanol and amyl alcohol. The ability to form tense density in beer only remains to be checked.

Source.—Soil.
Group 3. Oxidise ethyl alcohol to acetic acid only and not completely to CO$_2$; show pigmented and ropy growth.

Only three isolates could be placed in this group. One of these isolated from soil agreed well with *A. melanogenum*. It formed a deep brown pigment on glucose yeast extract—CaCO$_3$ agar after 2 weeks. The chromogenic property was enhanced in litmus milk. The culture formed 1-sorbose from 1-sorbitol and reduced Fehling's solution completely; formed dihydroxy acetone from glycerol: acid formed in glucose, lactose, sucrose, ethyl, propyl and butyl alcohols, but not in maltose, fructose, methyl, isopropyl, isobutyl or amyl alcohols. Only slight acid in glycerol.

Another strain could be tentatively identified as *Acetobacter viscosum*, again placed by Bergey *et al.*, in the appendix. This was described by Shinwell (1948) and characterised by the formation of ropiness or rendering the liquid media viscous. It produced also very slimy growth on solid media; thick white growth on Beijerinck's solid medium; clear ropiness in Hoyer's mineral solution; formation in 7 days of 2 cm. smooth moist and transparent colony on semi-solid medium and viscosity in liquid media containing ammonium cations as the sole nitrogen source are some other peculiarities of this species.

The third isolate belonging to this group agreed with some characteristics of *A. aceti* on the one hand and *A. roseum* on the other. Because of its distinct rose coloured colony formation on all media employed, it should be characterised as *A. roseum*. One strain utilized ammonium salts as sole source of nitrogen and formed dihydroxy acetone but we do not know if *A. roseum* actually does so. This was isolated from mango whereas the strain described in Bergey has been referred to have been isolated from fermenting mash of dried persimmons and figs and dates.

Group 4. Oxidise ethyl alcohol to acetic acid but not to CO$_2$; oxidise a large number of carbohydrates.

Of the five isolates in our collection placed in this group, two isolates from plum and banana respectively could be identified as *A. oxydans* whereas the rest secured from soil, neera (unfermented toddy) and berry (*Zizyphus rotundifolia*) were referred to as strains of *A. industrium*. It is necessary to point out here that *A. oxydans* has been given the primary status of a recognized species in *Bergey's Manual* whereas *A. industrium* is given only a secondary status in being placed among the 17 species mentioned in the appendix. Inasmuch as both are not well described in the *Bergey's Manual*, their characteristics are presented below.
Morphology.—The morphological form varied from the coccoidal form to rods nearly 2 μ in length, the cells being arranged singly, in pairs, or in short chains. Branched and swollen cell formation were observed with the 2 strains placed as *A. oxydans* but not with the other strains. All 5 strains were capsulated, non-sporulating, Gram negative and as a rule non-motile, though one strain each of *A. oxydans* (from banana) and *A. industrium* (from berry) in our collection exhibited slight motility. Though Bergey’s *Manual* describes *A. oxydans* as typically motile, this has been shown to be a variable character by Jorgensen (1939) and Shinwell (1948).

Growth on Beijerinck’s solidified medium.—All 5 strains showed poor growth on this medium, colonies being pin point and barely visible.

Growth on apple juice agar.—Round, smooth, glistening, opaque colonies with an entire to undulating margin. About 1–2 mm. in diameter. Four of the 5 strains showed a cream coloured to yellowish pigment, the strain of *A. oxydans* from banana being the only one showing no pigmentation.

Giant colonies on Beijerinck’s semi-solid agar.—Absent.

Growth in yeast extract broth.—The 2 strains placed as *A. oxydans* gave poor particulate growth while the three other strains gave a good uniform growth. In addition the strain isolated from berry produced a thin ascending film.

Growth in yeast extract—glucose broth.—Same as above with added turbidity.

Growth in yeast extract—ethyl alcohol broth.—Floccular growth and ring which is yellowish in the case of the *A. industrium* strains. Some strains also show a powdery deposit.

Growth in yeast extract—glucose—CaCO₃ agar.—Smooth, opaque, slightly raised glistening colonies ranging in colour from pale yellow to pink and orange.

Physiological characteristics.—Catalase positive, gelatine not liquefied; litmus reduced with the production of acid by all strains except *A. industrium* from soil which shows alkaline peptonisation and *A. oxydans* from banana which shows only reduction and peptonisation. No growth in Hoyer’s mineral solution. Ethyl alcohol tolerated upto a concentration of 5–7 per cent. (v/v), acid being produced in it after long incubation (5–20 days). Oxidation of ethyl alcohol incomplete. Glucose, maltose, sucrose, fructose, galactose, dextrin, arabinose, xylose and ethyl alcohol fermented with acid only. Lactose not fermented by one strain of *A. oxydans* (from banana) while others ferment it. One strain each of *A. oxydans* (from plum) and
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*A. industrium* (from berry) produce acid from propyl alcohol while isopropyl alcohol is not fermented by any strain. *A. oxydans* from plum also produces acid from butyl alcohol but not isobutyl alcohol while the strain from banana produces slight acid from isobutyl alcohol and none from butyl alcohol. All 3 strains of *A. industrium* fail to produce acid from butyl alcohol but the strain from neera and berry do so from isobutyl alcohol. Dihydroxy acetone was produced from glycerol by the 2 strains placed as variants of *A. oxydans* on this account. Traces of dihydroxy acetone were also produced by the strains of *A. industrium* isolated from neera and berry.

**Group 5. Produce acid and gas from carbohydrates.**

In this group showing the unusual property of producing gas from some carbohydrates were placed six strains from one collection isolated from such diverse sources as spoilt beer, soil, orange, apple and *drakshasa* (grape liquor). Inasmuch as no strain of *Acetobacter* is known to form acid and gas from carbohydrates or alcohols, our strains could not be placed in any of the known species and are described in detail here.

**Morphology.**—Cylindrical to ellipsoidal rods (average size 1–2 μ/0.5–0.8 μ or 0.5–0.6/0.7–0.9 μ) occurring singly and in pairs. Swollen cell formation shown by 4 strains only (from 2 soils, orange and apple) Gram negative, non-motile and non-sporulating.

**Growth on Beijerinck’s solidified medium.**—Round, smooth, moist, glistening translucent colonies with an entire margin, about 1–2 mm. in diameter.

**Growth on apple juice agar.**—Luxuriant growth. Colonies within 20 hours round, smooth, moist glistening and slimy with an entire margin and 1–3 mm. in size.

**Giant colonies on semi-solid apple juice agar.**—Smooth, slightly raised glistening, semi-transparent colonies varying in size from 0.5–3.2 cm. The strains from Nasik soil and *Drakshasa* produced more opaque colonies.

**Growth in yeast extract broth.**—The strain from spoilt beer showed only a heavy floccular deposit at the bottom while all other strains in addition showed growth with a tendency to ascend the glass wall.

**Growth in yeast extract–glucose broth.**—As above, but heavier growth and brownish coloured sediment.

**Growth in yeast extract–ethyl alcohol broth.**—White film or ring with a tendency to ascend glass wall. Uniform turbidity and floccular deposit.
Growth on yeast extract—glucose—CaCO₃ agar.—Abundant to moderate smooth moist, growth with gas bubbles at the butt of the slant.

Physiological characteristics.—Catalase positive; gelatine not liquefied; litmus reduced with acid and soft curd; good growth in Hoyer's mineral solution; alcohol tolerance ranged from 6-10%, acid being produced after a time. Oxidation of ethyl alcohol, incomplete. Acid and gas produced from glucose, lactose, maltose, sucrose, fructose, mannitol and glycerol; and acid only from ethyl alcohol. The strains from Nasik and Bombay soils produced no acid from propyl, isopropyl, butyl and isobutyl alcohols. Strains from spoiled beer, orange, apple and drakhsasa produced acid from butyl and isobutyl alcohols. In addition, the isolates from orange and drakhsasa also produced acid from propyl and isopropyl alcohols. A trace of dihydroxy acetone was produced by isolates from spoiled beer and Bombay soil whilst all other isolates did not do so.

The production of acetic acid by these 6 strains was confirmed by steam distilling the fermented liquid, neutralising the distillate with sodium hydroxide, evaporating to dryness, dissolving the dried mass in a small quantity of water and adding neutral FeCl₃ to it. The formation of a brown precipitate of basic ferric acetate on boiling confirmed the presence of acetic acid. Inasmuch as bacteria other than Acetobacter, such as the heterofermentative lactic acid bacteria, Coli-aerogenes group of bacteria and certain anaerobic sporeformers are also known to produce acetic acid, additional tests such as the methyl red and Voges-Proskauer tests were also carried out and found to be negative. Gas production was maximum in 1 week (0.8–2 ml.) from 10 ml. of liquid medium and the nature of the medium and age of culture had some effect on the amount of gas produced. The formation of CO₂ from glucose under certain conditions by A. oxydans (Butler, 1936, 1938) and from arabinose and xylose by A. xylinum and A. sorbose (Fred et al., 1923) has been reported in the literature but the fermentation of a large number of sugars and alcohols with the consistent production of gas has not been so far attributed to Acetobacter species, and deserve further investigations.

Summary

A study of 30 strains of Acetobacter species from diverse sources such as air, soil, various fruits, fermented liquors and animal excreta was attempted. By a detailed characterization of the isolates, they could be divided into 5 groups and identified as species of A. aceti, A. xylinum, A. pasteurianum, A. melanogenum, A. industrrium, A. oxydans, A. aceti viscosum, A. aceti roseum, A. rancens and their variants. Detailed descriptions of the less known species
are presented to supplement the information available in appendix to the genus given in the *Bergey's Manual*. The description of a group of 6 strains which possess the unusual property of fermenting several carbohydrates with the production of acid and gas—a property not thus far ascribed to *Acetobacter* species—is also given.

**References**


