STUDIES IN INTENSIVE BACTERIAL OXIDATION.

I. THE OXIDATION OF ALCOHOL TO ACETIC ACID.

By Gilbert J. Fowler and V. Subramaniam.

INTRODUCTION.

The conversion of alcohol into acetic acid is one of the simplest bacterial oxidations. It finds apparently an exact analogy in the conversion of alcohol to acetic acid by the action of oxygen in presence of platinum black, a discovery made by Edmund Davy [1] in 1821. So clear did this seem to be that the so-called 'quick vinegar process' in which alcohol is allowed to trickle over beech shavings with production of acetic acid was thought to be due to the purely physical effect of the extended surface of the shavings.

Pasteur [2] in 1862 showed the true cause of the fermentation to reside in the living organisms which form as a fine film on the surface of alcoholic liquids, and which had been termed 'mycoderma' by Persoon in 1822.

The 'quick vinegar process' bears a close analogy to the 'percolating filter' used in purifying sewage, in which one of the chief reactions is the oxidation of ammonia to nitric acid. Just as there are many side reactions taking place in the 'percolating filter' due in part to worms, insects and other living organisms, so in the 'quick vinegar process' considerable loss takes place, due to the development of vinegar eels, mites, etc. Moreover in both cases it is difficult exactly to distribute the liquid over each portion of the oxidizing surface, and consequently further loss occurs, in the case of the 'percolating filter' through reduction of the nitrates formed, and in the case of the 'quick vinegar process' through the oxidation of acetic acid to carbon dioxide.

[1] *Schweigger's Journal*, 1821, i. 340.
[2] *Compt. rend.*, 1862, 265.

Naturally the possibility suggested itself of accelerating the process of acetification by methods analogous to those used in the 'activated sludge' method for accelerating nitrification.

The method would consist in inoculating a suitable medium with acetifying organisms, and causing them to move through the liquid by a current of air. On acetification becoming complete, the bacteria would be allowed to subside or otherwise be removed from the liquid, and a fresh quantity of medium added to the bacteria thus retained. By a repetition of this process it was hoped eventually to accumulate a mass of bacterial growth which would bring about acetification very rapidly. In practice sundry difficulties were encountered. The conditions were not strictly comparable with those which obtain in the purification of sewage. In the latter case albuminoid matter is present and easily clots in the course of the process, affording a large surface for bacterial action. The alcoholic or saccharine medium used in the acetification process did not yield a colloidal precipitate of this nature. A remedy for this difficulty was sought by adding an artificial colloidal precipitate, viz., alumina cream, but the only effect of this appeared to be that the bacteria were enveloped in the precipitate and their action impeded. Precipitated carbonate of lime was then added, in the hope that the acetic acid produced would be neutralized as formed with ultimate production of concentrated calcium acetate; but, although it is stated that nitrifying organisms can withstand a 20 per cent. solution of potassium nitrate, it was found that concentrations of calcium acetate exceeding 2 per cent. were toxic to the acetifying organism in spite of efforts to acclimatize it to such conditions.

Further, of the various types of acetifying bacteria utilised, it is now evident that some were not suited for such a method, as they formed tough thick pellicles almost like flannel in appearance, which were not broken up on stirring with air.

Better results were obtained with an organism isolated from fermented toddy. The varieties of acetifying bacteria and their morphological changes under varying conditions are so great, that it is unwise to give a definite name to the organisms used, but it appears most probable that the organism forming thick wrinkled pellicles was allied to *B. Xylinum*, described by Adrian Brown,[1] while the organism eventually selected for the experiments described in this paper is a variety of *B. Aceti*.

It may be mentioned that although high strength acetic acid was not obtained in these early experiments, yet the experience gained

[1] *J. Chem. Soc.*, 1886, **99**, 172.

enabled a very fair vinegar of 5 per cent. strength to be produced by the ' quick vinegar process ', operated on a moderate working scale by Mr. Inuganti in Hyderabad, based on the preliminary experiments conducted by him in the Institute.

EXPERIMENTAL.

The present researches were taken up in continuation of some preliminary technical experiments made in the industrial laboratory at Coonoor by one of us (V. S.), under the supervision of Sir Frederick Nicholson. It was there found that better and more easily controlled results were obtained by the Pasteur slow vat process than by the ' quick vinegar ' or continuous process. In accordance with the general experience already referred to, the bacterial condition of the percolaters in the latter process is difficult to control. On transferring the inquiry to Bangalore, the various factors of a satisfactory yield by the slow process were systematically investigated, the effect of forced aeration was then tried, and ultimately concentrations of 10 per cent. and upwards were obtained in quantity.

In general terms the method consisted in building up considerable masses of a suitable culture, first in large test tubes, then in flasks, from which it was transferred to a large bell jar and finally to a ten gallon cask. Suitable methods of aeration and heating were devised as occasion required.

The experiments fall under the following heads :—

1. Preparation of satisfactory cultures of bacteria.

2. Small scale experiments on the effect of the following factors :—

   (*a*) Variation in proportion of inoculant.

   (*b*) Increase of scale of experiment, the proportion of inoculant and medium being retained.

   (*c*) Increase in concentration of alcohol.

   (*d*) Variation in air supply.

   (*e*) Presence of catalysts.

3. Larger scale experiments made on the basis of the foregoing results.

4. Miscellaneous observations.

I. PREPARATION OF SATISFACTORY CULTURES OF BACTERIA.

A large number of organisms have been found capable of oxidizing ethyl alcohol to acetic acid. Compounds such as hexoses which will yield alcohols by simple decomposition can also be acetified by such organisms. How far all of these should be looked upon as different species in a biological sense it is difficult to decide. It is frequently the case that strains developed from a pure culture display greater difference in chemical effect than members of different morphological species. From the point of view of the biochemist the organism is to be looked upon as a more or less effective chemical agent, and his efforts are directed to producing a maximum concentration of enzymic activity, the precise name or biological characteristics of the enzyme-carrier being of secondary moment.

There are, however, four main types of acetifying bacteria, viz. *B. aceti*, *B. Pastorianum*, *B. Kutzinianum*, and *B. Xylinum*. Of the first three, the cells of *B. aceti* are smaller and narrower than those of the other two, and the film which it forms is easily broken. It, therefore, lends itself most readily to a process in which the organism is diffused evenly throughout the medium.

B. Xylinum forms a very tough pellicle, sometimes resembling white flannel, and the early experiments of Mr. Inuganti showed it to be unsuitable for producing rapid acetification.

B. aceti was found by Mr. Inuganti to grow in sour toddy and therefore an attempt was made to isolate a pure culture from this liquid. About 20 cc. was taken in a small beaker and incubated at 35° for 1½ days; microscopic examination then showed the presence of yeasts and micrococci, as well as bacteria which agreed in appearance with the descriptions of *B. aceti* and *B. Xylinum*.

An unsuccessful attempt to subculture these was made in the following manner. A medium was prepared composed of alcohol (2 per cent. by volume), acetic acid (2 per cent.), and ·05 per cent. phosphates of calcium, magnesium and ammonium. The medium was made up with sterile water, incubated at 35° for seven days to confirm its sterility, and then divided among a number of sterile test tubes, plugged as usual. Another series of sterile tubes were prepared containing a 2 per cent. solution of agar. By inoculating the alcohol tubes from the toddy medium and pouring the melted 2 per cent. agar into the inoculated tubes, it was possible to obtain agar plates of the above medium without sterilising the whole at a high temperature. The agar plates thus prepared showed the development

of colonies in less than 24 hours. By a repetition of this process what appeared to be pure colonies were obtained, but on further microscopic observation it was found that even after nine subcultures *B. Xylinum* was still present together with *B. aceti.*

In consequence of this, toddy was abandoned as raw material, and instead the following medium containing ordinary vinegar was used :—Alcohol, 4 per cent. by volume. Acetic acid, 2 per cent. (vinegar containing acetic acid to this amount). Phosphates of calcium, magnesium and potassium, 0·05 per cent. Yeast water, 10 cc., prepared by boiling 7 per cent. by weight of dry yeast with water, and filtering until quite bright. The whole was made up with sterile water to 100 cc.

25 cc. of this mixture was exposed in a small flat beaker in the incubator for two days at 35°. Growths formed and on microscopic examination revealed the presence of *B. aceti* in characteristic form together with some other unknown bacteria. From this mixture *B. aceti* was cultivated in the manner attempted with the toddy cultures, and in this case the operations were more successful, the 9th subculture being pure.

A number of test tubes containing the liquid alcohol acetic acid medium was inoculated with the organism thus isolated, and incubated at 30°. Vigorous growth was obtained after about five days, developing on the sides of the test tubes. 10 cc. of this culture was then mixed with 100 cc. of the medium, and after about ten days a bacterial deposit was formed approximating to 1/10 of the volume in the flask. The whole was again mixed with a further 500 cc. of medium and so on till a large quantity of the organism was obtained as a clay coloured deposit which was used in the experiments to be described later.

Nutritional Requirements of the Acetifying Organism.

In the course of the preparation of the cultures used in the work, a number of observations were made as to the most suitable nutritive material for the bacteria. It should be understood that the requirements vary as the object in view is to produce growth in bulk or to encourage functional (i.e. acetifying) activity. The former condition obtains when preparing large masses of active material prior to production of acetic acid, the latter as soon as acetification begins.

Mineral phosphates such as those of calcium, magnesium and ammonium in presence of suitable quantities of glucose have been

found to increase the growth activity at the expense of the acetification. Accordingly phosphates of calcium and magnesium have been omitted in conducting acetification experiments and the proportion of ammonium phosphate and glucose has been reduced to the minimum necessary to prevent starvation.

Microscopical examination of the mixture.

B. aceti thus obtained forms a pellicle when inoculated upon the surface of a suitable medium. Its microscopical appearance under these circumstances is quite different from its appearance when obtained as a deposit by the procedure indicated above.

The bacteria forming the pellicle appear under the microscope as masses of cells, about 2μ in length, slightly contracted in the middle giving them a figure of eight appearance. These cells are united into chains of variable length, easily broken up by pressure of the cover glass. Frequently the cells are completely divided in the middle thus producing strings of micro-coccus-like forms; both forms of cells are sometimes found in the same chain. The bacteria occurring in the deposit develop much longer chains as well as long cells, which often swell out at one or two points along their length, giving them a very irregular appearance.

The above observations agree well with the drawings of *B. aceti* by Hanssen. The cell membrane of these bacteria when treated with dilute solutions of iodine, is stained yellow. The organism grows on glucose, peptone and starch peptone solutions, but does not liquefy gelatine, nor develop to any extent upon it.

In the course of the examination of the growths above mentioned occurring in fermenting toddy, characteristic cultures of *B. Xylinum* were obtained which agreed closely with the description given by Adrian J. Brown. The further study of this organism is reserved for another communication.

II. ACETIFICATION EXPERIMENTS.

The dependence of satisfactory yields upon approximately pure cultures is clearly seen from a number of preliminary experiments carried out with some of the impure cultures obtained from toddy.

Experiments were carried out in half-litre Erlenmeyer flasks containing 250 cc. of the alcohol-vinegar medium above mentioned. The flasks were incubated at 30–32° in an improvised incubator

consisting of a deal box heated by an incandescent electric bulb. The acid formed was determined by withdrawing 5 cc. of the fermented liquid, and titrating, after diluting with water against decinormal sodium hydroxide, using phenol-phthalein as indicator. This method is only approximately accurate but was sufficiently satisfactory for preliminary comparative tests. The results are summarised in Table I, from which it is seen that the purer the culture, the more rapid is the oxidation of the alcohol, thus the original mixed culture required thirteen days to reach the limit of acetification, which was reached by the third subculture in ten days, and by the sixth subculture in nine days. With increased purity of culture much better results were obtained.

Experimental Results in Absence of Artificial Aeration.

Experiments were undertaken to determine the conditions for maximum acetification with pure cultures prepared as described on p. 151.

For this purpose one-litre Erlenmeyer flasks were employed, well sterilized and plugged. . . . The medium to be acetified was made up as follows:—Alcohol by volume, 2-3-or 4 per cent. as the case may be, ammonium phosphate 0·01 per cent. glucose 0·05 per cent. bacterial emulsion 1/10 of total volume, the whole made up with sterile water to 500 cc.

The quantity of bacterial deposit or emulsion used in each experiment was determined as follows:—The bacterial deposit in the culture flasks was allowed to settle, the supernatant liquid decanted, the bacterial deposit well shaken, the required quantity withdrawn by means of a sterile pipette, and added to the medium under investigation. The plugged flasks were kept in the incubator, maintained at 30–32°. The flasks were covered with dark cloth to protect the contents from light.

Daily estimations of acid were made in the following manner:—5 cc. of the solution were removed and diluted with 50 cc. of distilled water boiled for some time under a reflux condenser to remove carbon dioxide, and then titrated against decinormal caustic soda, using phenol-phthalein as indicator. It was found later that this method of estimation was not entirely accurate, and more exact but more convenient methods of analysis were adopted. For comparative tests, however, it was useful and sufficient. It was found that a critical point exists for the addition of alcohol, beyond which if alcohol is not added oxidation to carbon dioxide takes place; alcohol was accordingly added as this became necessary.

Alcohol in the solution was determined as follows :—the acid in 10 cc. was neutralized with decinormal sodium hydroxide, and 60 per cent. of the liquid distilled at 90°, the distillate being made up to 100 cc. re-distilled, 40 cc. of distillate collected and made up to 100 cc. and 20 cc. of this liquid oxidized with half-normal potassium bichromate and sulphuric acid by heating for three hours on a waterbath under a reflux condenser ; the acetic acid was steam distilled, and the distillate titrated with decinormal sodium hydroxide and phenolphthalein. This method was found to be more accurate than determination of the alcohol by conversion to iodoform or by specific gravity, the error in blank experiments not exceeding 6 per cent. of the alcohol present as against a possible 10 per cent. error by these methods.

The results of the acetification experiments are given in Table 1*a*, in which :—

1. The alcohol quantities have been uniformly corrected to actual parts by volume, i.e., number of cc. in 100 cc.

2. The initial acidity of the culture medium, viz., about 0·5 to 0·7 per cent. has been subtracted in all cases, and the quantity of the acid given is that obtained from the alcohol added.

3. Correction has been made for slight quantities of alcohol in the culture medium added along with the bacterial emulsion.

4. The quantities of acetic acid are all given in grams per 100 cc.

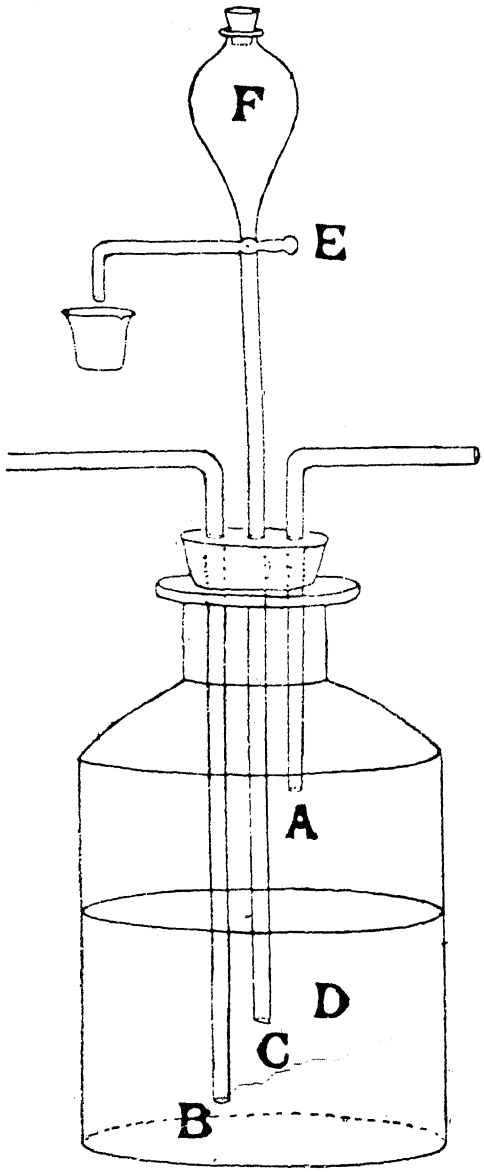
5. As small quantities of liquid were taken out at intervals for acid estimation, the percentage of added alcohol was calculated upon the quantity of medium actually present in the flask when the alcohol was added.

6. The yield of acetic acid obtained was calculated by noting the total quantity of alcohol added and determining the alcohol remaining unoxidized, the difference being the alcohol actually used. Thus, let there be $x$cc. in 100 cc.—then the sp. gr. of alcohol at 25° being 0·785, the weight of $x$cc. of alcohol is $x \times 0{\cdot}785$ gram. The yield of acetic acid from this amount of alcohol $= \frac{x \times 0{\cdot}785 \times 60}{46}$ gram. The percentage yield, if y represents the actual yield, is

$$\frac{y \times 46 \times 100}{x \times 0{\cdot}785 \times 60}$$

In the experiments set out in Table IIa, the chief factors studied were, the effect of increase of the concentration of alcohol, and the effect of the presence of catalyst.

DIAGRAM A



Experiments VII, and VIII show the effect of increasing the concentration of alcohol. It will be seen that while the initial alcohol present varied from 2 per cent. to 4 per cent., the rate of oxidation did not vary, and the strength of acid ultimately was the same, neither was there much effect noted if 2 per cent. is added in the middle as in Experiment VI. Other experiments showed that an initial concentration of more than 4 per cent. alcohol was prejudicial.

The effect of the addition of catalysts is seen clearly from experiments IV and V. The addition of ethyl acetate although not increasing the rate of acetification, enabled the process to be extended to a greater eventual maximum acidity. The addition of manganese sulphate on the other hand, both increased the rate of acetification and the acid strength ultimately obtained.

Experiments using forced aeration.

In experiment IX, Table II,[1] it will be seen that twice the usual volume both of inoculant and medium was used, the experiment, in other respects, being on the same lines as number V which gave a high yield of acid. In the case of experiment IX, however, the increased volume of liquid filled up the flask so that only a small surface was exposed to the air. The lack of adequate oxygen under these conditions is clearly seen in the results, the acetification ceasing after seven days at a maximum of 2·7 per cent., as compared with the production of over 7 per cent. in nine days in experiment V.

It was also evident from observation of sundry cultures kept without regard to aeration, that if large quantities of inoculant were to be used, it would be necessary to introduce air artificially in order to prevent the putrefaction of the bacterial masses.

The conditions of maximum efficiency to be aimed at would therefore be the employment of the highest quantity of inoculant which could be kept active by adequate food supply, and sufficient oxidation to prevent putrefaction, or even the production of intermediate products, but insufficient to encourage superoxidation to carbon dioxide.

Method of experiment.—The apparatus employed for this section of the work was similar to that used by Beesley in his experiments on nitrification,[2] and enabled samples to be withdrawn aseptically for examination during the course of the experiments. The apparatus is shown in Diagram A. D is a two-litre wide-mouthed bottle fitted with a rubber stopper through which were inserted three tubes, A, B and

[1] See graphs opposite page 165.
[2] *J. Chem. Soc.* 1914, 105, 1914.

C; A reaching to just below the stopper, B to the bottom of the bottle and C to just below the surface of the liquid. Tube C was connected to a three-way tap, E, provided with a bulb, F, by means of which samples could be withdrawn. A was connected to a filter pump by way of two wash-bottles containing concentrated (15 per cent.) caustic soda, in which any carbon dioxide or volatilised acetic acid could be absorbed. The air entering by B was first washed by passing through caustic potash, followed by lime-water to make certain that no carbon dioxide entered the apparatus. The end of the tube B was drawn out to a narrow jet so as to admit the air in a fine stream, cotton wool plugs were inserted at the inlet and outlet of bottle D, so that the whole could be sterilised at the outset of an experiment.

The medium used was the following:—Alcohol, 4 per cent. by volume; ammonium phosphate, 0·01 per cent.; ethyl acetate, 0·01 per cent.; glucose, 0·05 per cent; made up to 1000 cc. with distilled water.

The amount of bacterial emulsion added differed in different experiments. The bottle was placed in the incubator, in which the temperature from October, 1921, to January, 1922, ranged from 30° to 33°, and was protected by a dark cloth from the light of the electric bulb used for heating.

The quantity of air admitted could be roughly adjusted by observing the rate of passage of air bubbles, and controlling the filter pump accordingly. The results of these experiments are given in Table II*b*. In each case controls were kept under the same conditions, but without the presence of bacteria. As the amount of acetification in these controls was negligible, the maximum being 0·15 per cent., no correction was made on this account in calculating the amount of acid formed.

The first three experiments in Table II*b* indicate the effect of the proportion of inoculant on acetification, aeration remaining the same. In the first experiment it would appear that not enough inoculant had been added. In the second excellent results were obtained, while in the third there is a sign that the air supply is insufficient to maintain the culture in a healthy condition, the culture forming a sediment.

In Experiment XIII the quantity of medium was increased, the aeration remaining the same. The bacteria being diffused through a greater volume of liquid the proportion of sediment was diminished, and improved acetification resulted. It was only, however, on doubling the quantity of air (Experiment XIV) that the entirely satisfactory

results of Experiment XI were again obtained. It is clear that, as in Table II*a*, aeration must be increased in proportion to the total volume of the liquid. A further increase beyond this critical quantity again results in superoxidation (See Experiment XV). In the course of these experiments observation showed that, as might be expected on general grounds, increase in the quantity of growth in proportion to the medium in which it was diffused, was accompanied by an increased tendency to clumping; this is a point of practical importance.

Estimation of volatile acid and of carbon dioxide.—In the course of the acetification process some loss occurs owing to superoxidation to carbon dioxide and to volatilization of some acetic acid. It is important that these losses should be carefully estimated, the determination especially affording a means of scrutinizing the progress of the reaction. The carbon dioxide and acetic acid passing off from the acetification process were estimated in the following manner.

The air passing from the apparatus was led into gas washing bottles containing standard sodium hydroxide, the strength of which was estimated at the beginning of the experiment with decinormal sulphuric acid, using phenolphthalein as indicator. At the close of the experiment the alkali in the washing bottles was made up to 500 cc. with water free from carbon dioxide. A portion of this solution was again titrated with decinormal acid, this titration giving the value of the total alkali neutralised by carbon dioxide and by acetic acid.

The carbon dioxide was separately determined as follows:—10 cc. of the alkaline solution was taken, the carbon dioxide precipitated by freshly prepared baryta solution, and the precipitate immediately filtered, washed with water free from carbon dioxide and transferred to a weighed crucible. It was then ignited, cooled, treated with a few drops of sulphuric acid and finally ignited and weighed as barium sulphate. From this the amount of carbonate precipitated was calculated, and this amount subtracted from the total titration, gave the quantity of alkali neutralised by the acetic acid. The figures of the acetic acid determination were checked by taking 10 cc. of the liquid, treating with an excess of pure barium carbonate, and boiling to remove all carbon dioxide. The solution was then filtered and the filtrate treated with 30 cc. of a 20 per cent. solution of glacial phosphoric acid, steam distilled, and the acid distillate titrated with decinormal caustic soda and phenolphthalein.

It was found that the production of carbon dioxide coincided with the disappearance of alcohol from the acetifying medium, and thus served as a useful indication of the progress of the reaction.

The loss of acetic acid by volatilisation was found to be very small. The total loss, amounting to about 10 per cent., is probably to be explained by (*a*) superoxidation, (*b*) alcohol volatilisation, (*c*) subsidiary fermentation. Factor (*c*) can be satisfactorily determined only by analysing the products of a large scale fermentation.

III. EXPERIMENTS ON A LARGER SCALE.

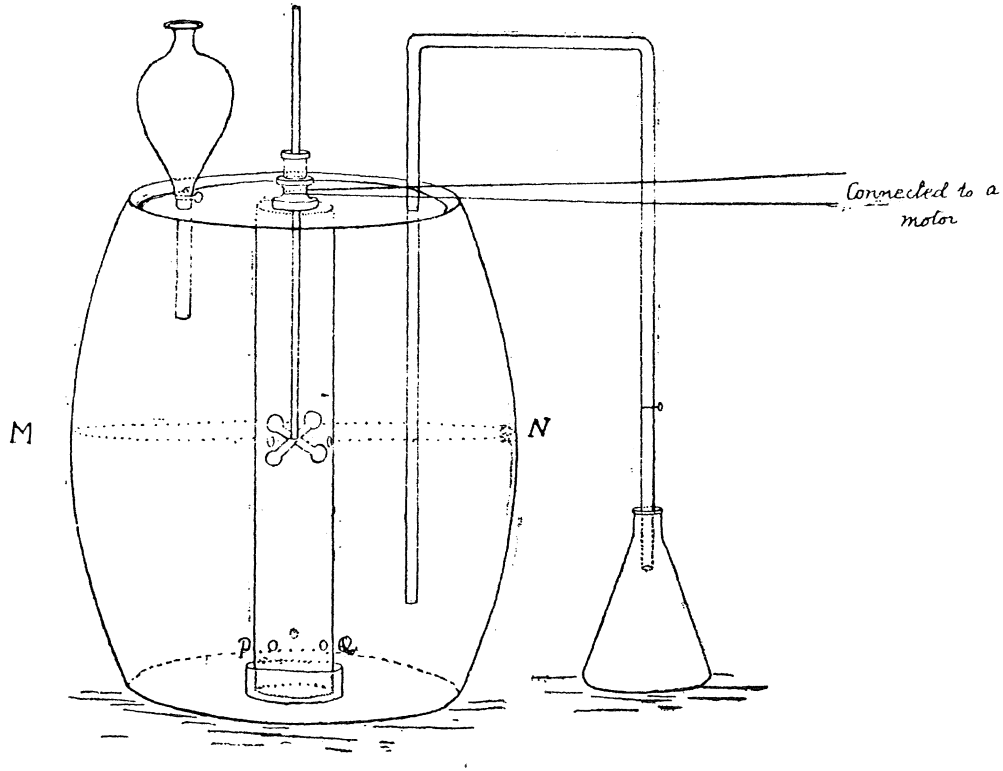
The foregoing experiments having shown the necessary conditions for obtaining satisfactory results, the scale was increased from a volume of one litre to five litres by employing a large inverted bell-jar with a lid made of aluminium provided with the necessary orifices. Arrangements were made for adding the alcoholic medium and siphoning off the acetic formed, and also for admission and escape of air. In this case the air was blown from a large gasholder through a finely drawn out glass tube.

The whole bell-jar and its connections was sterilised by heating for one hour at 5 lbs. pressure. It was then enclosed in a dealwood case heated by means of an electric bulb, the bell-jar being protected from light by means of a dark cloth. The temperature varied from 30° to 31·5°.

Two sets of observations were made (Table III). In the first it would appear that the volume of inoculant was hardly sufficient to obtain the best results as only about 85 per cent. of the total alcohol added was acetified, and the acid strength obtained was only 7 per cent. With increased proportion of inoculant, 86.4 per cent. of the alcohol was acetified with production of an eventual acid concentration of 9·6 per cent. as in Expt. II. Table IV.

The next step was to make use of a 10 gallon tub. Here the arrangement for heating and aeration was not quite so simple as when small vessels were used. In order to avoid the use of an air compressor for forced aeration a device was adopted on the lines of one recently employed for the treatment of sewage by means of activated sludge. A hollow wooden cylinder was fixed in the centre of the barrel by means of a wooden disc which was screwed up in the centre of the bottom of the barrel. Holes were bored in the cylinder below the surface of the liquid at M, N, and also round the portion of the cylinder just above the bottom of the barrel at P, Q. On revolving a wooden paddle vertically within the cylinder and just below the surface of the liquid, an excellent circulation was set up. Trials made with alumina-cream showed that a sediment of this material was finely distributed through the liquid in a few seconds, consequently

DIAGRAM B



no difficulty was anticipated in circulating the bacterial deposit, and in fact, none was experienced. Moreover, determinations of dissolved oxygen made during the actual acetification process always showed an ample supply to be present. The paddle was driven by a ¼ H.P. motor. A separating funnel and a siphon arrangement surmounted the barrel as in Diagram B. The wooden cover was quite air-tight and two or three holes were bored and closed with a cotton plug, the whole being steamed continuously for three days and then washed before the experiment began.

The results of the trials with this apparatus are given in Table III. Expts. III and IV. The first experiment was made without artificial heating to avoid the necessity for this if possible. Unfortunately under these conditions slimy growths made their appearance, attaching themselves to the sides and other projections in the barrel, and the production of acid ceased at 6.9 per cent. Later experiments showed that this growth tended to oxidise the acetic acid to carbon dioxide.

Accordingly, arrangements were made for heating the liquid. The obvious difficulty of using a steam coil under such conditions was overcome by the employment of a simple electrical device for heating the liquid. The length of nichrom wire calculated to develop the necessary temperature (30–35°) was 16 feet of diameter ·02 in. This was wound on two mica plates, 9 inches by 4 inches, which were sandwiched between two plain mica plates, the whole being held in a wooden frame, the grooves of which were filled up with a cement of magnesia, plaster of Paris and asbestos flour over which after it was in position, a thick solution of silicate of soda was finally poured. This mixture was found to be unattacked by the acid solution, the frame being immersed for 7/8 of its length. The free ends of the nichrom wire were connected to two terminals above the surface of the liquid and mounted on an extension of the framework holding the coil. The terminals were connected by flex to a wall plug. The temperature of the liquid heated by this arrangement varied from 30.7° to 32.5°, which approximates to the optimum temperature for acetification.

After installing this arrangement, and re-inoculating with culture as free as possible from the slimy growth already mentioned, good results were obtained as shown in experiment IV, Table III, where a strength of acid of 9 per cent. was reached, taking into consideration the initial acidity of 0.5 per cent.

After this the attempt was made to make the process as nearly continuous as possible by frequent addition of small quantities of the alcoholic medium, care being taken to keep the level of the liquid in

the barrel up to the holes bored in the central cylinder. To maintain this condition an amount of liquid was withdrawn equal in volume to the liquid to be added. The results of such continuous experiments are recorded in Table IV.

IV. MISCELLANEOUS OBSERVATIONS.

The Bacterial Oxidation of Acetic Acid.

General experience shows that in the absence of alcohol the acetic acid formed in the acetification process is itself oxidised to carbon dioxide and water. This process was systematically examined by stopping the addition of alcohol after a certain acidity was reached, and continuing the aeration as before. Determinations of acid and carbon dioxide showed that the former decreased in proportion to increase of the latter.

Experiments were carried out under artificial aeration. The initial acid concentration varied with different experiments, and the results are set out in Table V, from which it will be seen that the rate of decrease in acid concentration is greater when this is high, becoming less as the acid concentration decreases.

Study of the Slimy Growth.

As already mentioned in the first barrel experiment it was observed that after an acid concentration of 6.9 had been reached a white slimy bacterial deposit was formed on any support available in the interior of the barrel, and acetification ceased, although alcohol was present to the extent of 1 per cent fermentation liquor. Further aeration for one or two days resulted in a decrease in acidity to 6·7 per cent.

This trouble, as described on page 159, was overcome by re-starting the process with a fresh culture at a higher temperature. At the same time the opportunity was taken to investigate the slimy growth, from which the liquid was first decanted; it was then scraped into two sterile Petri dishes, washed well with sterile water and transferred to a medium of acid-alcohol and water, in which it could be examined at leisure. Under the microscope the white mucilage was seen to consist of regular films and chains with occasional protuberances, but could not be referred to any of the well-known types of acetic bacteria. It is likely that the growth consists, partly at any rate, of involution forms such as are described by Janke.[1]

[1] *Centr. Bakt. Par.*, 1916, **45**, 1; 1921, **53**, 81.

Several experiments have been carried out to determine the effect of this growth on acetic acid, and the results are given in Table VI. It is clearly seen that the activity of the organism is solely concerned with the oxidation of acetic acid to carbon dioxide, as even in the presence of as much as 4 per cent. of alcohol there is no sign of acetification taking place.

Janke (*loc. cit.*) has discussed the conditions of formation and growth of these mucus-like forms. He concludes that their production is favoured by (*a*) a temperature of 15–28° : (*b*) admission of air : (*c*) excess of nitrogen compounds. Our own experience leads to similar conclusions. The best remedy for the development of these growths lies in prevention rather than cure. If the temperature of the medium is kept in the neighbourhood of 32° from the outset these bacteria which grow best at this temperature and which are the most active acetifiers are favoured at the expense of the mucus-formations.

Experiments on the effects of 'Activated Silt'.

As already indicated, one of the chief difficulties in the intensive method of acetification is the building up rapidly of a sufficient volume of bacterial growth. The use of artificial colloidal precipitates such as alumina cream to obtain an artificial surface on which the bacteria can develop was not found satisfactory. As already mentioned, the precipitate seemed to smother the bacteria. It was thought that finely divided silt, such as was used in the experiments on water-purification by one of us (G.J.F.) and R. R. Deo, might offer the extended surface required. Accordingly, through the courtesy of a former student in the Institute, Mr. Edal-Behram, now chemist at the Shanghai Water Works, we received from the Whang Poo at Shanghai several pounds of silt which consists chiefly of fine light particles of mica, remaining suspended for a very long time, and through forming a nidus for the necessary bacteria may be a cause of the rapid self-purification of Chinese rivers.

About 500 grams of the silt as received was well washed with tap water and drained. An equal amount of water was then added, and the whole shaken and allowed to settle, when a colloidal suspension of fine particles of mica was thus obtained in the supernatent liquid. This was used instead of water in making up the acetifying media, and several trials were made with it. No increase however was observed in the rate of oxidation. It was also noticed that after it had been used a number of times, the silt settled rapidly bringing down the bacteria, and thus acted as a coagulating rather than dispersing agent. It would appear that the acidity of the solution

was adverse to the maintenance of the collodial suspension, consequently it is not possible to use silt for accelerating acetification as for assisting nitrification, the solution in the latter case being slightly alkaline.

Effects of Certain Salts on Acetification.

Attention has already been called (p. 148) to the fact that calcium acetate in concentrations beyond 2 per cent. had a retarding effect on acetification. Inasmuch as other salts such as manganese acetate had an accelerating effect, it would seem likely that the retarding effect of calcium acetate depends on the calcium ion. This conclusion is confirmed by experiments with acetates of calcium and ammonium and with calcium citrate, calcium acetate being the most toxic of the three salts. These results are summarised in Table VII.

SUMMARY AND CONCLUSIONS.

1. Pure cultures of acetifying bacteria have been prepared by combined plating and dilution methods, using as culture medium a suitable mixture of alcohol, acetic acid (vinegar) and mineral salts. Large volumes of these cultures have been built up by inoculation into increasing volumes of liquid. The nutritional requirements of the organisms have been carefully studied.

2. The oxidation of alcohol to acetic acid by intensive bacterial action has been quantitatively studied. The accelerating effect of certain catalysts, particularly ethyl acetate and salts of manganese has been observed.

3. The beneficial effect of forced aeration on the acetification process has been clearly shown. There is, however, a critical limit to the quantity of air used per given volume of liquid, depending on the amount of bacterial growth and of alcohol present. Excess results in superoxidation of the acetic acid formed, while a deficiency delays oxidation and interferes with the healthy growth of the organism.

4. It has been found that up to 4 per cent. the course of acetification is independent of alcohol concentration.

5. The process of acetification by forced aeration in presence of masses of the necessary bacteria has been operated successfully up to a volume of 5 gallons of liquid of 8.8 per cent. acidity, 8 per cent. by volume being acetified daily.

6. In order to protect the culture from extraneous growths it has been found necessary to maintain the liquid at a temperature approximating to 32°.

7. The oxidation of acetic acid to carbon dioxide by bacterial action has been systematically studied. The reaction appears to proceed quantitatively, and at a rate which increases with the increase of initial concentration of acid.

8. The action of extraneous organisms, 'mucous' bacteria, on the acetification process has been studied, and appears to consist mainly in oxidising acetic acid to carbon dioxide even in presence of sufficient quantities of alcohol.

9. The influence of 'activated silt' on the process has been examined, but has not been found advantageous.

10. The effect of calcium acetate on the reaction has been studied in comparison with other salts, and its marked inhibitory effect is found to depend on the calcium ion.

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TABLE I.

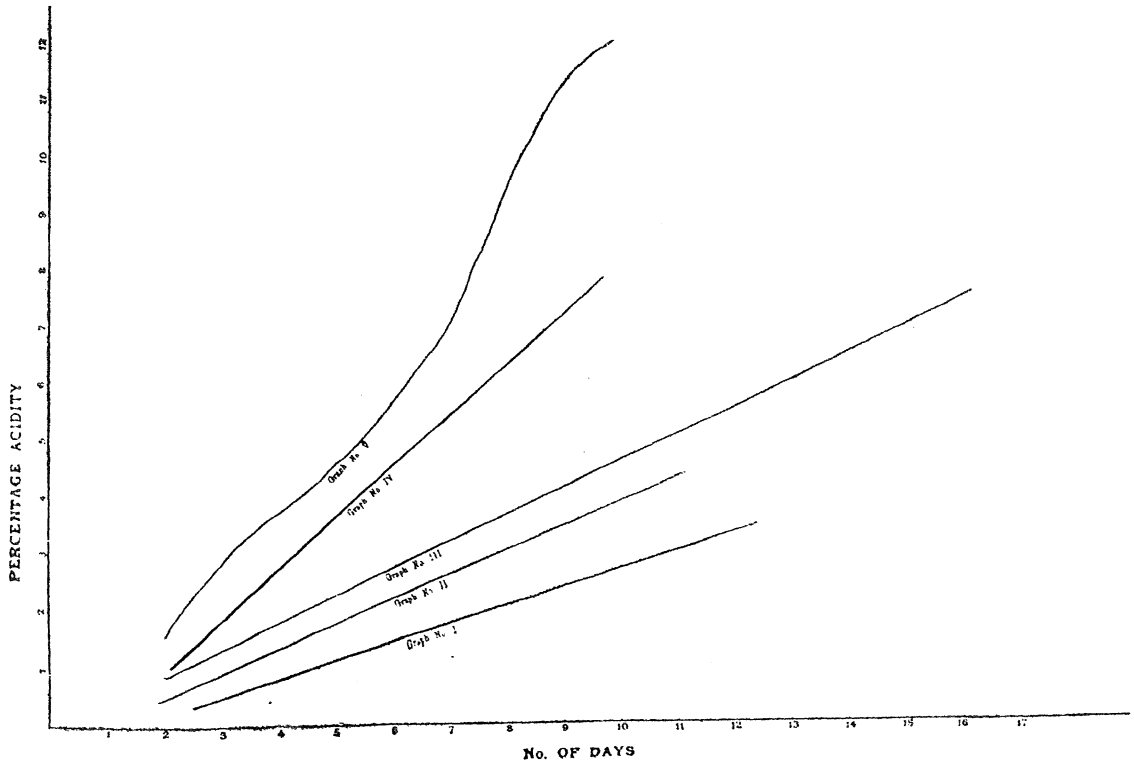
Impure Cultures of B. aceti, containing B. Xylinum, yeast and Cocci.

Percentage of acetic acid formed after :—

	Days													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
I. 10 cc. of toddy added to 250 cc. of nutrient medium composed as above.	2	2	2·1	2·1	2·3	2·5	2·5	2·8	2·8	3	3·2	3·4	3·4	3·4
II. *3rd Sub-Culture*, 10 cc. added to 250 cc. as composed above.	2	2·2	2·4	2·6	2·7	2·8	2·9	3	3·2	3·4	3·6	3·6	...	...
III. *6th Sub-Culture*, 10 cc. added to 250 cc. as composed above.	2	2·2	2·4	2·7	3	3·1	3·1	3·3	3·4	3·6	3·6	...	...	...

Remarks :—Even after further addition of alcohol amounting to 2 per cent. of the total volume there was no increase in acid formation.

ACETIFICATION CURVE



Note.—GRAPHS I, II, III, IV AND V REPRESENT EXPTS. I, III, IV, V AND XI OF TABLES II*a* AND II*b*.

TABLE II

(IIa—Without aeration, IIb—With aeration)

Percentage acidity and rate of acetification after :—

Description of acetifying liquid		Days															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
IIa.																	
I. 50 cc. of bacterial emulsion added to 500 cc. of culture liquid. Composition 2% alcohol, ·05% glucose and ·01% ammonium phosphate.	P.A.	...	...	0·5	0·9	1·2	1·5	*1·7	2·1	2·4	*2·6	3·1	*3·4	...	...	...	...
	R.A.	...	...	0·5	0·4	0·3	0·3	0·2	0·4	0·3	0·3	0·5	0·3	...	...	...	...
II. Do. † ·05% acetic acid.	P.A.	...	0·1	0·6	1·0	1·4	*1·8	2·3	*2·7	3·2	*3·6	4·1	*4·5	...	...	...	...
	R.A.	...	·05	0·5	0·4	0·4	0·4	0·5	0·4	0·5	0·4	0·5	0·4	...	...	...	...
III. Do. † ·5% acetic acid.	P.A.	...	0·4	0·8	1·3	*1·8	2·3	*2·7	3·2	*3·6	4·1	*4·5	...	...	...	...	...
	R.A.	...	0·4	0·4	0·5	0·5	0·5	0·4	0·5	0·4	0·5	0·4	...	...	...	...	...
IV. Do. † ·05% ethyl acetate instead of acetic acid.	P.A.	...	0·8	1·0	*1·8	2·3	2·7	3·2	*3·6	4·1	*4·6	5·0	*5·5	5·9	*6·4	6·4	*6·8
	R.A.	...	0·8	0·2	0·8	0·5	0·4	0·5	0·4	0·5	0·5	0·4	0·5	0·4	0·5	0·4	0·4
V. Same as IV † ·01% manganese sulphate.	P.A.	...	1·1	*1·9	*2·8	*3·7	*4·6	*5·5	*6·4	*7·3	...	...	...	...	...	...	...
	R.A.	...	1·1	0·8	0·9	0·9	0·9	0·9	0·9	0·9	...	...	...	...	...	...	...
VI. Same as V but add 2% alcohol in the middle.	P.A.	...	1·1	†1·9	2·8	†3·7	4·6	†5·5	6·4	†7·3	...	...	...	...	...	...	...
	R.A.	...	1·1	0·8	0·9	0·9	0·9	0·9	0·9	0 9	...	...	...	...	...	...	...
VII. Same as VI but 3% alcohol to start with.	P.A.	...	1·1	1·8	†2·8	3·7	†4·6	5·5	†6·4	†7·3	...	...	...	...	...	...	...
	R.A.	...	1·1	0·7	1·0	0·9	0·9	0·9	0·9	0·9	...	...	...	...	...	...	...
VIII. Same as VI but add 4% alcohol to start with.	P.A.	...	1·1	1·8	2·8	†3·7	4·6	†5·5	6·4	†7·3	...	...	...	...	...	...	...
	R.A.	...	1·1	0·7	1·0	0·9	0·9	0·9	0·9	0·9	...	...	...	...	...	...	...

Note :—P.A. denotes percentage of acetic acid formed.
R.A. denotes rate of acetification.
* Denotes an addition of alcohol equal to 1% of the total volume of the acetifying liquid.
† Denotes an addition of alcohol equal to 2% of the total volume of acetifying liquid.

TABLE II.—*contd.*

(IIa—Without aeration, IIb—With aeration)

Percentage acidity and rate of acetification after :—

Description of acetifying liquid		Days															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
IX. Same as VIII but twice the volume of both inoculant and cultural liquid. 100 : 1000.	P.A.	...	1·0	1·3	1·8	2·1	2·4	†2·7	...	...	...	...	...	...	...	...	...
	R.A.	...	1·0	0·3	0·5	0·3	0·3	...	...	...	...	...	...	...	...	...	...
IIb.																	
X. Same as IX but with aeration.	P.A.	...	...	0·4	0·6	1·5	1·6	1·2	1·2	...	...	...	...	...	...	...	...
XI. Same as X but doubling the quantity of inoculant. 200 : 1000.	P.A.	...	1·5	3·0	†3·8	4·8	†5·7	†7·6	†9·5	†11·4	12·1	...	...	...	...	...	...
	R.A.	...	1·6	1·5	0·8	1·0	0·9	1·9	1·9	1·9	0·7	...	...	...	...	...	...
XII. Same as X but with 400 cc. of inoculant. 400 : 1000.	P.A.	...	1·5	3·0	3·4	3·4	3·2	(No further acetification and much Co_2 began to be formed).									
XIII. Same as XII but with 2000 cc. of cultural liquid. Aeration as in XI. 400 : 2000.	P.A.	...	1·5	3·0	†3·8	4·8	†5·7	6·7	†7·6	8·6	†9·5	...	...	...	...	...	...
	R.A.	...	1·5	1·5	0·8	1·0	0·9	1·0	0·9	1·0	0·9	...	...	...	...	...	...
XIV. Same as XIII but double the quantity of air.	P.A.	...	1·5	3·0	†3·8	4·8	†6·7	†7·6	9·5	†11·4	12·1	...	...	...	...	...	...
XV. Same as XIV but double the quantity of air.	P.A.	...	1·5	3·0	3·2	3·2	3·4	3·0	3·9	(More Co_2 is formed even after further addition of alcohol).							

Note :—P.A. denotes percentage of acetic acid formed.
R.A. denotes rate of acetification.
† Denotes an addition of alcohol to 2% of the total volume of acetifying liquid.

TABLE III.

Large Scale Experiments.

Percentage of acetic acid formed after :—

Description of acetifying liquid	Days															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
I. Five litres of acetifying liquid † 750 cc. of culture † nutrient salts as in small scale experiment.	...	1·6	3·0	3·4	†3·8	5·0	†5·7	7·0	...	...	...	...	...	...	...	...
II. Repeated experiment I with 1100 cc. of culture.	...	1·6	3·2	†3·9	5·0	†5·8	6·8	†7·7	8·7	†9·6	...	...	...	...	...	...
III. Total volume of liquid 5½ gls. nutrient salts as in small scale experiments. 3 litres culture, 4% alcohol.	...	0·3	0·9	1·0	1·2	1·8	2·3	2·9	3·3	†3·3	3·8	4·5	†5·2	5·9	6·5	6·9
VI. Same as above but experiment done at 32°C., the liquid being heated with nichrome wire imbedding bath. 3 litres culture, 4% alcohol.	...	0·5	0·9	1·6	2·3	2·9	†3·6	4·4	†5·3	6·2	†7·1	8·0	8·9	...	...	...

Note :—† Denotes an addition of alcohol equal to 2% of the total volume.
In all cases the initial acidity due to culture addition has been subtracted.

TABLE IV.

Continuous Experiments.

Experiment No.	Initial acidity. Per cent.	Volume and composition of liquid added.	Percentage of acetic acid after addition.	No. of hours taken to reach initial acidity given in column 2.	Percentage addition on the total volume of liquid.
I	8·9	Alcohol—20 cc. Ammonium phosphate—·02 gm. Glucose—·05 gm. Make up to 220 cc. with water.	8·8	(*a*) 4, (*b*) 3½	1
II	8·8	Alcohol—40cc. Ammonium phosphate ·04 gm. Glucose—·10 gm. Make up to 430 cc. with water.	8·6	(*a*) 6½, (*b*) 6	2
III	8·8	Alcohol—80 cc. Ammonium phosphate ·08 gm. Glucose ·10 gm. Make up 860 cc. with water.	8·4	(*a*) 12, (*b*) 12	4
IV	8·8	Acohol—160 cc. Ammonium phosphate ·16 gm. Glucose—·10 gm. Make up to 1720 cc. with water.	8·1	(*a*) 24, (*b*) 24	8

NOTE.—Volume of liquid has been maintained at 22 litres and before fresh addition is made an exact quantity has been withdrawn.

TABLE V.

The Bacterial Oxidation of Acetic Acid after :—

Description of the acetifying liquid.		Days.													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
I. Repeated experiment XI, Table II*b*, stopping alcohol addition when 3·8 per cent. of acid had been formed.	P.A.	...	1·5	3·8	3·6	3·3	3·3	3·0	2·7	2·5	2·2	1·9	...	...	...
	R.D.	...	...	...	0·2	0·3	...	0·3	0·3	0·2	0·3	0·3	...	...	...
II. Same as above but alcohol addition stopped at 5·7%	P.A.	...	1·5	3·0	†3·8	4·8	5·7	5·2	4·8	4·4	4·0	3·6	...	...	...
	R.D.	...	...	...	...	...	...	0·5	0·4	0·4	0·4	0·4	...	...	...
III. Same as above but alcohol addition stopped at 7·6%	P..A	...	1·5	3·0	†3·8	4·8	†5·7	7·6	7·4	7·0	6·6	6·2	6·0	...	...
	R.D.	...	...	...	...	...	...	...	0·2	0·4	0·4	0·4	0·2	...	...
IV. Same as above but alcohol addition stopped at 9·5%	P.A.	...	1·5	3·0	†3·8	4·8	†5·7	†7·6	9·5	9·0	8·6	8·2	7·8	7·4	...
	R.D.	...	...	...	...	...	...	...	...	0·5	0·4	0·4	0·4	0·4	...

Note.—P.A. denotes percentage of acetic acid formed.
R.D. denotes rate of decrease in acid concentration.
† denotes an addition of alcohol equal to 2 per cent. of the total volume of liquid.

TABLE VI.

Experiments on the 'Mucous' Bacteria.

Percentage of acetic acid formed after :—

Description of acetifying liquid.	Days							
	1	2	3	4	5	6	7	8
I. 450 cc. of the barrel liquid Expt. III, Table III, aerated with 50 cc. of the mucous bacteria on a small scale. Initial composition 6·9 per cent. acid and 1 per cent. alcohol.	6·9	6·5	6·3	6·2	5·7	5·4	5·2	4·9
II. 50 cc. of the mucus with 450 cc. of liquid of the following composition :—4 per cent. alcohol + nutrient material as in the small scale experiments. Aerated.	...	0·5	0·8	1·2	1·5	1·3	1·0	0·7
III. *Same as II but with 1% addition of acetic acid.	1·0	1·4	1·2	0·9	0·6	0·3	...	...
IV. Same as II but with 2% addition of acetic acid.	2·0	2·0	1·8	1·5	1·2	0·8	...	...
V. Same as II but with 3% addition of acetic acid.	3·0	3·0	2·8	2·4	2·1	1·8	1·6	...

* Note.—On examination of the culture bottle and potash wash bottles for CO_2, an amount of CO_2, equivalent to 1 per cent. of acid was formed in this experiment.

TABLE VII.

Experiment. No.	Description of Salts.	Concentrations used in percentages.	Effect.
I.	Calcium Acetate.	·01, ·05	No appreciable effect.
II.	Do.	·05, ·07	Slight retarding effect. Time taken to reach 3·8 of acid concentration is six days instead of normal four days. Fermentation quite regular afterwards.
III.	Do.	·5 —1	Appreciable retarding effect. Time taken to reach 3·8% is eight days. Course of acetification irregular afterwards.
IV.	Do.	1, 1·5, 2·1	Very great retarding effect. Acetification in all cases does not proceed beyond 2% which is attained in six days.
V.	Ammonium Acetate.	·5, 1	No appreciable effect.
VI.	Do.	1 , 1·5, 2·1	Slight retarding effect. Acetification to the extent of 3·8% acid concentration is attained in six days. Afterwards a slight tendency to mucus formation.
VII.	Calcium citrate.	·01, ·05	No appreciable effect.
		·5 —1	Great retarding effect. Acetification stopped at 3·4%