

THE FERMENTATION OF TODDY AND AN ACCOUNT OF THE MICRO-ORGANISMS PRODUCING IT.

By M. Damodaran.

Apart from the more purely biological and theoretical aspects of the subject a study of the fermentation of toddy is of interest on account of its important industrial bearing. Palm-toddy, tapped either from the coconut, date or palmyra palm, as the case may be, forms the basis of three industries in this country which are already of considerable importance and offer scope for much further development if conducted scientifically. In the first place toddy, before fermentation, forms the raw material for the manufacture of 'jaggery' or crude sugar. Secondly, fermented toddy is the commonest alcoholic beverage in the country and is also the source of distilled liquor in the form of 'arrack'. Finally this alcoholic liquid is itself a raw material for the manufacture of vinegar. A knowledge of the microflora of toddy is of obvious importance in all these industries, especially in the alcohol and vinegar fermentations where the nature of the products is qualitatively and quantitatively determined by the type of organism responsible for the action. It is surprising that little or no data on this subject are available.

The juice of the palm as it freshly flows from the tree is a saccharine liquid containing in the case of the coconut an average of about 15 per cent. cane-sugar and 0.1 per cent. glucose (Norris, *Agricultural Journal of India*, 1922, 17, 353). The usual method of toddy-making is to allow this freshly tapped juice to ferment spontaneously, this being also the plan adopted for the further conversion of the alcoholic liquid into vinegar; in common practice the vendor leaves in a dark place any surplus toddy for indefinite periods till a certain amount of acetic acid is formed. Consequently in either case the nature of the organisms, of the yeasts which produce alcoholic fermentation as well as the bacteria which are responsible for its conversion into acid, that find their way into the exposed liquid is purely fortuitous, and probably differs according to the locality and the source. Very little seems to be known as to the characters of these organisms.

Fermentation with pure cultures of special yeasts has been adopted with great success in the brewing industry, mainly as a result of the extensive researches of Hansen, and with some success in the preparation of wines. The preparation of vinegar also is now a scientifically regulated process and the importance of using pure cultures of particular species of bacteria for acetification, so as to produce vinegars

with different flavours, and for rapid working with different types of apparatus is well recognised. Investigations are at present being carried out in England on the fermentation of cider with a view to similar artificial control over the nature and amount of the products.

In view of these facts, the present work was undertaken, as the starting point in a more comprehensive scheme, the objects of which are to study the alcohol- and vinegar-producing organisms found in toddy from different sources and in different localities of India, to determine how far the indigenous methods of conducting these fermentations are efficient, and to ascertain if these could not be improved by such modifications as the use of pure cultures. This paper gives a description of the yeasts and bacteria isolated from samples of coconut and date-toddy obtained in Bangalore, and gives analytical results of examining the fermentation products.

As the immediate object of the investigation was to study those micro-organisms which play a part in the fermentation of toddy under the customary conditions of tapping, no special precautions were taken in collecting the toddy except to bottle the liquid as soon as it was brought down from the tree and to prevent extraneous organisms in the air from gaining entrance later. Samples were obtained from different suburbs of Bangalore to see if difference in locality influenced the character of the micro-flora.

METHODS OF ISOLATING AND PURIFYING THE ORGANISMS.

The different varieties of micro-organisms present were isolated by the method of fractional plate cultures on beer-wort-agar. The first objective was to make a count, by the dilution method, of the number of organisms present so as to ascertain the approximate dilution at which distinct and well-separated colonies were obtained on plating. By making the dilutions in sterile beer-wort a 10 c.c. tube of wort containing the freshly inoculated organisms at the proper dilution was obtained. The whole volume of this infected liquid was used for making a series of ten fractional plate-cultures on wort-agar. After incubation for four days the plates were examined and every colony which, from general external characters and the microscopic appearance of the vegetation, might be supposed to represent a distinct species was demarcated. Streak-cultures were made from these colonies on wort-agar slants, taking care that well separated colonies were chosen from plates containing a comparatively small number of colonies, each of which might be taken to be a growth from a single cell.

The purity of streaks thus obtained was tested by making three fractional plate-cultures from each and carefully examining the external

morphology as well as the nature of the vegetation. When these were identical for all three fractional cultures it was assumed that a pure specimen had been isolated and a fresh streak was made on wort-agar and retained as a stock-tube for study. Frequent sub-cultures were subsequently made to ensure that the cultures did not become impure through infection from the air. In this way two distinct species of yeasts and one of bacteria were isolated from the coconut-toddy examined.

CULTURAL METHODS FOR THE YEASTS.

As the only satisfactory method of establishing the identity of yeast is by complete study of its morphological and biochemical characters, the following cultural methods were adopted not only for ascertaining the specific characters of each form, but also for distinguishing the varieties from one another. In fact, it was only towards the end of the examination that the number of varieties was established as two only, the external appearance of the colonies on plate cultures first giving the impression that there were four forms concerned. On detailed study the pure specimens believed to represent four distinct varieties reduced themselves to two, each of which on plate-cultures produced two colonies slightly differing in appearance according as the growth was aerobic or anaerobic, depending on whether the colony was at the surface of the agar or beneath it.

The characters studied were as follows:—

I. *Budding* of the organisms was observed in hanging drop-cultures in beer wort. The differences in the budding were not marked enough to distinguish between the two varieties.

II. *Form of colonies* on plate-cultures, this being the criterion for the separation of types in the first instance.

III. *Streak-cultures* of the two varieties did not show much difference in external appearance.

IV. *Form of vegetation* served as a valuable aid in differentiation under comparable conditions, although liable to changes according to circumstances. The vegetation was studied under the following conditions:—

(a) Cultures (24 hours old) in beer-wort.

(b) Young cultures on wort-agar.

(c) Cultures (3 months old) on wort-agar.

V. *Film-formation* was not observed on inoculating each yeast into flasks half-filled with beer-wort and allowing to remain for some time.

VI. *Spore-formation* was a characteristic which proved of the greatest value in differentiating the two species. Two or three methods were tried and spore-formation took place readily as follows :-

(a) The ordinary plaster-block method.—A few drops of a young and vigorous culture in beer-wort kept at the laboratory temperature for 24 hours were poured over the block placed in a deep Petri dish containing a little sterile water.

(b) By cultures on potato slants.

(c) The easiest and most satisfactory method was to keep cultures on agar-slants during 3-4 months when the cells were found to have passed into a sporing condition.

VII. *Thermal death-point*.—Sterile beer-wort tubes were inoculated with the organisms from 24 hours old cultures and placed in a thermostat for five minutes, removed, incubated, and subsequently examined to see if growth had occurred.

VIII. *Action on sugars*.—The fermenting action of the yeasts was tried on Mayer's solution, prepared with different sugars from the percentage formula: Sugar, 1.5; H_2KPO_4 , 0.5; $MgSO_4$, 0.5; $Ca_3(PO_4)_2$, 0.05; NH_4NO_3 , 0.075. Solutions containing dextrose, lævulose, saccharose, maltose and lactose were used. The action on a solution in which the sugar was replaced by soluble starch was also examined. The different solutions were placed in test tubes with suitable arrangements for observing gas-formation.

IX. *Liquefaction of gelatine*.—Stab-cultures in solid gelatine media were made and kept for a long period, but neither yeast liquefied the gelatine.

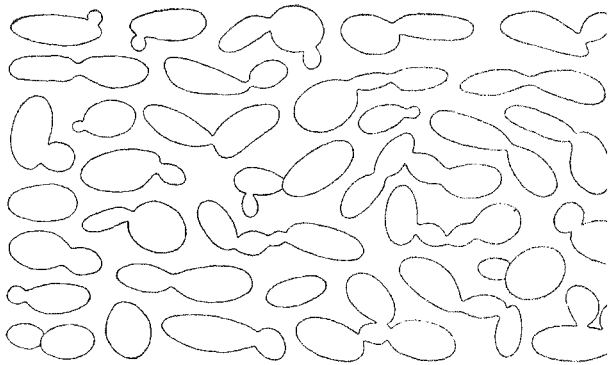
DETAILED DESCRIPTION OF THE ORGANISMS.

Yeast I.—(Figs. I and II).

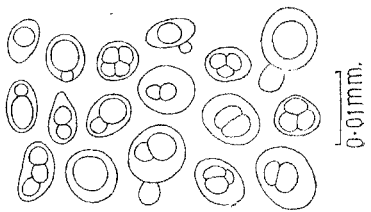
Morphology.—Yeast No. I appeared on plate-cultures as moist round colonies with smooth edges, a convex surface and milky white opaque appearance. It gives a smooth solid white streak on agar-slants.

Vegetation is not a constant characteristic, but is liable to changes according to circumstances. Young cultures in beer-wort and

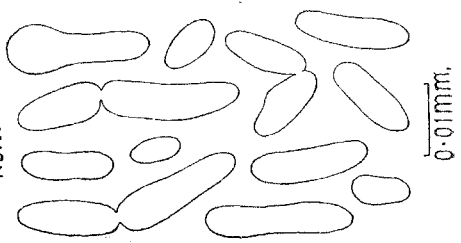
No. III.



No. II.



No. I.



on agar slants are predominantly spherical, showing that the yeast belongs to the *Cerevisiæ* type. In old streak-cultures however the appearance is considerably changed, the commonest forms being the elongated cells shown in Fig. I; a large number of granules also make their appearance inside the cells.

Spore-formation is abundant on old agar-slants, on plaster-block and potato; the appearance is shown in Fig. II.

Budding takes place in a simple manner, multiple budding being common.

Thermal death-point, 52–53°.

Fermentation of sugars takes place with glucose, lævulose and saccharose, but not with arabinose, maltose, lactose or starch.

Film-formation, negative.

Yeast II.—(Figs. III and IV).

Morphology.—The colonies in plate-cultures have a spindle-shaped appearance at the beginning of growth, but after a few days and on sub-culturing the colonies become spherical and resemble those of yeast I; the streak also has the same appearance given by I. Yeast II does not liquefy gelatine.

Vegetation.—Young cultures on beer-wort consist of ellipsoidal cells which however assume various forms in old streak-cultures as shown in Fig. III.

Spore-formation takes place as readily as with I, but the spores have an entirely different and characteristic appearance (Fig. IV).

Budding takes place in the usual way.

Thermal death-point, 52–53°.

Fermentation of sugars takes place with glucose, lævulose and saccharose, but not with arabinose, maltose, lactose or starch.

Film-formation, negative.

ISOLATION AND STUDY OF THE BACTERIA.

The bacteria present were isolated from the mixture with yeasts by the appearance of colonies on plate-cultures. This was the only method available, the usual one of eliminating bacteria from a mixture with yeasts by making the medium acidic, or of eliminating the yeasts

by rendering the medium alkaline not being applicable, because both yeasts and bacteria thrive in an acid medium, and both are incapable of growth in an alkaline medium. The bacteria were easily identified as belonging to the species of *acetobacter*. The following characteristics were studied :—

Morphology.—The bacteria appeared on plate-cultures as moist round colonies much smaller than those of the yeasts; there was a difference in colour also, the bacterial colonies inclining to a slight brownish tint. On agar-slopes a beaded growth was obtained. Though ordinarily aerobic, the organism is capable of anærobic growth, frequently thriving below the agar in plate-cultures.

Vegetation.—Young cultures in alcoholic liquids (fermented beerwort or toddy) consist of short rods occurring singly and in chains, the chains being rather exceptional (Fig. V). Old cultures show involution forms consisting of much larger cells.

Scum-formation.—In fluid cultures this is one of the most noticeable characters. A rather thick, moist, folded pellicle, with a tendency to spread upward at the edges, is formed. On shaking the liquid, the pellicle breaks, producing turbidity.

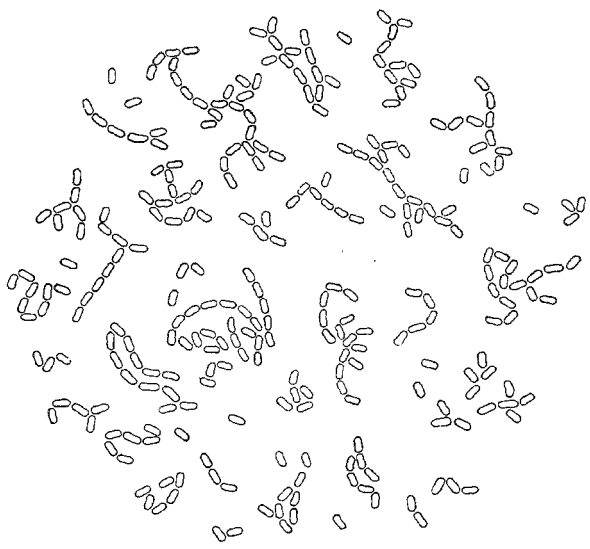
Stains.—The organism readily stains by Löffler's method. When the scum is stained with iodine and sulphuric acid the cells are stained yellow, but a test for cellulose with iodine did not give a blue colour.

Thermal death-point, 38–39°.

A study of these properties and comparison with the recognised types of *acetobacter* show that these bacteria share the general properties of the genus *acetobacter* but do not entirely coincide in character with any definite species. Its closest parallel is *A. kutzin gianus* (Hansen), which it resembles in the shape of the cells, their occurrence singly, the nature of the skin formed in fluid culture and the turbidity imparted to media. It differs from *kutzin gianus*, however, in that the pellicle cannot be stained blue by iodine. From *A. pasteurianus* (Hansen) it differs in many points, particularly in the absence of chain-formation and the characteristic involution forms which this species gives. Similar differences of one sort or other are to be found with the eight other recognised species of *acetobacter* so that it seems right to conclude that these bacteria form a hitherto undescribed species.

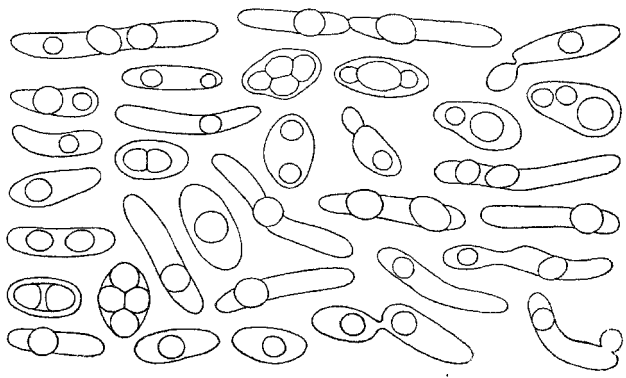
Chemical action.—That the bacteria present were able to produce acetic acid was proved by chemical analysis, as described later with (a) The volatile acid from toddy which was shown to consist of pure

No. V.



0.01mm.

No. IV.



0.01mm.

acetic acid, (*b*) Toddy which had been preserved after the death of the yeasts for a considerable time with only the bacteria active, when it was found to be converted into vinegar containing a large percentage of acetic acid, and (*c*) Artificial culture media, into separate portions of which a mixture of yeasts with bacteria was inoculated.

The bacteria were found to thrive quite as vigorously on sugar media as in alcoholic ones, the difference however being that in the former case little acetic acid was produced. Thus two flasks containing the same volume of a sugar solution were inoculated, one with a 24-hours old culture of bacteria alone, the other with a mixture of bacteria and yeasts, and allowed to stand for a week. After this time the two were analysed for total acid and volatile acid. The following values were obtained in terms of $N/10$ sodium hydroxide used for every 100 c.c. of the sugar solution:—

Fermentation by	Bacteria	Bacteria and yeasts
Total acid	c.c. 42.2	c.c. 104.8
Volatile acid	0.6	53.7

It is seen that little volatile acid is to be found when the sugar is acted on directly by the bacteria, but where yeasts are also present to convert the sugar first into alcohol there is abundant production of acetic acid.

In view of the fact that no care was taken to collect the toddy under sterile conditions it may seem rather surprising that only one species of bacterium was ever found in the many specimens of toddy examined. This is however explained by the acidity imparted to the toddy by the growth of the yeasts and the *acetobacter*, and the inability of ordinary bacteria to survive in such strongly acid media.

DATE TODDY.

On submitting toddy from the date-palm to the method of fractional plate-cultures already described the same two yeasts and the one type of bacterium as found in coconut-toddy were isolated. Toddy from different localities in Bangalore was also studied in the same way and with the same results, showing that for slight differences in locality no change in the nature of the organism is to be observed, irrespective of the source from which the toddy arose.

ANALYTICAL.

The analytical part of the work consisted in (1) Detection of the various products of toddy-fermentation, (2) Quantitative estimation of the more important constituents in a number of samples and (3) Study of alcohol- and acid-production by pure cultures.

Qualitative detection.—Toddy was fermented for two days and then centrifuged to remove the precipitated protein matter; the following substances were identified:—

Acetaldehyde.—A small volume of toddy was exactly neutralised with alkali and then distilled when traces of acetaldehyde were revealed by (a) the slight precipitate of silver from ammoniacal silver nitrate, (b) reduction of Nessler's solution and (c) the characteristic blue-violet colour in the cold with concentrated nitric acid containing 0.5 per cent. potassium dichromate.

Furfurol was recognised in the above distillate by the red coloration with aniline acetate.

Acetone could not be detected either by means of *p*-nitrophenylhydrazine or by Deniges' agent (solution of mercuric sulphate in sulphuric acid and water).

Non-volatile acids.—*Lactic acid* was isolated by evaporating the centrifuged toddy to a pasty consistence, extracting the strongly acid syrup thus obtained four or five times with water-saturated ether and distilling the ether from the extract. The identity of the syrupy residue was established by (a) the yellow coloration produced with Uffelmann's agent, ferric chloride and phenol, (b) conversion to aldehyde by oxidation with acid permanganate, (c) Hopkins' reaction, the cherry-red coloration obtained with alcoholic thiophene in presence of concentrated sulphuric acid and saturated copper sulphate and (d) formation of zinc lactate in prismatic crystals under the microscope.

Succinic acid was isolated as calcium succinate by Pasteur's method.

Tartaric acid was identified as potassium bitartrate by adding potassium chloride in presence of glacial acetic acid and potassium acetate.

Volatile acids.—From the industrial point of view it is of much greater importance to know the exact nature of the volatile acids produced than of the fixed acids, and a systematic study of these volatile acids was therefore made. A solution for qualitative examination was obtained by steam distilling the centrifuged toddy, exactly neutralising the distillate with sodium hydroxide, boiling off the volatile matters and concentrating the solution of the sodium salts. The solution was examined in the separate portions according to Bertrand as follows:—(a) Boiling with silver nitrate did not give a black precipitate, showing absence of formic acid, (b) Mixed with an equal volume of ether and then drop by drop, with continual shaking a two per cent. solution of

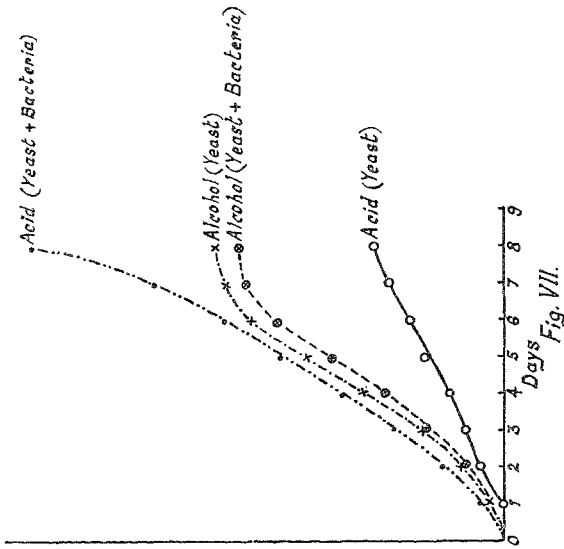


Fig. VII.

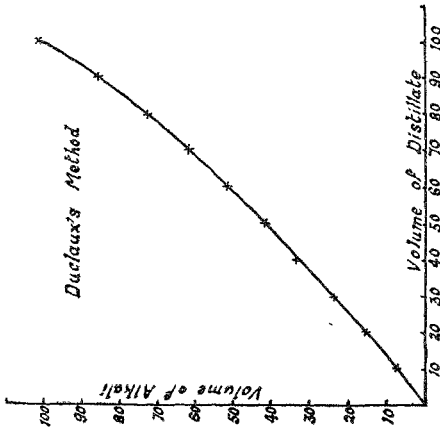


Fig. VI.

copper sulphate, a blue colour was not produced, showing absence of butyric acid and (c) Ferric chloride in presence of acetic ester also did not produce any colour change, thus indicating the absence of propionic acid which produces a yellow coloration.

These observations were further tested by Duclaux's method. This consists in making up the solution of the free volatile acids to a definite volume (110 c.c.), distilling this, collecting the distillate in ten successive fractions of 10 c.c. each and determining the acidity of each fraction by titration against alkali. The amount of alkali required to neutralise the first 10, 20, 30, 40, etc. c.c. of distillate is expressed as a percentage of the total required to neutralise all the ten fractions. The sequence of numbers thus obtained and the nature of the curve they give is characteristic of the volatile acids present.

As the accuracy of this method is dependent on working with a solution containing as little as possible of any substance other than the volatile acids concerned, a suitable solution was first obtained. A large quantity of the centrifuged toddy was distilled with steam till the liquid passing over was no longer acidic; the distillate, consisting of all the volatile bodies was neutralised with excess of calcium carbonate to fix the volatile acid. The mixture was now vigorously boiled to drive off other volatile products such as aldehyde, filtered, evaporated to dryness and the salt thus obtained purified by recrystallisation. From this salt the free acid (or acids) present was liberated by dissolving in a small quantity of water, precipitating the calcium by adding just sufficient amount of concentrated tartaric acid solution and allowing to stand over night. The solution was then filtered from the precipitated calcium tartrate, made up to 110 c.c. and distilled, the distillate being collected in successive quantities of 10 c.c. each and each fraction titrated against lime-water.

The following figures (c.c.) were obtained :—

Distillate	Lime-water for neutralisation	Lime-water calculated to total 100
10	12.55	7.4
20	25.71	15.2
30	39.77	23.4
40	54.95	32.5
50	69.37	41.0
60	85.51	50.6
70	102.91	60.8
80	121.83	72.0
90	142.93	84.5
100	169.18	100.0

The numbers thus obtained and the curve drawn from these (Fig. VI) is in close accord with that of pure acetic acid, and the same

results with two other samples examined thus confirmed the above qualitative examination.

Glycerine.—After evaporating the toddy to a syrup, glycerine was easily identified by the ordinary reactions such as formation of acrolein with potassium bisulphate, etc.

Phosphate was identified in the ash obtained on ignition of the evaporated toddy by extraction with nitric acid and applying the ammonium molybdate reaction.

Quantitative estimation.—The chief constituents of both coconut- and date-toddy were determined quantitatively in a number of samples. The procedure followed the official methods of the American Association of Agricultural Chemists, except in the determination of sugars.

The clear liquid obtained by centrifuging toddy after one day's fermentation, and from which the carbon dioxide was removed as far as possible by pouring a number of times from one vessel to another, was used for all the analyses excepting the protein determination, the values of which are given for the toddy before centrifuging. Nitrogen was determined by Kjeldahl's method and expressed as crude protein by the factor 6.25. Total acids were estimated by direct titration of the liquid against *N/10* caustic soda using azolitmin as indicator and expressed as tartaric acid; the volatile acid, by distilling in steam and titration of the distillate. Alcohol was estimated by density; 100 c.c. were neutralised with alkali and distilled till almost the whole had passed over, the distillate being made up to 100 c.c. and the density determined by a pycnometer. The necessary temperature corrections were made and the ratio, density of distillate at 20° to density of water at 4°, calculated and applied to the percentage of alcohol in the alcohol tables. The sugars before and after inversion were determined by titration against Pavy's solution.

The results are tabulated below :—

Analysis of Toddy in grams per 100 c. c.

I

Nitrogen	Total acid	Volatile acid	Alcohol	Reducing sugar	Total sugar
0.008	0.483	0.247	3.45	0.093	0.271
0.052	0.730	0.296	8.95	0.282	0.566
0.035	0.574	0.487	5.40	0.182	0.531
0.009	0.457	0.227	3.67	0.089	0.233
0.034	0.654	0.374	4.85	0.204	0.552

0·012	0·412	0·188	3·25	0·041	0·135
0·009	0·400	0·207	2·98	0·034	0·165
0·035	0·566	0·246	4·95	0·193	0·374
0·013	0·386	0·241	3·05	0·056	0·147

ALCOHOL AND ACID FORMATION.

Since it is important to know if action of the yeast is influenced by the presence of bacteria, alcohol and acid-formation in beer-wort by pure cultures of Yeasts I and II, and of these in presence of bacteria was studied. Vigorous young cultures for this purpose, distributed in a sufficiently small amount of liquid were obtained by making streak-cultures of the organisms concerned on large agar-slants and washing off the cultures when growth was at its best (after four days) with small measured amounts of sterile water. Mixtures were made of (1) the two yeast cultures and (2) the yeasts and the bacteria, in such a way that the concentration of yeasts in both was approximately the same. These two cultures were then inoculated into two large flasks of beer-wort, the liquid from both taken out daily under sterile conditions and analysed for alcohol and acetic acid. Acetic acid being expressed in terms of alkali required for neutralisation, the results are given in the following table :—

Days	Alcohol		Acetic acid	
	Yeasts only	Yeasts and bacteria	Yeasts only	Yeasts and bacteria
1	0·2	0·2	0·0	0·6
2	0·55	0·5	0·6	1·6
3	1·05	1·0	1·0	2·8
4	1·8	1·5	1·4	4·1
5	2·5	2·2	2·0	5·7
6	3·25	2·9	2·4	7·2
7	3·55	3·3	2·9	9·0
8	3·7	3·4	3·3	12·2

The results are expressed graphically in Fig. VII, and show that (1) the presence of bacteria does not seriously interfere with alcohol-production, at any rate in the first few days, and (2) the conversion of alcohol into vinegar by mere keeping, the ordinary method followed in this country, is a very slow process.

This second fact is further illustrated by periodic analyses of a sample of toddy kept for a period of over four months.

It was found that a period of about four months must elapse before the quantity of acetic acid reached a maximum.

At the same time determination of the volatile acid after this period showed 100 c.c. of the liquid to contain volatile acid corresponding to 543.75 c.c. of $N/10 \times 1.1419$ alkali, i.e., an amount equivalent to 3.73 gms. of acetic acid per 100 c.c. An even approximately quantitative conversion of the alcohol present would involve a larger amount of acetic acid.

SUMMARY.

1. The bacteria and the yeasts found in toddy have been isolated and described.

2. It has been shown that slight changes in locality have no influence on the nature of the organisms. This is true irrespective of the source of the toddy, as shown by the fact that date-toddy contains the same micro-organisms as coconut-toddy in neighbouring localities.

3. Some analytical results for the chief constituents in coconut and date-toddy are given.

4. From a study of the bacteria present it is shown that though they exercise no appreciable effect on the percentage of alcohol present in fermenting toddy, they are responsible for certain other factors, viz., (1) scum-formation and turbidity in toddy, (2) the unpleasant smell associated with toddy, for cane-sugar solutions and wort when fermented by yeasts alone have a pleasant odour during the first few days of fermentation, giving place to the characteristic toddy smell when bacteria also are present; and (3) a large percentage of acetic acid.

5. The suggestion is tentatively put forward that these unpleasant results due to bacteria may possibly be eliminated by taking advantage of the difference in thermal death-points between the yeast and bacteria.

6. It has been shown that the prevailing method of making vinegar by mere keeping is extremely inefficient.

*Department of Bio-Chemistry,
Indian Institute of Science,
Bangalore.*

[Accepted, 25-11-27.]