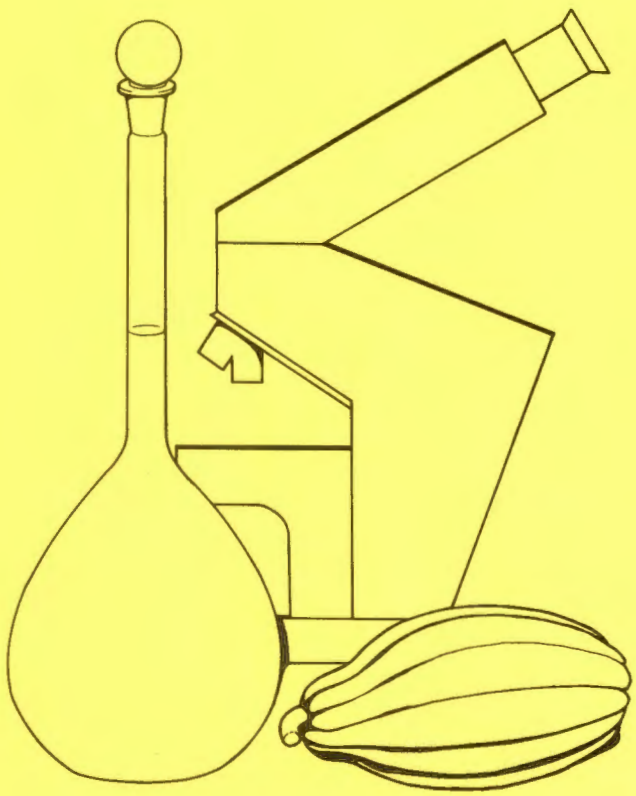
Cocoa Fermentation in Ghana and Malaysia



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Part 2

COCOA FERMENTATION IN GHANA AND MALAYSIA

(Part 2)

Further Microbiological Methods and Results

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INTRODUCTION

The first part of this report dealt mainly with the chemical phenomena observed in the cocoa bean heaps of West Africa and the boxes of Malaysia. Where necessary microbiological information was given to complement the chemical findings and to make the story of the changes during these fermentations complete.

Details of some chemical methods were not included in the first part of the report. Likewise, much microbiological information was omitted. It is the objective of Part 2 to deal with those chemical and microbiological aspects of the investigation not previously recorded. This should enable future investigators to see how we approached the problem and to use any of this information should they find it useful.

ISOLATION

Choice of medium

One medium was chosen to isolate cocoa organisms, namely, apple juice yeast extract (AJYE) prepared in the manner described by Carr (1953). This medium was chosen because past experience has shown that it supports the growth of organisms in acid fermenting environments extremely well. It is a medium made routinely and is, therefore, readily available. It was thought that organisms living in an environment ranging from pH 3.5-5.2 should be isolated on a medium within that pH range and since AJYE is adjusted to pH 4.8 this was another strong reason for choosing it. It is realised, however, that the raw material for this medium is not universally available and other media were investigated to see if they would support the growth of the lactic acid bacteria, acetic acid bacteria, yeasts and species of *Bacillus* isolated from cocoa. Three media were tested, namely, MRS medium (DeMan *et al*, 1960), malt medium and tomato juice medium, all adjusted to pH 4.8. Bakker (private communication) showed that the MRS medium supported the growth of all cocoa strains except *Bacillus* spp. and that the malt medium enabled all the test organisms to grow but less vigorously than on AJYE. Tomato juice medium was in an intermediate position between the other two in its support of growth. These three media have the advantage that they are commercially available in dehydrated form.

Method of isolation

The bean samples which weighed 20 gm were transferred to a stomacher bag containing 100 ml sterile water. After being crushed in the stomacher the resultant suspension was regarded as a 10° dilution and from it were prepared greater dilutions usually ranging from 10^{-1}-10^{-6}. A spread plate technique was employed using 0.1 ml of appropriate dilution per plate. Plates were incubated aerobically and anaerobically at ambient temperature which ranged from 25°-30°C. Anaerobic incubation was carried out in BBL sealable jars, either by the internal release of CO_2 or hydrogen combined catalytically with the available oxygen. Both methods proved satisfactory for growing the more microaerophilic organisms. In later experiments some plates were incubated at 45°C in an attempt to recover the thermophiles. Plates prepared in this manner were observed daily for mould overgrowth and after 3 days, counts were made and recorded. The results of such counts may be seen in Part 1 of this report (Carr,

Davies and Dougan, 1979).

In addition to the straight count an estimate of the different kinds of organism present was also made, thus giving an idea of which sort of organism was in the majority at any one time and site in the heap or box. Colonies were also examined, described and recorded, then picked off into liquid AJYE and finally prepared as a stab culture in AJYE agar contained in small bijou bottles. When organisms had grown along the line of the stab they were removed to a refrigerator and stored until transported to Britain. Most organisms survived this treatment well, but one of the heat tolerant strains which occurred in very large numbers died in these conditions. There was little opportunity to examine this organism before it died but in view of the large numbers in which it occurred both in Africa and Malaysia future investigators should endeavour to investigate its role. Because of its shape and size it is referred to in Part 1 as an irregular punctiform (Carr, Davies and Dougan, 1979).

IDENTIFICATION

Methods and Materials

Organisms from both countries fell into four major categories, with smaller numbers of others also occurring. The primary divisions were made by examining the colonies, describing them and staining the organisms with Gram's stain. It is not intended to publish details of morphology in this report but these are available if further facts are required.

Gram's stain

The organisms can easily be recognized by the use of this stain and appear as follows. Lactic acid bacteria are Gram positive rods of varying size usually situated in palisades and irregular clumps. Individual cells have parallel straight sides and rounded ends. The appearance of bacilli is somewhat similar as their cells give the same staining reaction as lactic rods, but bacilli are usually larger. They are also spore formers and the presence of unstained refractile oval bodies either on their own or within vegetative cells is strongly diagnostic. In the absence of such evidence the colony shape of bacilli is characteristic. Yeasts are Gram positive when stained but this is generally not necessary because their colonies are easy to recognize. Yeasts may be recognized microscopically by their cells which are generally ovoid, reproduce by budding and are usually about 10 times the size of bacterial cells. The main group of Gram negative bacteria present are the acetic acid bacteria which vary from sausage shaped to ovoid and can even be sickle shaped but this is rare. These are the organisms about which there is least certainty of their identity on grounds of morphology and usually require biochemical tests to confirm their identity. Allocation of micro-organisms to various groups on the grounds of their appearance is somewhat inexact whereas the application of biochemical tests allows organisms to be more precisely assigned to the right group.

Biochemical tests for lactic acid bacteria

The lactic acid bacteria were subjected to the following tests: ability to ferment some 23 sugars and related compounds, the ability to metabolize malic, citric, quinic and succinic acids, the ability to produce catalase, to produce mannitol from fructose, gas from glucose,

ammonia from arginine, to split aesculin. The organisms' optimum pH and tolerance of 5% NaCl were investigated as were the ability to grow at 15, 30, 40 and 50°C. The media and methods for these tests are described by Carr (1970) and it is not intended to describe them in detail. It is, however, intended to describe what each test can achieve and the importance of the information it gives us.

Significance of the biochemical tests.

The fermentation of sugars is a very useful test because lactic acid bacteria are able to ferment a wide range of these substances. Many of the sugar fermentations, e.g. glucose and fructose, are common to all lactic acid bacteria whereas the ability to ferment the rarer sugars helps to sort the organisms into different species. Both the lactic rods and cocci are divided into two metabolic types called homo and heterofermentative. The former is so called because their metabolism of glucose leads to lactic acid almost as an entire end-product. In contrast the heterofermenters produce in addition to lactic acid, ethanol, acetic acid and CO_2. The test for gas from glucose indicates whether CO_2 is evolved and the production of mannitol from fructose also indicates the type of fermentation since heterofermenters not only utilize this sugar as an energy source producing lactic acid, but also as a hydrogen acceptor reducing it to mannitol. Thus, the study of sugar fermentations by lactic acid bacteria indicates the relationship of one strain to another and also shows the internal energy gaining pathways within different strains as well as indicating how these organisms might behave towards various sugars in their normal environment.

Biochemical tests for acetic acid bacteria

The acetic acid bacteria were tested as follows: for the production of acid from ethanol and its subsequent metabolism (over oxidation), for production of catalase, growth in a medium containing an inorganic source of nitrogen, other mineral salts and ethanol as a sole carbon source (Hoyer's medium), production of acid from glucose, production of dihydroxyacetone from glycerol, production of cellulose and also brown pigment. In addition they were tested for their ability to metabolize citric, quinic and succinic acids, also their ability to grow at 15, 30, 40 and 50°C. It is not intended to detail these procedures since they have been described by Carr (1968), Carr and Passmore (1979).

Significance of the biochemical tests

The production of acetic acid from ethanol is a very important feature of acetic acid bacteria; indeed if they ceased to carry out this function they would cease, by definition, to be acetic acid bacteria. There are, however, two types of these organisms, both of them associated with low pH fermented products. There are the true *Acetobacter* spp. which are the organisms found in association with vinegar making. They are powerful oxidisers of ethanol, being able to oxidise large quantities of this substrate and to produce high concentrations of acetic acid. In doing so these bacteria produce much energy which is partly dissipated as heat during this exothermic reaction. It is this oxidative activity which is mainly responsible for the rise in temperature in a mass of fermenting cocoa beans. These organisms can also metabolize the resultant acetic acid to CO_2 and H_2O, especially when the prime substrate, ethanol, has disappeared. This reaction is called 'over oxidation'. There is a group of other bacteria able to acetify ethanol but only to a limited extent and these cannot over oxidize the end products. Such organisms were once called *Acetomonas* but have now been changed to the name *Gluconobacter*. These are more likely to metabolize sugars, a characteristic that will be mentioned later. The production of catalase is a common feature of aerobic organisms but there are certain species of acetic acid bacteria that surprisingly do not produce this enzyme. The ability or inability to produce this substance helps to identify certain species. Growth in Hoyer's medium helps to identify certain species of *Acetobacter* and distinguishes them from *Gluconobacter*, none of which can grow in this medium. Acid is produced from glucose by some *Acetobacter* spp. When they do produce acid the primary one is gluconic acid but in addition some strains give rise to 2-ketogluconic acid, 2,5-diketogluconic acid and 5-ketogluconic acid. The ability to produce these acids from glucose varies with the strain (Frateur, 1950). The production of dihydroxyacetone from glycerol varies amongst *Acetobacter* spp. whereas all *Gluconobacter* spp. bring about this change. The production of brown pigment is the result of further metabolisms of 2,5-diketogluconic acid or of 5-ketofructose. This activity is mainly confined to *Gluconobacter* spp. Such activities as those with the sugars and glycerol are usually referred to in the literature as ketogenic activity. The production of cellulose is usually regarded as a function

of green plants and its occurrence in the bacterial kingdom is very rare. One species of *Acetobacter* produces true cellulose and this is *A. xylinum*. A positive cellulose is, therefore, very specific.

LACTIC ACID BACTERIA

Identity of the lactic acid bacteria from Ghana

Tables 1 and 2 show the results of the tests done on the lactic acid bacteria while Table 3 shows the sites of isolation. These organisms were compared according to our own methods and the strains in the present study can be directly compared with the organisms described by Carr and Davies (1970, 1972, 1974). It should be noted that in order to keep the number of isolates down to a manageable size a bacterium was usually isolated only once. These results do not, therefore, reflect the distribution of a particular strain throughout the fermentation. Such information would have been highly desirable, but to acquire it would have required the isolation of many hundreds of cultures and involved a project of several years' duration. Table 1 shows the results of sugar fermentations, and Table 2 other biochemical and physiological tests. We have included with each group the results of named species so that a direct comparison can be made of the behaviour of the named species in our media and the isolates from cocoa.

Sugar fermentations

The points to notice about these are first that within one species group there is some variation in sugar fermentation pattern which is generally to be expected. Secondly some of these bacteria can ferment an extremely wide range of sugars and related compounds. This applies particularly to *L. plantarum* which is probably the most ubiquitous lactobacillus of them all. Table 1 shows that all of these bacteria ferment two of the sugars most likely to be present, namely, glucose and sucrose and only the two somewhat odd unknowns failed to utilize fructose. This in itself is unusual since most heterofermentative bacteria thrive better on fructose than glucose. Other sugars may also be present in cocoa, such as small amounts of galactose derived from the pectin; also the pentoses, ribose, arabinose and xylose which are often to be found in plant materials. It is noteworthy that out of the six sugars utilized by strains A17 and A31 three of them are pentoses. Thus, cocoa probably provides these bacteria with a fair range of sugars from which to derive energy.

Other tests

It was perhaps slightly surprising to find that all the lactic acid bacteria isolated were rods and that no *Leuconostoc* spp. (heterofermentative lactic cocci) were present since these often occur in acidic fermenting situations. This is probably accounted for by the greater heat sensitivity of this type of organism. The fact that the heterofermenters were in excess of the homofermenters is typical of the usual situation in mixed fermentations.

The test for ability to grow at various temperatures showed that most of these organisms could not tolerate 50°C since none was able to carry out any significant growth at this temperature. As Table 3 shows not one of the lactic acid bacteria was isolated from a site where the temperature exceeded 46°C and it would therefore seem that the cut off point of growth for the more heat tolerant lactic acid bacteria lies between 45 and 50°C. There is, however, no evidence to suggest that these organisms cannot survive these elevated temperatures even though they may not grow. It can be concluded from the foregoing tests that the longer a heap or box remains at a temperature between 30-40°C the more readily will the lactic acid bacteria grow. Thus organisms being suppressed in a position of high temperature could start to grow in a lower temperature zone when moved by the process of turning.

The ability to grow at various pH levels shows that these organisms are nearly all able to grow over a wider range than that which occurs naturally before and after fermentation. The ability to grow in 5% NaCl indicates that these organisms are osmotolerant, all being able to grow in an environment with an osmotic pressure higher than that found in the fermentation of cocoa. Table 3 indicates that these lactic acid bacteria can grow either under aerobic or anaerobic conditions. It is true to say that these organisms are microaerophilic, having a fermentative system as their major energy gaining mechanism but for many growth is only slightly impaired in the presence of air.

Metabolism of organic acids

Almost all of these organisms can metabolize malic and citric acids. It can be seen in Part 1 of this report that these acids do occur in cocoa in fairly significant quantity along with other non-volatile acids. Malic acid is decarboxylated to lactic acid and CO_2 whereas citric acid is dissimilated to lactic and acetic acids plus CO_2 by heterofermenters.

In contrast citric acid is broken down to acetoin and CO_2 by homofermenters (Whiting and Coggins, 1964). All of these activities reduce the acidity during cocoa fermentation but the one with the most effect is the breakdown of citric acid by the homofermenters since the end products of their activities are non-acidic. It is interesting to note that the homofermenters also metabolize quinic acid. It is not known whether this acid is present in cocoa but it seems likely since cocoa leaves are reported to contain p-coumaryl quinic and chlorogenic acids, both of which are related to quinic acid and might also be associated with the fruit and seeds. Although the loss of this acid would affect acidity less than the loss of malic or citric acid, the formation of the unsaturated intermediate shikimic acid could have the effect of enabling some lactic acid bacteria to metabolize lactic acid (Whiting and Coggins, 1971), thereby reducing the acidity further.

Identity of the lactic acid bacteria from Malaysia

Sugar fermentation

A comparison of Tables 1 and 4 shows that there were less identifiable species in Malaysia than Ghana, but there were more unknowns, some of which behaved in a very atypical manner; for example, M42 did not ferment any sugars at all in our tests and although it looked like a lactic acid bacterium there is some doubt as to its true identity. If the number of strains of a particular species isolated in any way reflects the number of organisms present, then the proportion of *L. plantarum* and *L. collinoides* are the opposite in the two countries.

Other tests

Table 5 shows the physiological tests and it can be seen that there are a higher proportion of NT (not tested). This is because more of the Malaysian organisms failed to grow on our standard test media and this applies most especially to the unknowns. However, the overall picture presented in Table 5 is somewhat similar to Table 2. The Malaysian organisms seem to be less osmotolerant that the African ones, whereas the latter can withstand higher temperatures. In other respects they are similar both in the pH range at which they grow and their dissimilation of organic acids. It is particularly interesting to note that the strains of *L. plantarum* in both countries can metabolize quinic

acid, a function carried out by only one other organism, namely, M42, the isolate that was apparently unable to utilize any sugars.

Table 6 shows that these organisms were isolated from all Trials except No.4. This simply reflects the policy, stated earlier, that if an organism had been isolated previously it was not isolated again. Table 25 (Part 1, p.73) shows clearly that lactic acid bacteria were present during the two days that samples were taken. Furthermore, there seems little doubt about their viability since Table 26 (Part 1, p.74) shows that there was a marked increase in lactic acid during the time this trial was examined.

The lactic acid bacteria, therefore, have two major activities, the first being the production of lactic acid from available carbohydrates. In this activity they would, in part, be in competition with the yeasts that are also contenders for available carbohydrates as an energy source. This activity of the lactic acid bacteria would increase acidity. The second role for these bacteria is the dissimilation of the naturally occurring organic acids causing a drop in acidity. It seems likely, however, that the breakdown of carbohydrates contributes more to the general acidity than the breakdown of organic acids removes. This, however, is not proven.

The fact that lactic acid bacteria occur in the environment of fermenting cocoa beans is not surprising since the conditions are right for their development. The occurrence of various carbohydrates to provide energy, and several metabolisable organic acids and a low pH, all combine to produce conditions in which lactic acid bacteria can flourish. What is perhaps remarkable is the similarity of the lactic acid bacteria that are found in the heaps of Ghana and boxes of Malaysia. Although the conditions in the two countries generally are similar one might have expected the two places to give rise to a 'local flora'. This is not so since the major groups are the same and only the metabolically deficient organisms differ from one another.

TABLE 1

Sugar fermentations of *Lactobacillus* spp. from Ghana

Designation	Ribose	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Cellobiose	Sorbose	Raffinose	Melezitose	Glycerol	Mannitol	Sorbitol	Dulcitol	Salicin	Inositol	α Meth.gluc	Melibiose	
L. plantarum	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	NT	-	+	Homofermenters
A34	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	
A50	+	+	+	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	
A70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	
A84	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	
A88b	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	
L. mali	-	-	±	-	+	+	-	+	-	+	-	+	±	±	-	-	-	-	-	-	+	NT	+	-	
A54a	-	-	+	-	+	+	+	+	-	+	-	+	+	+	-	-	-	-	-	+	+	-	NT	-	
L. collinoides	+	+	-	+	+	+	+	-	∓	∓	+	-	-	-	-	-	-	∓	-	-	∓	NT	∓	+	Heterofermenters
A15	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-	W	-	-	-	-	+	+	
A36	+	+	-	+	+	+	+	-	-	W	+	-	-	-	-	-	-	W	-	-	-	-	+	+	
A42	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-	W	-	-	-	-	+	+	
L. fermentum	+	±	-	±	+	+	+	W	+	+	+	±	-	-	+	-	-	-	-	-	-	-	-	+	
A40	+	+	W	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	W	-	-	-	+	
A52	+	+	-	+	+	+	+	+	+	+	+	+	W	-	+	W	W	W	W	W	-	-	-	+	
A59	+	+	-	+	+	+	+	+	+	+	+	+	W	-	+	W	W	W	W	W	-	-	+	+	
A83	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	+	
A85	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	+	-	-	W	+	
A90	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	W	W	W	W	W	-	-	+	+	
Unknowns																									
A17	W	+	-	+	W	-	+	-	-	-	+	-	-	-	-	-	-	W	-	-	-	-	-	-	
A31	+	+	-	+	+	-	W	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	

KEY

+ Positive
W Weak positive
- Negative
± 80% or more positive
∓ 80% or more negative
NT Not tested
R Rod shape

TABLE 2

Other tests on *Lactobacillus* spp. from Ghana

Designation	Shape	Gas from glucose	Mannitol from fructose	Catalase	Aesculin	Arginine	NaCl (5%)	Growth at °C 15	30	40	50	Growth at pH	Malic	Citric	Quinic	Succinic	Lactic
L. plantarum	R	-	-	-	+	-	±	+	+	+	NT	3.4-8.0	+	-	+	-	
A34	R	-	-	-	+	-	W	+	+	+	-	3.4-8.0	+	+	+	-	
A50	R	-	-	-	+	-	+	+	+	+	-	3.0-8.0	+	-	+	-	-
A70	R	-	-	-	+	-	-	+	+	+	W	NT	+	+	+	-	
A84	R	-	-	-	+	-	+	+	+	+	-	3.0-8.0	+	+	+	-	-
A88b	R	-	-	-	+	-	+	+	+	+	W	NT	+	+	+	-	
L. mali	R	-	-	W	+	-	-	±	+	+	NT	3.4-7.4	+	W	-	-	
A54a	R	-	-	-	-	-	+	+	+	+	-	4.0-6.0	+	-	-	-	
L. collinoides	R	+	+	-	+	+	-/+	+	+	±	NT	3.0-7.0	±	±	±	-	
A15	R	+	+	-	+	+	+	+	+	+	-	3.4-8.0	+	+	-	-	
A36	R	+	+	-	+	+	+	+	+	+	-	3.4-8.0	+	+	-	-	+
A42	R	+	+	-	+	-	+	+	+	+	-	3.4-8.0	+	-	-	-	
L. fermentum	R	+	+	-	NT	+	NT	-	+	+	NT	NT	NT	NT	NT	NT	
A40	R	+	+	-	-	+	W	NT	+	+	-	3.0-7.0	+	+	-	-	-
A52	R	+	+	-	-	+	+	NT	+	+	-	2.4-7.0	+	+	-	-	-
A59	R	+	+	-	-	+	+	+	+	+	-	2.4-7.0	+	+	-	-	-
A83	R	+	+	-	-	+	W	NT	+	+	-	3.0-7.0	+	+	-	-	-
A85	R	+	+	-	-	+	+	NT	+	+	-	3.0-7.0	+	+	-	-	-
A90	R	+	+	-	-	+	+	NT	+	+	W	2.4-7.0	+	+	-	-	-
Unknowns																	
A17	R	+	-	-	-	-	+	W	+	+	-	NT	-	-	-	-	
A31	R	+	-	-	-	-	+	NT	+	+	-	2.4-5.0	-	-	-	-	

KEY - see Table 1

TABLE 3

Locations from which the *Lactobacillus* spp. were isolated in Ghana

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
L. plantarum				
A34	Bottom of box	24	25	Anaerobic
A50	Bottom of box	48	28	Anaerobic
A70	Heap 1 drying stage	72	38	Anaerobic
A84	Bottom heap 2	24	33	Aerobic 45°C
A88b	Middle heap 2	24	42	Aerobic 45°C
L. mali				
A54a	Bottom heap 1	72	41	Aerobic
L. collinoides				
A15	Bottom heap 1	0	28	Aerobic
A36	Top of box	24	36	Aerobic
A42	Bottom heap 1	48	41	Aerobic
L. fermentum				
A40	Top of box	24	36	Anaerobic
A52	Middle of box	48	35	Aerobic
A59	Top of heap 1	96	46	Anaerobic
A83	Bottom heap 2	24	33	Aerobic 45°C
A85	Bottom heap 2	24	33	Aerobic 45°C
A90	Top of heap 2	24	44	Anaerobic
Unknowns				
A17	Middle heap 1	24	36	Anaerobic
A31	Bottom of box	24	25	Aerobic

* Unless otherwise stated plates were incubated at room temperature (25-30°C)

TABLE 4

Sugar fermentations of *Lactobacillus* spp. from Malaysia

Designation	Ribose	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Cellobiose	Sorbose	Raffinose	Melezitose	Glycerol	Mannitol	Sorbitol	Dulcitol	Salicin	Inositol	α Meth.gluc	Melibiose	
L. plantarum	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	NT	-	+	Homofermenters
M68b	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	W	+	+	-	+	-	-	+	
M94	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	W	+	+	-	+	-	-	+	
L. collinoides	+	+	-	+	+	+	+	-	∓	∓	+	-	-	-	-	-	-	∓	-	-	∓	NT	∓	+	Heterofermenters
M13b	+	+	-	W	+	+	+	+	-	+	+	+	+	-	∓	-	-	-	-	-	+	-	-	+	
M25	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	
M35	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	W	+	+	+	W	+	
M43	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+	
M56a	+	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	
M56b	+	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	
M59	+	+	-	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-	+	-	-	+	
Unknowns																									
M32	+	+	-	+	+	+	+	+	-	+	+	-	+	-	+	-	-	-	+	-	+	-	-	+	The information contained here and in Table 5 is insufficient to be able to decide the fermentation type of these unusual organisms
M48	-	-	-	-	+	-	-	-	-	+	W	-	W	-	W	-	W	W	-	-	-	-	-	-	
M90a	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	W	-	-	-	-	-	-	
M96	+	-	-	-	+	+	+	+	-	-	W	-	+	-	-	-	-	-	-	-	+	-	-	-	
M36	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	+	
M42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

KEY - see Table 1

TABLE 5

Other tests on *Lactobacillus* spp. from Malaysia

Designation	Shape	Gas from glucose	Mannitol from fructose	Catalase	Aesculin	Arginine	NaCl (5%)	Growth at °C 15	30	40	50	Growth at pH	Malic	Citric	Quinic	Succinic
L. plantarum	R	-	-	-	+	-	±	+	+	+	NT	3.4-8.0	+	-	+	-
M68b	R	-	-	-	+	-	+	+	+	+	W	3.4-8.0	+	+	+	-
M94	R	-	-	-	+	-	+	+	+	+	+	3.4-8.0	+	+	+	-
L. collinoides	R	+	+	-	+	+	W	+	+	±	NT	3.0-7.0	±	±	±	-
M13b	R	+	+	-	+	+	W	NT	+	+	-	3.4-6.0	+	+	-	-
M25	R	+	+	NT	+	+	W	NT	+	+	-	3.4-6.0	+	+	-	-
M35	R	+	+	NT	+	+	W	NT	+	+	-	3.4-6.0	+	+	-	-
M43	R	+	+	-	+	+	+	+	+	+	W	3.4-8.0	+	-	-	-
M56a	R	+	+	-	+	+	W	NT	+	+	W	3.4-7.0	+	+	-	-
M56b	R	+	+	-	+	+	W	NT	+	+	W	3.4-6.4	+	+	-	-
M59	R	+	+	-	+	+	W	NT	+	W	W	3.4-6.0	+	+	-	-
Unknowns																
M32	R	NT	+	-	+	+	-	NT	NT	NT	NT	3.4-6.0	W	+	-	-
M48	R	-	W	-	-	-	W	NT	+	+	W	3.4-6.4	-	W	-	-
M90a	R	-	NT	-	W	-	W	+	+	+	W	NT	+	W	-	-
M96	R	-	-	-	+	-	W	W	NT	NT	NT	NT	+	-	-	-
M36	R	-	+	-	-	-	-	NT	+	+	-	NT	+	+	-	-
M42	R	-	+	NT	+	NT	NT	NT	+	+	-	NT	+	+	+	-

KEY - see Table 1

TABLE 6

Locations from which the *Lactobacillus* spp. were isolated in Malaysia

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
L. plantarum				
M68b	Top of Trial 1	96	48	Anaerobic
M94	Trial 3 lorry	-	-	Aerobic
L. collinoides				
M13b	Sweatings	-	-	Aerobic
M25	Bottom Trial 1	4	31	Aerobic
M35	Middle Trial 1	24	33	Aerobic
M43	Middle Trial 1	24	33	Anaerobic
M56a	Top Trial 2	24	NT	Aerobic
M56b	Top Trial 2	24	NT	Aerobic
M59	Top Trial 2	24	NT	Aerobic 45°C
Unknowns				
M32	Top Trial 1	24	33	Aerobic
M48	Sweatings Trial 2	-	-	Aerobic
M90a	Top Trial 2	144	50.5	Aerobic 45°C
M96	Middle Trial 3	72	40	Anaerobic
M36	Middle Trial 1	24	33	Aerobic
M42	Top Trial 1	24	33	Anaerobic

* Unless otherwise stated plates were incubated at room temperature (25-30°C)

ACETIC ACID BACTERIA

Identity of acetic acid bacteria from Ghana

Three distinct species of *Acetobacter* were found in the cocoa fermentations of Ghana. These were identified using the first eight tests shown in Table 7. These tests originated by Frateur (1950) and later modified by Carr (1968) sorted the acetic acid bacteria into four species, namely, *Acetobacter rancens*, *A. ascendens*, *A. xylinum* and *Gluconobacter oxydans*. Of these *A. rancens* and *A. xylinum* are commonly found in situations such as vinegar generators, whereas *A. ascendens* is a rare species. The other acetic acid bacterium called *Gluconobacter oxydans* belongs to a different group of organisms but is regarded as an acetic acid bacterium. It is capable of oxidizing ethanol to acetic acid and can only produce about 5% of the acid as an upper limit, whereas the true acetobacters can produce considerably higher concentrations than this. There is a further difference since *Acetobacter* spp. can also oxidize acetic acid when the ethanol is depleted. There are a number of other differences which make it easy to distinguish these two types of acetic acid bacterium.

Metabolism of organic acids

It is interesting to note that although all strains of *A. rancens* were identified as that by the conventional tests their behaviour towards malic and citric acid in particular divided them into four groups. Furthermore, their ability to oxidize these acids makes them capable of reducing acidity as well as creating acidity by the production of acetic acid. Both citric and malic acids are strong acids in contrast to acetic acid, which is weak. The destruction of a strong acid will affect the pH more profoundly than the generation of a weak acid such as acetic. Acetic acid bacteria can also metabolize lactic acid which is plentiful in cocoa fermentations as a result of the activities of lactic acid bacteria. Thus, the activities of acetic acid bacteria are quite complex, for they not only produce acetic acid which is weak, but destroy other acids which are much stronger.

Growth at various temperatures

Many activities of microbes produce energy and generally this is taken back into the various biological systems during the chemical changes that keep the micro-organisms alive. The oxidation of ethanol to acetic acid is exothermic and that is the main cause of heating in a mass of fermenting cocoa beans. Other biochemical activities can also produce heat, e.g. alcoholic fermentation, but the actual amount of heat evolved in this activity is not so great as in acetification. The production of heat and the gradual increase of temperature has the effect of killing off all heat sensitive micro-organisms and this applies in particular to the fermenting yeasts although these are also suppressed by acetic acid. Like the other organisms so far considered the acetic acid bacteria do not show any marked growth at 50°C but most of the strains shown in Table 7 survive this elevated temperature. This means that in those parts of the fermenting mass where the temperature is high microbial activity will be minimal and it is in the cooler parts that most activity occurs.

Table 8 shows the locations and conditions under which acetic acid bacteria were isolated. It shows clearly that they are present at a very early stage since they were isolated from the baskets used to convey the beans after pod splitting to the heaps or boxes. It also illustrates a fact not usually mentioned in connection with these organisms, namely, that although aerobic they can withstand quite anaerobic conditions. This is borne out by the fact that several were isolated from the bottom of the heap and quite a number were isolated from anaerobic plates. There is no doubt of their heat resistance since several isolates were from locations where the temperature had reached 44°C and several where even higher temperatures had been reached. It is interesting to see that the three strains of *Gluconobacter oxydans* occurred within the first four hours of the fermentation and were then not seen again. These organisms lack a Krebs Cycle and are thus deficient in having a good energy gaining mechanism. Competition from the much more efficient acetobacters and lactobacilli would inevitably limit them to the early stages.

Identity of acetic acid bacteria from Malaysia

Examination of Table 9 shows that the dominant acetic acid bacterium is *Acetobacter rancens* which is a very common member of this group. Like the ones from Africa they can be divided according to the organic acids they metabolize. They correspond with the African organisms in the combinations + malic and succinic, + malic, citric and succinic but the other two combinations found in Africa do not exist amongst the Malaysian organisms. There is, however, a third category of organisms amongst the Malaysian ones, namely, a group which would otherwise be classed as *A. rancens* except that they can grow in Hoyer's medium. The ability to grow in a medium with inorganic nitrogen, other mineral salts and ethanol is fairly rare amongst the acetic acid bacteria. Under the system of Frateur's (1950) classification this organism would be classified as a new species. As far as we are aware an *Acetobacter* sp. with this combination of characters has never hitherto been recorded. The other species of *Acetobacter*, namely, *A. xylinum*, is quite common in association with fermentation products in temperate climates. It takes its name from the fact that the organisms make cellulosic threads which are matted to form cartilaginous buoyant sheets giving it a great advantage in a liquid medium. This would not, however, give the organism any special advantage on the solid substrate of cocoa beans.

Metabolism of organic acids

The same remarks apply to these organisms as to those isolated in Africa, namely, that there is a conflict of function between the production of acetic acid on the one hand the the destruction of other acids on the other.

Growth at various temperatures

The growth of these organisms is much the same as those of Africa, namely, that they mostly grow well up to 40°C and that between this temperature and 50°C the growth tends to weaken. It should be noted, however, that these organisms are tested for heat resistance in a semi-defined medium and in pure culture. It is possible that in natural conditions where other organisms are growing temperature relations may be different. If the average isolation temperature of all the organisms so far discussed are compared they read as follows: lactic acid bacteria from Ghana 35.3°C, Malaysia 36.5°C. Acetic acid bacteria from Ghana

36.9°C, Malaysia 44°C. There is a significant difference between the last figure and the other three. Does this merely reflect the times and the places at which acetic acid bacteria were isolated in Malaysia or does it reflect a different behaviour pattern between the acetic acid bacteria from the two countries? A look at the temperature graphs in Part 1 suggests this was a factor that did not vary much between the two countries and that this higher temperature simply reflects the locations and times from which the Malaysian organisms were isolated.

The *Gluconobacter* spp. were only few in number and like their African counterparts were isolated only within the first 24 hours of the fermentation.

TABLE 7

Tests on *Acetobacter* and related spp. from Ghana

Designation	Gram stain	Over oxidation of acetic acid	Catalase	Growth in Hoyer's medium	Acid from glucose	Dihydroxyacetone from glycerol	Pigment	Cellulose	Malic	Citric	Quinic	Succinic	Lactic	15°	30°	40°	50°
Acetobacter rancens									+ malic + succinic								
A1b	-	+	+	-	+	-	-	-	+	-	-	+		NT	+	W	W
A2	-	+	+	-	+	-	-	-	+	-	-	+		NT	+	W	W
A51a	-	+	+	-	+	-	-	-	+	-	-	+		+	+	+	+
									+ citric + succinic								
A20	-	+	+	-	+	-	-	-	-	W	-	+		+	+	+	W
A37	-	+	+	-	+	-	-	-	-	+	-	W		+	+	+	W
A60	-	+	+	-	W	-	-	-	-	W	-	W		+	+	+	-
									+ malic + citric + succinic								
A19	-	+	+	-	+	-	-	-	+	+	-	+		+	+	+	W
A43b	-	+	+	-	+	-	-	-	+	+	-	+		+	+	+	W
A89	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+	-
A57	-	+	+	-	+	-	-	-	W	W	-	+		+	+	+	W
A77	-	+	+	-	+	-	-	-	-	+	-	+		+	+	+	+
A79	-	+	+	-	+	-	-	-	+	+	-	+	+	NT	+	+	+
A87	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+
									± malic + citric + quinic + succinic								
A51b	-	+	+	-	+	-	-	-	+	W	+	W		+	+	+	+
A78	-	+	+	-	+	-	-	-	+	+	+	+		+	+	+	-
A. ascendens																	
A46	-	+	+	-	-	-	+	-	-	W	-	W	+	+	+	+	-
A56	-	+	+	-	-	-	+	-	-	+	-	W		+	+	+	W
A91a	-	+	+	-	-	-	-	-	+	+	-	+		+	+	+	W
A. xylinum																	
A32	-	+	+	-	+	+	-	+	-	-	-	W		+	+	-	-
A49	-	+	+	-	+	+	-	+	-	-	-	W	+	+	+	-	-
Gluconobacter oxydans (Acetomonas)																	
A4	-	-	+	-	+	+	W	-	-	-	-	-		+	+	+	W
A5	-	-	+	-	+	+	+	-	NT	NT	NT	NT		NT	NT	W	-
A24	-	-	+	-	+	+	+	-	-	-	-	-		+	+	W	-

TABLE 8

Locations from which the *Acetobacter* spp. were isolated in Ghana

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
Acetobacter rancens				
A1b	Basket	NT	NT	Aerobic
A2	Basket	NT	NT	Aerobic
A51a	Bottom of box	48	28	Anaerobic
A20	Top of heap 1	24	44	Aerobic
A37	Top of box	24	36	Aerobic
A60	Top of heap 1	96	46	Anaerobic
A19	Top of heap 1	24	44	Aerobic
A43b	Middle of heap 1	24	36	Aerobic
A89	Top of heap 2	24	44	Aerobic 45°C
A57	Bottom of box	72	43	Aerobic
A77	Bottom of heap 2	0	26	Aerobic 45°C
A79	Top of heap 2	0	24	Aerobic 45°C
A87	Bottom of heap 2	24	33	Aerobic 45°C
A51b	Bottom of box	48	28	Anaerobic
A78	Bottom of heap 2	0	26	Aerobic 45°C
A. ascendens				
A46	Top of heap 1	48	45	Aerobic
A56	Top of heap 1	72	48	Aerobic
A91a	Heap 1 drying stage	168	32	Anaerobic
A. xylinum				
A32	Bottom of box	24	25	Aerobic
A49	Bottom of box	48	28	Aerobic
Gluconobacter oxydans				
A4	Basket	NT	NT	Aerobic
A5	Basket	NT	NT	Aerobic
A24	Middle box	4	29	Aerobic

* Unless otherwise stated plates were incubated at room temperature (25-30°C)

TABLE 9

Tests on *Acetobacter* and related spp. from Malaysia

Designation	Gram stain	Over oxidation of acetic acid	Catalase	Growth in Hoyer's medium	Acid from glucose	Dihydroxyacetone from glycerol	Pigment	Cellulose	Malic	Citric	Quinic	Succinic	15°	30°	40°	50°
Acetobacter rancens									+ malic + succinic							
M4b	-	+	+	-	+	-	-	-	+	-	-	+	W	+	+	-
M6b	-	+	+	-	+	-	-	-	+	-	-	+	NT	+	W	W
M44	-	+	+	-	+	-	-	-	W	-	-	+	+	+	+	W
M46	-	+	+	-	+	-	-	-	W	-	-	+	+	+	+	W
M61	-	+	+	-	+	-	-	-	W	-	-	W	+	+	+	W
M65	-	+	+	-	+	-	-	-	W	-	-	W	+	+	+	+
M83	-	+	+	-	+	-	-	-	W	-	-	+	+	+	+	-
M99	-	+	+	-	+	-	-	-	+	-	-	+	+	+	+	W
M105	-	+	+	-	+	-	-	-	+	-	-	+	+	+	+	-
									+ malic + succinic + citric							
M6c	-	+	+	-	+	-	-	-	+	+	-	+	+	+	W	-
M26	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	W
M73	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	w
M75b	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	W
M86	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	W
M87	-	+	+	-	+	-	-	-	+	+	-	+	NT	+	+	-
M90b	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	W
M97	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+
M106	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	W
Acetobacter lovaniensis									+ Hoyer							
M13a	-	+	+	+	+	-	-	-	+	-	-	+	+	+	+	-
M53	-	+	+	+	+	-	-	-	W	-	-	W	+	+	+	W
M74b	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	W
M78	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	W
M92	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	W
M98b	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	W
M101	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+	W
M102	-	+	+	+	+	-	-	-	+	-	-	+	+	+	+	W
A. xylinum																
M71a	-	+	+	-	+	+	-	+	-	-	-	-	+	+	W	-
M71b	-	+	+	-	+	+	-	+	-	-	-	-	+	+	W	-
Gluconobacter oxydans (Acetomonas)																
M20	-	-	+	-	+	+	+	-	-	-	-	-	+	+	+	W
M27	-	-	+	-	+	+	+	-	-	-	-	-	+	+	W	-
M29	-	-	+	-	+	+	+	-	-	-	-	-	+	+	W	-

TABLE 10

Locations from which the *Acetobacter* spp. were isolated in Malaysia

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
Acetobacter rancens				
M4b	Trial 1	0	NT	Aerobic
M6b	Polybag	NT	NT	Aerobic
M44	Trial 1 top	48	45	Aerobic
M46	Trial 1 bottom	48	45	Aerobic
M61	Trial 1 middle	72	40	Anaerobic
M65	Trial 1 top	96	48	Aerobic
M83	Trial 2 middle	72	48	Anaerobic
M99	Trial 3 top	48	36	Anaerobic
M105	Trial 3 top	168	45	Aerobic
M6c	Polybag	NT	NT	Aerobic
M26	Trial 1 top	24	33	Aerobic
M73	Trial 1 top	144	47	Aerobic
M75b	Trial 1 top	120	50	Anaerobic
M86	Trial 1 pre-dry	168	47	Anaerobic
M87	Trial 2 top	120	46	Anaerobic
M90b	Trial 2 top	144	51	Aerobic 45°C
M97	Trial 3 top	48	36	Aerobic
M106	Trial 3 top	168	45	Anaerobic

/Contd

Table 10 contd

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
Acetobacter lovaniensis				
M13a	Trial 1 sweatings	NT	NT	Aerobic
M53	Trial 1 top	72	46	Aerobic
M74b	Trial 1 top	120	50	Anaerobic
M78	Trial 1 bottom	120	45	Anaerobic
M92	Trial 2 bottom	144	47	Anaerobic
M98b	Trial 3 middle	48	47	Aerobic 45°C
M101	Trial 2 middle	192	49	Aerobic
M102	Trial 3 top	72	44	Anaerobic
A. xylinum				
M71a	Trial 2 bottom	48	46	Aerobic
M71b	Trial 2 bottom	48	46	Aerobic
Gluconobacter oxydans				
M20	Trial 1 top	4	31	Aerobic
M27	Trial 1 top	24	33	Aerobic
M29	Trial 1 sweatings	NT	NT	Anaerobic

* Unless otherwise stated plates were incubated at room temperature (25-30°C)

YEASTS

Their role and identity

Of all the micro-organisms occurring in cocoa the yeasts are the most important since it is their activity that initiates the series of biochemical events that follow. The fermenting yeasts found early in the process are of prime importance because they ferment the available sugars to ethyl alcohol and CO_2. The alcohol then becomes the substrate for the acetic acid bacteria which oxidize it to acetic acid thus producing the substance that is said to be essential for the development of chocolate flavour.

While the biochemical activities of the genus *Saccharomyces* are well known, similar information is not available for other yeast genera. The weak fermenters, and in particular the non-fermenters, probably respire away some of the available sugar with the production of CO_2 in a manner similar to that found in animals. Other substrates may also be utilized. It is known, for example, that species of the genus *Schizosaccharomyces* (so called because of their fission instead of budding) can produce acetic acid as an end product. It is not known how significant this might be in the overall cocoa fermentation scheme, although the indications are that it is not important since these organisms were detected only in Africa.

Identification of yeasts from Ghana and Malaysia

Tests applied

We (J.G. Carr and Patricia A. Davies) are particularly grateful to our colleague, Dr R.R. Davenport, an acknowledged expert on yeasts, for undertaking a preliminary examination of these organisms from both countries. His identification down to the level of the genus gives enough information to describe the kind of biochemical activities these organisms can perform. The tests applied were as follows:

(i) The production of carotenoid pigment. Some yeasts are orange in colour and this is a very constant diagnostic feature.

(ii) Colony morphology. The shape and size of colony is very varied amongst yeasts and can be used as a means of identification more easily for yeasts than bacteria.

(iii) Budding. Most yeast cells bud when reproducing vegetatively and the form in which they do this is strongly diagnostic. Some divide by fission.

(iv) Ascospores. These spores vary considerably in shape and size, and their appearance can be used for diagnosis of genera.

(v) Ability to ferment glucose and to assimilate nitrate.

	+ Fermentation	+ Nitrate	=	*Hansenula*
	+ Fermentation	- Nitrate	=	*Saccharomyces*
If lemon shaped	+ Fermentation	+ Spores	=	*Hanseniospora*
	- Nitrate	- Spores	=	*Kloeckera*

(vi) Details of yeasts' habitats, and the production of special end-products such as esters are very helpful in determining the kind of yeast.

A comparison of yeasts found in Ghana and Malaysia

It is interesting to note when comparing the yeasts isolated in the two countries that five genera were common to both (Tables 11 and 13), and that the predominant ones were *Candida*, *Kloeckera* and *Saccharomyces*. Of these *Candida* spp. tend to be weakly fermenting whereas *Kloeckera* and *Saccharomyces* spp. are strong fermenters. Of these two groups members of the genus *Kloeckera* could always be found earlier than *Saccharomyces* (Tables 12 and 14) which would indicate that the latter are probably the main fermenting organisms. All other yeasts were found less frequently, suggesting they were present in the role of 'passengers' and that their activities contributed only slightly to the overall chemical changes. Less yeasts were isolated from the African fermentations than those in Malaysia, which suggests that smaller numbers might have been involved in the former. However, the isolation of organisms from a mixture such as in fermenting cocoa is largely a matter of the preference shown by the operator, although it is fairly reasonable to assume that if there is less of one kind of organism than another this is reflected in the proportion of organisms actually isolated.

Most yeasts occur in the early stages of fermentation and this is probably due to the conditions becoming increasingly hostile. Sugars disappear, ethanol increases and as a consequence of this acetic acid increases. At the same time the temperature increases and it is,

therefore, surprising to see in Table 14 that strain M75a, a *Saccharomyces* sp. was isolated at 120 hours when the temperature was 50°C and the concentration of acetic acid 1.05% (Part 1). Members of this genus are particularly sensitive to acetic acid and to find one surviving such conditions is exceptional. In contrast, members of the genus *Schizosaccharomyces* are particularly resistant organisms since they were found on dried beans some 264 hrs after the start of fermentation.

The yeasts' reaction to high temperatures is much the same as most micro-organisms. While they only appear to grow weakly or not at all at 50°C they are tolerant of quite warm conditions and are able to grow when the temperature is slightly lower. Apart from their initial role in fermentation we do not know the subsequent function of the yeasts. The questions remain - what substrates do they use and what are the end products? Until we know the answer to these questions, the post fermentation role of yeasts in cocoa remains obscure.

TABLE 11

Tests on yeasts isolated in Ghana

Designation	15°	30°	40°	50°	Carotenoid pigment	Colony type	Budding form	Fission	Ascospores (2-4)	Ascospores (1)	Pseudomycelium	Fermentation	Nitrate assimilation	Ester formation	Lactic
Hansenula spp.					-	R†	M	-	H	-	+	+	+	+	
A1a	NT	+	+	W											
Kloeckera spp.					-	S	B	-	-	-	-	+	-	-	
A23	+	+	W	-											
A71	+	+	-	-											
Torulopsis spp.					-	S	M	-	-	-	-	±	±	-	
A80	+	+	W	W											
Saccharomyces spp.					-	S	M	-	C	-	-	+	-	-	
A44a	+	+	+	-											+
A88a	+	+	+	-											-
Candida spp.					-	R	M	-	-	-	+	±	±	-	
A12	+	+	-	-											
A43a	+	+	+	-											
A48	+	+	+	W											+
A72	+	+	+	W											
A76	+	+	W	W											
Pichia spp.					-	R	M	-	H	-	+	W	-	-	
A45	+	+	W	W											+
Schizosaccharomyces spp.					-	S	-	+	C	-	-	+	-	-	
A55	+	+	+	W											-
A96a	+	+	+	+											
A98a	+	+	+	+											
Saccharomycopsis spp.															
A11	+	+	-	-											

† For the key see Table 13

TABLE 12

Locations from which the yeasts were isolated in Ghana

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
Hansenula spp.				
A1a	Basket	NT	NT	Aerobic
Kloeckera spp.				
A23	Middle of box	4	30	Aerobic
A71	Bottom of heap 2	0	26	Aerobic
Torulopsis spp.				
A80	Box-drying stage	72	NT	Aerobic
Saccharomyces spp.				
A44a	Middle of heap 1	48	45	Anaerobic
A88a	Middle of heap 2	24	42	Aerobic 45°C
Candida spp.				
A12	Basket	NT	NT	Aerobic
A43a	Middle of heap 1	24	36	Aerobic
A48	Bottom of box	48	28	Aerobic
A72	Bottom of heap 2	0	26	Aerobic
A76	Bottom of heap 2	0	26	Aerobic 45°C
Pichia spp.				
A45	Middle of heap 1	48	45	Anaerobic
Schizosaccharomyces spp.				
A55	Top of heap 1	72	48	Aerobic
A96a	Box drying stage	264	NT	Aerobic
A98a	Heap 2 mechanical drying	192	NT	Anaerobic
Saccharomycopsis spp.				
A11	Basket	NT	NT	Aerobic

* Unless otherwise stated plates were incubated at room temperature (25-30°C)

NT Not tested

TABLE 13

Tests on yeasts isolated in Malaysia

Designation	15°	30°	40°	50°	Carotenoid pigment	Colony type	Budding form	Fission	Ascospores (2-4)	Ascospores (1)	Pseudomycelium	Fermentation	Nitrate assimilation	Ester formation
Hansenula spp.					-	R	M	-	H	-	+	+	+	+
M1	+	+	-	-										
M2	+	+	+	W										
M6a	+	+	+	-										
M98a	+	+	+	W										
Kloeckera spp.					-	S	B	-	-	-	-	+	-	-
M3	+	+	W	W										
M5	+	+	+	W										
M7	+	+	+	-										
M15a	+	+	W	-										
M18	+	+	W	-										
M31	+	+	+	-										
M80	+	+	+	W										
Torulopsis spp.					-	S	M	-	-	-	-	±	±	-
M52	+	+	+	W										
M81	+	+	W	W										
Saccharomyces spp.					-	S	M	-	C	-	-	+	-	-
M37	+	+	+	W										
M60b	+	+	+	W										
M64	+	+	+	W										
M68a	+	+	+	+										
M75a	+	+	+	W										
M76	NT	+	W	-										
M95	+	+	+	+										

/Contd

Table 13 contd

Designation	15°	30°	40°	50°	Carotenoid pigment	Colony type	Budding form	Fission	Ascospores (2-4)	Ascospores (1)	Pseudomycelium	Fermentation	Nitrate assimilation	Ester formation
Candida spp.					-	R	M	-	-	-	+	±	±	-
M4a	+	+	+	-										
M8	+	+	+	W										
M14	+	+	+	-										
M17	+	+	+	-										
M24	+	+	+	-										
M40	+	+	+	W										
M49	+	+	+	+										
M50	+	+	+	W										
M51	+	+	+	W										
M55	+	+	+	+										
M60a	+	+	+	W										
M74a	+	+	+	-										
Rhodotorula spp.					+	S	M	-	-	-	-	-	-	-
M57	+	+	W	-										
M77	+	+	+	W										
M82	+	+	-	-										
Debaryomyces spp.					-	S	M	-	-	R	+	W	-	-
M84	+	+	NT	W										
Hanseniospora spp.					-	S	B	-	H	-	-	+	-	-
28a	+	+	W	W										
28b	+	+	W	W										

KEY: S = Smooth
R = Rough
M = Multipolar
B = Bipolar
H = Hat shaped
C = Circular
± = Some species + and some -
W = Weak

TABLE 14

Locations from which the yeasts were isolated in Malaysia

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
Hansenula spp.				
M1	Sweatings	NT	NT	Aerobic
M2	Sweatings	NT	NT	Aerobic
M6a	Polybag	NT	NT	Aerobic
M98a	Trial 3 middle	48	36	Aerobic
Kloeckera spp.				
M3	Trial 1	0	31	Aerobic
M5	Polybag	NT	NT	Aerobic
M7	Sweatings	NT	NT	Aerobic
M15a	Polybag	NT	NT	Aerobic
M18	Trial 1 top	4	31	Aerobic
M31	Trial 1	0	31	Aerobic
M80	Trial 2 bottom	72	44	Aerobic
Torulopsis spp.				
M52	Trial 2 start	0	32	Aerobic
M81	Trial 2 bottom	72	44	Aerobic
Saccharomyces spp.				
M37	Trial 1 middle	24	33	Aerobic
M60b	Trial 2 bottom	24	NT	Aerobic 45°C
M64	Trial 2 middle	24	NT	Anaerobic
M68a	Trial 1 top	96	48	Anaerobic
M75a	Trial 1 top	120	50	Anaerobic
M76	Trial 1 middle	120	48	Anaerobic
M95	Trial 3 top	32	45	Aerobic

/Contd

Table 14 contd

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates*
Candida spp.				
M4a	Polybag	NT	NT	Aerobic
M8	Sweatings	NT	NT	Aerobic
M14	Sweatings	NT	NT	Aerobic
M17	Polybag	NT	NT	Aerobic
M24	Trial 1 bottom	4	31	Aerobic
M40	Trial 1 bottom	24	33	Aerobic
M49	Sweatings trial 2	NT	NT	Aerobic
M50	Trial 2 start	0	32	Aerobic 45°C
M51	Trial 2 start	0	32	Anaerobic
M55	Trial 1 middle	72	40	Aerobic
M60a	Trial 2 bottom	24	NT	Aerobic 45°C
M74a	Trial 1 top	120	50	Aerobic
Rhodotorula spp.				
M57	Trial 2 middle	24	NT	Aerobic
M77	Trial 1 middle	120	48	Anaerobic
M82	Trial 2 top	72	51.5	Anaerobic
Debaryomyces spp.				
M84	Trial 2 top	96	51	Aerobic 45°C
Hanseniospora spp.				
M28a	Sweatings	NT	NT	Anaerobic
M28b	Sweatings	NT	NT	Anaerobic

* Unless otherwise stated plates were incubated at room temperature (25-30°C)

NT Not tested

THE GENUS *BACILLUS*

Since Part 1 of this report was published these organisms have been re-examined according to the methods of Gordon *et al* (1973) and it has been found necessary to modify the names slightly. It is now considered that the African species consist only of those that can be included in the species *B. subtilis*. There is, however, a distinction between those that can grow weakly under anerobic conditions and those unable to do so. Amongst those isolated in Malaysia there is a single strain (M67) named *B. licheniformis* and the remainder are in the species *B. subtilis*.

From the numbers isolated it would seem that there were less of these organisms present than those already mentioned. They are, however, significant because of their ability to produce highly resistant spores and thus survive under conditions that cause other organisms to die. This is exemplified by strains A68, A69, A81, A92 that occurred on dried beans and, therefore, might grow as opportunists at a later date should the beans become moist.

Since the metabolism of these bacteria differs virtually from one species group to another it is not possible, as with the other organisms, to make a general statement about their behaviour in cocoa. Only experimentation with these organisms and the substrates available to them in cocoa would give a clue to the importance of their role in this habitat.

TABLE 15

Tests on *Bacillus* spp. isolated in Ghana and Malaysia

Designation	Spore shape	Spore position	Sporangium distension	Motility	Gram stain	Catalase	Anaerobic growth	Growth in 7% NaCl	Growth at pH 5.7	Acid from glucose	Arabinose	Mannitol	Gas from glucose	Voges Proskauer	Lecithinase	Casein decomposition	Gelatin decomposition	Tyrosine decomposition	Starch hydrolysis	Nitrate to nitrite	Indole	Utilisation of citrate	Propionate	Hippurate	Malic	Citric	Quinic	Succinic	Lactic	30°	40°	50°
Bacillus licheniformis	E	CT†	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	+	-								
M67											As above														+	+	+	+	NT	+	+	+
Bacillus subtilis (ability to grow anaerobically)	E	CT	-	+	+	+	W	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	-	-								
A58																									+	+	+	W	-	+	+	+
A68																									+	+	-	-	-	+	+	+
A69																									+	+	-	-	-	+	+	+
A81																									+	+	+	+	-	+	+	+
A92											As above														+	+	+	+	-	+	+	+
M79																									+	+	-	+	NT	+	+	+
M91																									+	+	-	+	NT	+	+	+
M100																									+	+	-	-	NT	+	+	W
M107																									+	+	-	+	NT	+	+	W
Bacillus subtilis	E	CT	-	+	+	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	-	-								
A62																									+	+	+	+	-	+	+	+
A64											As above														+	+	+	+	-	+	+	+
M85																									+	+	+	+	NT	+	+	+

†KEY: E = Ellipsoidal
CT = Central or terminal
W = Weak reaction

TABLE 16

Locations from which *Bacillus* spp. were isolated in Ghana and Malaysia

Designation	Location	Time after the start of fermentation (hrs)	Temperature at the time of isolation (°C)	Method of incubating plates
Bacillus licheniformis				
M67	Trial 1 top	96	48	Aerobic
Bacillus subtilis (ability to grow anaerobically)				
A58	Box middle	72	41	Aerobic
A68	Box drying stage	168	NT	Aerobic
A69	Box drying stage	168	NT	Aerobic
A81	Box drying stage	216	39	Aerobic
A92	Box drying stage	284	NT	Aerobic
M79	Trial 1 middle	144	45	Anaerobic
M91	Trial 2 top	144	51	Aerobic
M100	Trial 2 top	168	47	Aerobic 45°C
M107	Trial 3 middle	168	51	Aerobic 45°C
Bacillus subtilis				
A62	Heap 1 middle	120	43	Anaerobic
A64	Box top	120	50	Aerobic
M85	Trial 1 pre-dry	168	NT	Aerobic

OTHER ORGANISMS

The medium and conditions chosen for these isolations were designed to select for the acid tolerant yeasts, lactic and acetic acid bacteria and bacilli. In addition there were a number of organisms that grow on the plates that do not fall into the categories already mentioned. These usually occur sporadically and it is not thought that they contribute greatly to the general changes brought about during cocoa fermentation. There was, however, one group of Gram positive cocci that occurred in the African samples at the drying stage. It is difficult to see what changes they might bring about on the dried testa of the bean. From their appearance they seemed to be micrococci. No such coherent group was found on the beans in Malaysia.

CONCLUSIONS

In the relatively short time we devoted to the microbiology of cocoa we have posed as many questions as we have answered. For example, we do not know very precisely how the various micro-organisms interact with each other and the available substrates. Indeed we do not, as yet, have a comprehensive list of the possible substrates available to the micro-organisms. Having investigated these it would then be necessary to see how specific substrates are metabolized by particular organisms.

It has been said earlier that we slanted the conditions to isolate particular groups of organisms. Were we right to do this? Have we by this restricted approach perhaps missed something important? Should the next microbial examination use different methods and media for isolation to ascertain whether something different develops?

One point that causes some concern is our method of sampling. A handful of beans taken from a box containing several tonnes cannot be truly representative of the whole. Thus, any chemical or microbiological conclusions drawn from such a limited sample can only be an indication of what is happening within the box. Sampling methods that have some statistical validity would probably require taking a relatively large number of samples. Such a requirement would need a team of people to operate it. This should, however, be considered if future work were to be done.

The study of microbiological and chemical phenomena in cocoa should be done simultaneously since they are respectively a measure of cause and effect. It is recognised that the highly sophisticated technology now applied to chemistry enables the production of results rapidly, accurately and in great numbers. Microbiology has not reached the same degree of automation as chemistry and, therefore, still relies upon individual observations and manipulations so that the production of results takes longer. While it is recognised that the application of the two disciplines separately can yield a great deal of information about the process, a combined attack is much more effective. The empirical manipulation of the fermentation process in Malaysia has only been partially successful in reducing acidity. It is our contention that with a full understanding of the cause and effect at work in cocoa

fermentation, modification in processing methods could be carried out with far more understanding than at present.

Future investigations into acid cocoa

Experience with other mixed microfloras has shown that if an off flavour is produced one of the most effective means of curing it is first to identify the substance or substances causing the disorder and then to ascertain the chemical and/or microbiological conditions that give rise to the off flavour.

It seems to us that acid tainted chocolate may not all be from the same cause because it is sometimes described as acidic and sometimes as fruity. It therefore needs the application of a flavour profile with the necessary vocabulary. When an expert panel of tasters has decided what is acidic chocolate and what is not then a comparison between the two kinds can be made and the substance identified. Having identified the substance the opportunity for ascertaining the cause is much greater.

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COCOA FERMENTATION IN GHANA AND MALAYSIA

(Part 2)

Methods for the Analysis of Cocoa Pulp and Cotyledons

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METHODS FOR THE ANALYSIS OF COCOA PULP AND COTYLEDONS

Sampling

(a) Cured cocoa

Procedures laid down by national standard organisations should be consulted.

(b) Fermenting cocoa

Generally in our work we have sampled from three sites daily: the top, middle and bottom positions of the fermenting mass. The sites are selected to represent three different aeration conditions. Sample sizes were approximately 400 gms.

Sample Preparation

The analysis of cocoa pulp presents some difficulty. It is all too easy to express pulp juice during the peeling process. An alternative procedure is to analyse whole bean samples (i.e. unpeeled) and cotyledons. The concentration of pulp constituents may then be obtained by difference. All of the early work was carried out by peeling the beans.

Reagents

0.2% Benzoic acid solution
6% Barium hydroxide solution
5% Zinc sulphate solution
Cation exchange resin

Reagent Preparation

The barium hydroxide solution is conveniently prepared a day prior to use by adding 6 gms to 100 mls of water in a stoppered reagent bottle. The bottle is shaken at intervals throughout the day and filtered into a stoppered reagent bottle when required.

Pipette a 10 ml aliquot of the barium hydroxide into a conical flask containing 200 mls of freshly boiled distilled water and titrate with 5% zinc sulphate solution using phenolpthalein as indicator. Adjust the solution strengths by appropriate dilution as indicated by the titration result.

Ion Exchange Resin

Wash the cation exchange resin with 2M hydrochloric acid followed by repeated distilled water washings until neutral and salt free as determined by silver nitrate solution. The resin is then dried on filter paper until it is free flowing.

Procedure

Remove the pulp and testa from 50 beans. Weigh 20 cotyledons to the nearest 0.01 gm and homogenize in a blender for 3 minutes with 200 ml of 0.2% benzoic acid solution. Weigh 20 gms of pulp and testa to the nearest 0.01 gm and homogenize in a blender for 3 minutes with 200 mls of 0.2% benzoic acid solution.

Centrifuge the solutions, pipette a 10 ml aliquot into a centrifuge tube followed by 5 mls of 5% zinc sulphate solution and 5 mls of 6% barium hydroxide.

Shake vigorously, centrifuge and decant into a sample bottle containing 1 gm of the prepared cation exchange resin. Aliquots are taken from this prepared solution for the determination of acetic acid, lactic acid, ethanol and total sugars. Citric acid is removed in the clean up procedure. Commercial kits are available for the estimation of alcohol,acetate and lactate by enzymic procedures.

Recoveries

100% recovery is achieved during the clean up procedure for ethanol, acetic acid, glucose and lactic acid. Final results should, however, be corrected for the initial moisture content of the samples which results in the solution volume being greater than 200 mls. This problem can be avoided if the solutions are made up to a fixed volume of 500 ml.

DETERMINATION OF THE pH OF COTYLEDONS AND PULP

Reagents

1. Standard acid potassium pthalate buffer at pH 4.00 at 20°C
2. Phosphate buffer at pH 6.88 at 20°C

Reagent Preparation

0.05M - Potassium hydrogen pthalate. Dissolve 10.21 g of the solid (dried below 130°C) in water and dilute to 1 litre. The pH is not affected by atmospheric carbon dioxide. The solution should be replaced after 5-6 weeks, or earlier if mould growth is apparent.

0.025M - Phosphate buffer. Dissolve 3.40 g of KH_2PO_4 and 3.55 g of Na_2HPO_4 (dried for 2 hours at 110-130°C) in carbon dioxide - free water and dilute to 1 litre. The solution is stable when protected from undue exposure to the atmosphere.

Procedure

(a) Cotyledons

Grind 12 g of cotyledons in a coffee grinder. Weigh 10 g of ground sample into a 150 ml beaker and slowly add, with stirring, 90 ml boiling water. The suspension must be free from lumps. Filter, cool filtrate to 20-25°C and immediately determine the pH using electrodes and potentiometer standardized with buffers at pH 4.00 and 6.88. Report to the nearest 0.1 pH unit.

(b) Pulp and testa

Weigh 10 g of sample into a 150 ml beaker and then proceed as above for cotyledons.

Enzyme Solution 4 - a preparation of 5 mg/ml acetate kinase

Standards: 136 mg sodium acetate → 100 mls (i.e. 10 mls soln)

1) 2 mls → 100 (1.2 mg/100 mls)
2) 6 mls → 100 (3.6 mg/100 mls)
3) 10 mls → 100 (6.0 mg/100 mls)
4) 15 mls → 100 (9.0 mg/100 mls)
5) 20 mls → 100 (12.0 mg/100 mls)
6) 25 mls → 100 (15.0 mg/100 mls)

Procedure

Wavelength 340 mm, light path 1 cm, temperature 29°C

Pipette into test tube	Blank	Sample
Buffer soln I	3.0 ml	3.0 ml
Soln 2	.10 ml	.10 ml
Enzyme soln 3	.01 ml	.01 ml
Sample	-	.10 ml
Distilled water	.10 ml	-
Mix and read absorbence (E_1) after 3 mins		
Enzyme soln 4	.01 ml	.01 ml

Incubate mixed contents at 29°C for 40-65 mins then read final absorbence (E_2). Calculate change in absorbence i.e. (E_1 - E_2)

Calculation

Prepare a standard curve using suggested dilutions.

Standard Dilutions

Day			
1	0.1 ml sample	(F = 1)	
2	.04 ml	"	0.6 ml H_2O (F = 2.5)
3	.03 ml	"	.07 ml H_2O (F = 3.33)
4	.02 ml	"	.08 ml H_2O (F = 5)
5	.01 ml	"	.09 ml H_2O (F = 10)
6	.01 ml	"	.09 ml H_2O (F = 10)
7	.02 ml	"	.08 ml H_2O (F = 5)

DETERMINATION OF ETHANOL BY ENZYMIC ASSAY

Principle

Enzymic method using alcohol dehydrogenase and NAD. Several methods have been published; this one is based on that of Tn. Bücher and H. Radetzti. *Klin. Wschr.* 29, 615 (1951).

Reaction

$$C_2H_5OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

Apparatus

1 Spectrophotometer suitable for accurate measurements at 340 nm

Reagents

1. Sodium pyrophosphate decahydrate
2. Semicarbazide HCl, alcohol free
3. Glycine
4. Potassium hydroxide 4N
5. Nicotinamide - adenine nucleotide (NAD) free acid
6. Alcohol dehydrogenase (ADH) from yeast, lyophilized
7. Ethanol standard solutions: .1, .2, .3, .5, .7 mg/ml

Preparation of Solutions

I - Buffer 10 g sodium pyrophosphate + 2.5 g semicarbazide HCl + 0.5 g glycine dissolved in 250 mls H_2O. Adjust to pH 8.7 with about 5 mls 4N KOH and dilute to 300 mls with distilled H_2O. Check pH, 2 day intervals.

II - NAD 50 g NAD in 3 mls distilled H_2O

III - ADH 50 mg ADH in 1 ml distilled H_2O

Store all solutions at 0-4°C; do not leave at room temperature longer than necessary. Solution I stable for 3 weeks, Solution II stable for 4 weeks, Solution III stable for 2 weeks. Do not use Solutions II and III immediately after preparation, wait approximately 2 hours at 0-4°C before carrying out analysis.

Pipette into separate test tubes:

	Blank	Standards	Sample
Buffer I	4.80 ml	4.80 ml	4.80 ml
NAD II	.10 ml	.10 ml	.10 ml
Mix and read absorbence at 340 nm against water; always carry out two blanks; read these also against water (E_1)			
ADH III	.02 ml	.02 ml	.02 ml
Mix, incubate stoppered tubes, for 70 mins in a water bath at 25°C. Read final absorbence at 340 nm against water (E_2)			

Calculation

Calculate (E_2-E_1) for all blanks, standards and samples, subtract average (E_2-E_1) for blanks, from standards and samples and prepare a calibration curve. Read off concentrations of samples in mg/ml.

Sample Dilution

Days 1 and 7 use .10 ml of sample as provided.

Days 2-6 use .05 ml sample provided + .05 ml H_2O. Multiply result by a factor of 2.