STUDIES RELATING TO THE BACTERIA ASSOCIATED WITH RICE AND OTHER CEREALS.

By Gilbert J. Fowler, and Dhiresh Lobhan Sen.

Introduction.

The researches described in the following pages originated through a request received from the Indian Munitions Board to investigate the bacterial factors of influence in the manufacture of starch.

This in the first place meant the determination of the sources of infection in a starch factory and of the conditions which might lead to fermentation and consequent loss of starch.

Among raw materials for starch production, rice was chosen as suitable for special study and it was found that the chief source of bacterial infection was the grain itself.

The most satisfactory methods of cleaning and sterilising the grain for use or for storage were consequently examined and from this arose naturally the special study of the most resistant bacteria.

Among these was found to be a bacillus similar at any rate to the bacillus isolated by Weizmann from grain, and employed by him for the fermentation of starch with production of acetone and butyl alcohol.

The attempt to isolate this bacillus, according to the method patented by Weizmann *i.e.* by successive sub-cultures into nearly boiling paddy mash lead to the general study of the fermentations produced in rice by the bacteria surviving under different conditions of selective sterilisation. This work has a bearing on the question of possible transmutation of species by selective sub-cultures.

It was found incidentally that ordinary methods of cooking rice by no means attained sterility but that the more nearly sterility was attained the more nearly a pure acetone fermentation resulted.

A comparative study of polished and unpolished rice in this connection revealed the fact that the former was less easily

sterilised than the latter. On examination polished rice was found to carry more bacteria than unpolished rice, for the reason that the process of polishing not only removes the protecting epidermis of the grain but also gets rid of an alkaloid present in the unpolished grain (as already noted by F. Hofmeister) which acts as a selective antiseptic.

This natural process of selective antiseptic action of a substance secreted by the grain has been noted in connection with another seed (*Cassia tora*) which is used as a fermenting agent in the indigenous indigo dye-vat and which is the subject of a research shortly to be published by Mr. Srinivasiah and one of us.

These observations lead to the conclusion that there is a definite symbiosis between certain seeds and certain bacteria, analogous in some respects to that which has been long observed in regard to the roots of leguminous plants and the organisms concerned in nitrogen fixation.

The subject obviously affords a large and important field of study and the present paper is in many respects of a preliminary character only. It appears however desirable to place on record the results so far obtained.

The work falls into five parts :—

Part I. Some bacterial factors in the starch industry.
,, II. Methods for sterilisation of grain.
,, III. Conditions of sterility of cooked rice.
,, IV. Fermentation conditions of starch, with special reference to the acetone fermentation.
,, V. Biochemical observations on polished and unpolished rice.

The work described in part I and a portion of part II was done in collaboration with Mr. M. B. Roy, to whom our best thanks are due.

PART I.—Some bacterial factors in the starch industry.

The process of preparing starch from either grain or tubers may be briefly outlined as follows :—

The raw material is washed, steeped and ground with water, so that the starch separates from the husk, germ and other constituents of the grain. The milky suspension of starch is then

passed through a fine sieve leaving most of the remaining material behind. A certain amount of fine debris of the grain will pass through the sieve, including nitrogenous matter capable of fermenting in the settling tanks in which the starch is deposited. This nitrogenous matter is removed either by means of an acid fermentation in the vat or by treatment with alkali. The deposited and purified starch is well washed, filter pressed and dried.

Loss of starch by fermentation is likely to occur during the preliminary steeping process and the later settlement process.

Serious loss will of course occur if germination of the grain takes place during storage not only if a large proportion of the grain germinates, but as will be seen later if only a small proportion begins to sprout, as sugar is produced which forms a pabulum for bacteria to thrive upon and so increase the likelihood of further infection.

As already stated the investigation was taken up at the request of the Munitions Board, with a view to determine the main sources of infection in a starch factory, the conditions leading to fermentation and consequent loss of starch, and the best methods for minimising such losses.

The two most probable sources of bacterial infection in a starch factory are:—

(a) Bacteria in the air of the factory,

(b) ,, on the surface of the grain.

(*a*) *Bacteria in the air.*

In order to study the possibilities of infection from the air, plates of (1) starch agar (2) peptone starch agar (3) nutrient (lemco peptone salt) starch agar were prepared and exposed to the atmosphere of different rooms in the Department of Applied Chemistry for a few hours.

The following table indicates the results obtained:—

Room.	*Growth on various media.*		
	Starch Agar.	Peptone starch Agar.	Nutrient starch Agar.
Balance room.	nil.	10 colonies.	Numberless.
Incubator room.	,,	Numberless.	,,
Machine room.	Mould.	,,	,,

On examining these colonies under the microscope, it was found that they consisted mainly of cocci with a few rod

forms of baccilli. Very dilute iodine solution was spread on the colonies and no starch reaction was observed.

Comparing this result with the fact that plates consisting only of starch agar showed no growth, it is clear that although bacteria from the air do not grow and develope on starch, yet when they grow freely on ordinary nutrient media a sufficient number develope which secrete amylase and so bring about the destruction of the starch.

This conclusion was confirmed by similarly exposing various solutions containing starch.

(1) *2% starch solution.*

It was found that some moulds appeared after 5 days but the starch was not appreciably affected.

(2) *2% starch solution with 0·5% glucose.*

After two days' exposure it was observed that the liquid was fermented and gave an alcoholic odour. The fermented solution examined under the microscope showed the presence mainly of cocci.

(3) *2% starch solution with 0·5% malt extract.*

Fermentation started after 48 hours and the organisms present in the liquid were chiefly cocci.

(*b*). *Bacteria on the surface of the grain.*

In order to study the bacteria present on the grains, paddy, ragi, jawar, wheat and maize were taken as examples.

After the mechanical removal of dust and other foreign solid particles by sieving and winnowing, these grains were washed three times with sterile distilled water and the last washing was tested for its bacterial contents. The third washing was taken every time because in practice the grains are washed in starch factories before use.

The media which were used for the growth of these organisms were the following:—

(1) Pure starch agar,

Starch	...	2%
Agar agar	...	1·5%

and remaining distilled water.

(2) Nutrient starch agar.

Peptone	...	1·0%
Agar Agar	...	1·5%
Lemco	...	0·5%
Sodium chloride	...	0·5%
Starch	...	2·0%

and remaining distilled water.

(3) Peptonised starch agar.

Peptone	...	0·5%
Agar Agar	...	1·5%
Starch	...	2·0%

and remaining distilled water.

In each case blanks were kept as control.

After inoculation the plates were incubated at 37°C. The results are tabulated below :—

Grains.	Growth on starch agar.	Growth on peptonised starch agar.	Growth on nutrient starch agar.	Time of incubation.
Bangalore paddy	Nil	Nil	10 colonies.	24 hrs.
	,,	4 colonies.	Enlargement of these colonies and more colonies had appeared.	48 ,,
	Mould	Colonies enlarged and moulds appeared.	Numberless colonies with the appearance of moulds.	72 ,,
Ragi	Nil	10 colonies.	Numberless colonies	24 ,,
	Moulds	Colonies enlarged.	Do	48 ,,
	Do	More colonies appeared and the plates were covered with moulds.	Do but moulds appeared.	72 ,,
Jawar.	Nil	13 colonies.	Numberless.	24 ,,
	,,	Numberless.	Do	48 ,,
	,,	Do	Do	72 ,,

With wheat and maize almost the same results were obtained as with jawar. Here also no colony was found on starch agar.

All colonies were carefully examined under the microscope and it was found that three distinct kinds of micro-organisms could be seen *viz.*, (a) involution forms (b) motile termo and (c) motile cocci.

Streak cultures were made from each plate and then a very dilute solution of iodine was added to half the plate. All plates except the starch agar gave no reaction showing that the starch was decomposed by bacteria.

From a careful study of the tabulated experimental results we can draw the following conclusions :—

Definite species of organisms seem to infect the grains. Pure starch agar is not affected since no nitrogenous pabalum is present in the medium.

The bacteria reside on the surface of the grains and live long in the form of spores which take some days to develope on the plates. Moulds also are present along with them.

In order to prevent the growth of moulds on the plates, the grains were washed with 0·5% copper sulphate solution. The copper sulphate was then removed from the grains by washing three to four times with sterile water and the final wash was plated out on all the three media. It was found that the growth of moulds was prevented and at the same time the number of bacterial colonies was appreciably reduced. The peptonised starch agar plates which were similarly inoculated, grew colonies which on examination were found to be mostly cocci. This important observation leads us to believe that copper sulphate destroys first moulds and then the bacteria, while the elimination of cocci could only be affected by a longer treatement with copper sulphate solution or with solutions of higher concentration (*cf.* Part 2).

Streak cultures were made from every plate that was inoculated and all of them were allowed to grow under strictly anærobic conditions.

After nine months the tubes were taken out and sub-cultures were made out of them in nutrient starch agar medium.

After 24 hours' growth to allow the transformation of spores into adult organisms, the colonies were examined and only cocci could be found — a significant abservation showing the frequently observed phenomenon that the cocci is the last surviving organism when grain is washed or treated with a mild antiseptic like dilute copper sulphate.

These cocci were inoculated into 3·6% rice mash to which 0·5% ammonium phosphate had been added. No fermentation took place, owing possibly to the conditions not being strictly anærobic.

Conclusion to Part I.

Having regard to the fact that considerable growths of bacteria were obtained from a third washing of the grain, it is clear that the main source of infection in a starch factory where cereals are employed as raw material, is the grain itself. This in its turn will help to infect the air.

Starch freed from sugar or nitrogenous material is not readily attacked by bacteria. If incipient germination takes place in even a portion of the grain on storage, fermentation of the starch in the remaining unchanged grain is greatly facilitated.

Fermentation and loss of starch is likely to take place in steeping and settling vats so long as nitrogenous matter is present.

The remedy against loss in this way lies in (a) sterilisation and disinfection of the stored grain (b) the addition of antiseptics to steeping and settling vats together of course with cleanliness and rapidity in carrying out the general operations.

PART II – Sterilisation of Grain

Having found the sources of bacterial infection the problem of preserving grain was next undertaken.

The preservation of grain becomes a difficult task when the grain is meant for seed or for human consumption. These requirements narrow the possibilities very considerably and put out of court most common insecticides and non-volatile chemical disenfectants. The sterilisation process designed to prevent bacterial infection will be also effective in preventing the damage due to the insects called 'weevils' which feed upon the starch contents of the grain. (J. H. Barnes and A. Grove, J. Agric. India, 1916).

Methods of sterilising grains may be classified under two heads:—(1) wet method (2) dry method.

In the wet method, solutions of copper sulphate, chlorine water and sulphurous acid were tried.

The dry method was tried with sulphur dioxide, carbon dioxide, chlorine, ozone and naphthalene.

General procedure "wet" method. A known weight of grain was steeped in a definite volume of antiseptic solution for a known time. It was then freed from the antiseptic by washing with sterile distilled water. 1 cc. of the third washing was inoculated into agar, nutrient starch agar and pure starch agar (as described in part 1). and incubated at 37°C.

The results are given in the following tables :—

WET PROCESS.

Grain.	Weight of grain taken.	Antiseptic.	Volume of antiseptic.	Time of steeping.	Washing.	Media infected.		Time of incubation.	Nature of micro-organisms.
						Peptonised starch agar.	Nutrient starch agar.		
Paddy.	10 gms.	Chlorine water.	5 cc.	20 min.	3rd	Growth.	Growth.	24 hrs.	Rod shaped and cocci.
,,	do.	do.	10 cc.	do.	do.	do.	do.	do.	Few rods and cocci.
,,	do.	do.	15 cc.	do.	do.	Growth diminished.	Growth diminished.	do.	do.
,,	do.	do.	20 cc.	do.	do.	Slight growth.	Slight growth.	do.	Cocci.
,,	do.	do.	25 cc.	do.	do.	Nil.	Nil.	do.	Nil.
N. B.—Available chlorine per cc. of the antiseptic solution is 0·0042 gm.						None after even 72 hours incubation.			
,,	30 gms.	0·5% copper sulphate.	50 cc.	2 hrs.	do.	Growth.	Growth.	do.	Rod shaped and cocci.
,,	do.	1·0% copper sulphate.	do.	1 hr.	do.	Slight growth.	Slight growth.	do.	Mostly cocci.
,,	do.	2·0% copper sulphate.	do.	½ hr.	do.	Nil.	2 Colonies.	do.	Cocci.
,,	do.	do.	do.	1 hr.	do.	Nil.	Nil.	do.	Nil.

WET PROCESS.—*Continued.*

Grain.	Weight of grain taken.	Antiseptics.	Volume of antiseptic.	Time of steeping.	Washing.	Media infected.		Time of incubation.	Nature of micro-organisms.
						Peptonised starch agar.	Nutrient starch agar.		
Paddy.	10 gms.	Dilute sulphurous acid.	5 cc.	20 min.	3rd	Growth.	Growth.	24 hrs.	Rods and cocci.
,,	do.	do.	10 cc.	do.	do.	do.	do.	do.	do.
,,	do.	do.	15 cc.	do.	do.	Slight reduction.	Slight reduction.	do.	do.
,,	do.	do.	20 cc.	do.	do.	do.	do.	do.	do.
,,	do.	do.	25 cc.	do.	do.	More diminished.	...	do.	Few rods and cocci.
,,	do.	do.	30 cc.	do.	do.	do.	do.	do.	do.
,,	do.	do.	35 cc.	do.	do.	Appreciably diminished.	...	do.	Mostly cocci.
,,	do.	do.	40 cc.	do.	do.	Considerably diminished.	...	do.	Cocci.
,,	do.	do.	45 cc.	do.	do.	Almost none.	...	do.	do.
,,	do.	do.	50 cc.	do.	do.	Nil.	...	do.	Nil.

N. B.—The strength of sulphurous acid is 0·44 normal.

General Procedure "dry" method.

Miniature tin receptacles or bins were constructed with inlet and outlet tubes one at the top and the other in the side. They were thoroughly washed dried and sterilised. The tubes were provided with a cork and a glass tube, which after filling with the gas could be easily sealed up, and the cork covered with sealing wax.

To begin with, the grains were put into the bins and the gas passed in. Then after filling, the glass tubes were sealed up and kept for a number of days, after which the bins were opened, emptied and the grains washed a number of times with sterile distilled water till free from the antiseptic. The final wash was used for inoculation. The results are tabulated in the next page.

Effect of antiseptics on germination.

In connection with the sterilisation of the grain by the dry process, the experiment was utilised to determine the effect of antiseptics on germination. Washed grain was placed for this purpose on wet sand for germination. The germination table is given in the next page.

The whole question of the possible relation of bacteria to the power of germination is one of great interest and will be referred to more in detail in Part V.

Dry Process.

Grain.	Weight ol grain.	Antiseptic.	Time of keeping.	Washing.	Media infected		Time of incubation	Nature of micro-organisms
					Peptonised starch agar.	Nutrient starch agar.		
Paddy	50 gms.	Chlorine gas	37 days	3rd	No growth	No growth 1 mould	24 hrs. 48 hrs.	Nil
do	do	Sulphur dioxide gas	29 ,,	do	do	No growth	48 hrs.	do
do	do	Carbon dioxide gas	11 ,,	do	Growth	Growth	24 hrs.	Few rods and cocci
do	do	Ozone	2 ,,	do	do	do	do	Mostly cocci
do	do	Naphthalene flakes	47 ,,	do	nil	do	do	Few rods and cocci
						do	48 hrs.	do

Germination table of the grain sterilised in the above dry process.

Antiseptic		Nature of germination.
Chlorine gas	...	Vitality lost.
Sulphur dioxide	...	do
Carbon dioxide	...	Germinated.
Ozone	...	do
Naphthalene	...	Slow germination.

N. B.—In each of the above cases the grains were washed with sterile water to remove the antiseptic and then kept on sterile sand soaked with sterile water for germination.

Conclusions to Part II.

It will be seen from the tables that on the whole, the dry method of sterlisation is more efficient and convenient than the wet method. The inefficiency of the wet method is not so much due to the character of the antiseptic used but to the greater difficulty in obtaining closeness of contact between the grain and the antiseptic, by this method.

The presence of an oil (ref. analysis page 140) on the husk repels water and consequently the antiseptic solution does not come in close contact with the husk on which the organisms reside.

"*Wet method*"—Increasing the volume of chlorine water for a given weight of paddy progressively diminishes the number of micro-organisms. The same is observed in the case of dilute sulphurous acid. The kinds of bacteria present initially seem to disappear in the following order.

First moulds, second bacteria and lastly cocci. This is clearly brought about in the case of chlorine water and also in the case of copper sulphate and dilute sulphurous acid.

An increase in the concentration of antiseptic was tried with copper sulphate and as might be expected higher concentrations act more quickly but there is the disadvantage that the copper sulphate penetrates into the grains if they are unsound. Some observations on the time factor have been made in the case of copper sulphate. Increasing the period of contact means increase of antiseptic effect but greater danger of absorption of copper sulphate by the grain.

The 'wet method' has the further disadvantage of making the grains wet and for a further preservation of the sterilised, grains, it will be necessary to dry them again completely prior to their being stored. This means extra cost and labour and some risk of germination. This method however can be successfully used in the case of grains which are going to be immediately manufactured into starch.

The '*dry method*' has many advantages over the 'wet method'. Easy penetration, easier handling of cereals and the saving of cost and labour involved in the drying of grains—are some of the merits of the dry method.

With regard to the efficiency of the dry method the results with chloride and sulphur dioxide as shown in the table speak for themselves. In the case of ozone only a single experiment was done, circumstances preventing further trials.

One great disadvantage of the dry method is that the vitality of the grain considerably diminishes and paddy kept in an atmosphere of chlorine or sulphur dioxide and the diminishing of the period of sterilisations, may allow the vitality or the germinating power of the grain to be maintained. Rough comparative estimates of the cost of sterilising one ton of paddy by each of the antisepties used are given in the following statement:—

Cost of sterilisation of one ton of paddy according to current market prices.

Copper sulphate	£ 1	6	5
Chlorine (calculated from bleaching powder)...	£ 3	6	5
Sulphur dioxide (calculated from sulphur) ...	£ 0	14	0

It will be seen from the figures given above that sulphur dioxide is the cheapest of the chemicals that could be used succesfully for the sterilisation of starch grains. Copper sulphate though comparatively cheap could be used only in the wet process while chlorine is out of all question being about four times as costly as sulphur dioxide. We have shown the advantage of this particular dry process as affecting a more complete sterilisation. It is easy to handle and has all the advantages of chlorine, as the sulphur dioxide gas can be blown through a whole bin containing grain and effects sterilisation. Thus with respect to all factors, sulphur dioxide recommends itself.

PART III.—Conditions of sterility of cooked rice.

In view of the resistant character of the organisms occurring on rice and the great importance of this grain as a food, a bacterial examination of rice cooked under various conditions is of special interest.

The difficulty of completely sterilising masses of cooked grain was impressed upon one of us in the course of experimental work on the fermentation process for producing acetone.*

Rice comes on to the market in four principal forms.

Paddy. This is the raw unhusked grain as it comes from the rice fields.

* Research Notes on the Acetone Fermentation Process in India—Fowler, Wad & Gokhale, Journ. Ins. Sci. Vol. IV p. 1

Husked paddy. This is paddy from which the husk only has been removed by simple agricultural methods. This is termed *unpolished rice.*

Polished rice is the product of modern machinery. The polishing process removes both husk and the underlying red skin which is left intact in the preparation of unpolished rice. The germ is also removed in the polishing process.

Boiled rice. This is a fourth from which is obtained by boiling paddy with water till it becomes soft. On again drying the husk can be very readily removed. In Burmah this product is known as "steamed rice". We were unable during the time of our experiments to obtain any of this product.

Two kinds of rice from different localities, one Bangalore polished rice, and the other Malabar unpolished rice were however examined.

The rice was placed in a sterile flask, a definite amount of water added and the mixture heated at a definite pressure for a definite time. The sample thus cooked was kept in the incubator at 37°C and examined after every 24 hours to see if any fermentation had set in.

The results of these experiments are tabulated below :—

SAMPLE I. POLISHED RICE.

Boiled under Pressure.	Quantity of rice.	Time of boiling.	Observation.	Remarks.
0·0 lb.	25 gms.	20 min.	Fermented after 48 hrs.	Bad smell.
5·0 lbs.	do.	do.	do. 72 hrs.	Agreeable smell.
10·0 lbs.	do.	do.	do. 144 hrs	Pleasant smell.
15·0 lbs.	do.	do.	Slight frothing after 11 days.	Faint good smell.
20·0 lbs.	do.	do.	Remained sterile for 68 days when the flask was accidentally broken.	

SAMPLE II. UNPOLISHED RICE.

Boiled under Pressure.	Quantity of rice.	Time of boiling.	Observations.	Remarks.
0 0 lb.	25 gms.	20 min.	Fermented after 72 hrs.	Bad smell.
5·0 lbs.	do.	do.	do. 10 days.	Pleasant smell.
10·0 lbs.	do.	do.	Not fermented.	Completely sterile.

It will be seen from the tables that when cooked at atmospheric pressure for 20 minutes, polished rice goes bad after 48 hours, while unpolished rice remains sweet up to 72 hours. With increase of pressure the rice becomes more nearly sterile.

In the case of the polished rice the optimum temperature for complete sterilisation is 126°C which corresponds to 20 lbs pressure the heating being continued for 20 minutes but in the case of unpolished rice a temperature of 115°C maintained for 20 minutes corresponding to 10 lbs pressure, is quite enough to make it completely sterile. This shows that polished rice organisms are more numerous and more resistant than the organisms of the unpolished rice.

This interesting observation was followed up by further investigation of the organisms present.

An equal weight *viz.* 25 gms of each kind of rice was washed with a litre of sterile water. 1 cc of the washings from each of the two kinds of rice was plated out on nutrient agar.

After 24 hours incubation, the plates from the polished rice presented a mass of colonies which could not be counted whereas only 20 colonies grew during the same time on the plates made from the unpolished rice washings.

The organims from the polished rice were of different kinds, among which long spirals were present, which were absent from the plates prepared from unpolished rice. The organisms on these were almost all of one kind, in fact the culture was practically pure.

The foregoing observations indicate (a) that the ordinary processes of cooking rice cannot be relied upon to ensure sterility, (b) that the fermentation is of a less objectionable character the nearer the mass approaches sterility and (c) that polished rice is more difficult to sterilise than unpolished rice.

The meaning of these results is more fully investigated in the following pages.

PART IV.—Fermentation conditions of starch with special reference to the acetone fermentation

On discussing with Sir Alfred Bourne the difficulty as shown by the foregoing experiments of sterilisation by ordinary cooking processes, he pointed out the possible pathological significance of the results if it should prove that the fermentation gave

rise to the production of acetone. In this connection he drew our attention to a recent American patent by Weizmann (No. 1315585) in which a method is given for isolating a bacillus from maize meal capable of fermenting starch to acetone and butyl alcohol.

One of us had had considerable experience with this fermentation (*loc cit*) using cultures brought from England which had been developed on maize.

It was by no means necessarily the case that a bacillus present on maize in England should also occur on paddy in India. It was of interest therefore to see if the acetone bacillus could be isolated from paddy by the Weizmann method. It was accordingly searched for in the local paddy, available in the Bangalore market, according to the Weizmann method which may be summarised as follows :—

A number (say 100) of cultures are prepared by inoculating *e.g.* hot (say 90°C to 100°C) dilute (say 2%), sterile maize mash with some maize meal, and then they are allowed to ferment at about 35°C to 37°C for about 4 or 5 days.

A number of tubes are selected from these above cultures, which show most vigorous fermentation. These tubes are then heated up to 90°C for a period of one to two minutes. Many of the bacteria are destroyed but the desired resistant spores remain. Next some sterile maize tubes are inoculated with the culture which has been heated as aforesaid so obtaining sub-cultures. When these sub-cultures are completely fermented, further sub-cultures are made by inoculating these fermented tubes into maize mash at a temperature of nearly 100°C, and cooled quickly just one minute after inoculation. This operation is repeated a number of times, by which almost all the bacteria will be eliminated except the bacillus *Granulobacter pectinovorum*. These bacteria can than be used in the production of acetone and butyl alcohol under aerobic conditions, by inoculating with the final culture, a cooled solution or suspension of the selected substrate *e.g.* maize, which has been previously sterilised for 3 to 4 hours at a temperature of 130°C to 140°C and a pressure of 2 to 3 atmospheres. The whole thing is kept at about 37°C and allowed to ferment. After completion the fermented mash is distilled and fractionated.

In our case 'paddy' was taken as raw material instead of maize and preparations were made as follows :—

EXPERIMENTAL MATERIAL.

1. *Paddy mash* :—This was prepared in the following ways :—

Bangalore paddy was powdered to a fine flour in a sterile coffee grinder. 40 gms of this paddy powder were mixed with two litres of distilled water and then boiled in an autoclave for 5 hours at 15 lbs. pressure. To each of four dozen sterile tubes 20 cc of this sterile mash was added and they were further sterilised in a steamer for three successive days. The sterile mash contained 2% paddy powder.

2. *Paddy meal* :—100 gms of the powdered paddy were thoroughly mixed with 1500 cc of sterile water.

Now six paddy mash tubes were inoculated with 2 cc of the above freshly prepared paddy meal, at a temperature of boiling water and quickly cooled just one minute after inoculation. These inoculated cultures were incubated at 37°C for fermentation.

The contents of all the tubes were fermented after two to three days. When the fermentation was complete, another set of paddy mash tubes were inoculated with the corresponding fermented material at a temperature of nearly 90°C to 100°C and quickly cooled just one minute after inoculation as above. These series of inoculated tubes were distinguished as sub-culture I or SI. When these sub-cultures were completely fermented another set of paddy mash tubes were inoculated as above and they were designated as sub-culture II or SII. It was found that tube No. IV and its descendant sub-cultures showed vigorous fermentation. In this above process, the ninth sub-culture was arrived at from tube No. IV within two months.

The accompanying fermentation tables will make the above procedure clearer :—

Fermentation Table.

Date 1920	Tube No. I	Tube No. II	Tube No. III	Tube No. IV	Tube No. V	Tube No. VI
14—2	Inoculated	Inoculated	Inoculated	Inoculated	Inoculated	Inoculated
17—2	+ S1	+ S1	+ S1	+ S1	+ S1	– Nil
19—2	+ S2	– Nil	+ S2	See special next table	+ S2	– Nil
23—2	+ S3	– Nil	– Nil		+ S3	– Nil
26—2	+ S4	– Nil	– Nil		– Nil	– Nil
1—3	+ S5					
7—3	– Nil	– Nil	– Nil		– Nil	– Nil
8—3	+ S6	– Nil	– Nil		– Nil	– Nil
		– Nil	– Nil		– Nil	– Nil
		Rejected				Rejected

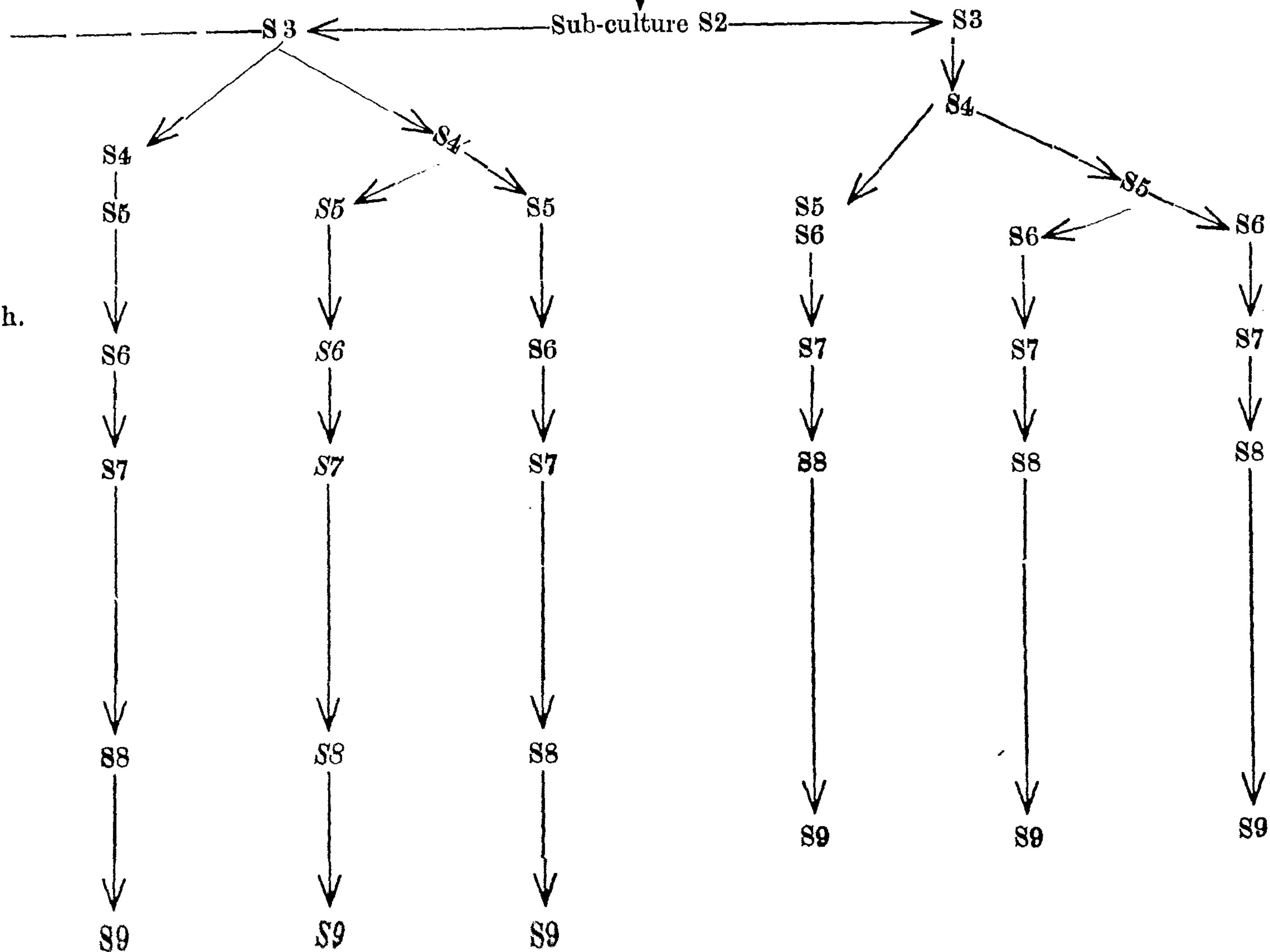
N. B.+Means—fermented. –Means—not fermented.

S1, S2 &c mean—sub culture 1, sub culture 2 &c, of the corresponding tube. In the subsequent pages the various cultures will be represented as S1. I, S2. II, S2. III &c. and so on indicating their number of sub-culture and their origin. For example S5. IV will mean the fifth sub culture from the feurth tube.

Fermentation Table.—*Continued.*

TUBE NO. IV.

Sub-Culture S1.



The most promising sub-cultures were now inoculated into larger volumes of mash, so that sufficient quantities of fermentation products might be obtained for identification and quantitative estimation.

In the experiments to be described, acetone was estimated by the following technical method :—

5 cc of the acetone containing solution is added to 25 cc of normal caustic soda solution. The mixture is well shaken and allowed to stand 10 minutes. N/5 iodine solution is slowly run from a burette drop by drop, shaking thoroughly till the upper portion of the solution on standing for a minute becomes quite clear. A few more cc of the iodine solution are run in so as to have an excess of about 25% and the whole solution is allowed to stand 10 to 15 minutes. 25 cc of normal sulphuric acid are added and the iodine which is liberated is titrated with N/10 sodium thiosulphate solution using starch as indicator.

1 cc of N/10 iodine solution = 0·00967 gm acetone. In experiments where the strength of the paddy mash is not given, it is to be understood that it contains 2% unless otherwise stated.

The experimental details are given in below for each sub-culture examined.

Culture S6 Tube No. I.

(a) The contents of the tube S6. I were inoculated into a litre of sterile paddy mash and allowed to ferment at 37°C in an incubator. Fermentation was very slow.

Duration of fermentation—96 hours.
Acidity of 100 cc in terms of N. Alkali—1·5 cc—0·073 % acid.

When the fermentation was complete, 10% of the volume of the fermented liquor was distilled off. The distillate contained 1·2% acetone on the weight of the mash.

Tube IV. The contents of the tubes S_1IV, S_2IV, S_3IV and S_4IV were each inoculated into one litre of paddy-mash and incubated at 37°C. The results of the various fermetations are summarised in the following statement :—

	S_1IV,	S_2IV,	S_3IV.	S_4IV.
Duration of fermentation (hours)	96	72	72	72
Acidity of 100 cc in terms of N alkali ...	2	2·1	2·9	2·4
Acetone (per cent)	0·195	0·458	1·17	1·4

In no case could any butyl alcohol be detected.

In the case of the ninth sub-culture of tube IV the original tube fermented vigorously and on inoculating into 250 cc of sterile mash a strong smell of acetone was obtained. It was therefore determined to use this culture for the fermentation of larger quantities of material.

The organism present in this sub-culture was plated out on to nutrient agar. After 24 hours incubation nearly 100 colonies were obtained. Of these two typical varieties were selected and streak cultures made on nuttrient agar slants, growth was obtained in 48 hours. Stained preparations showed masses of well defined bacilli similar in appearance to the bacillus of Weizmann, although its ready growth on nutrient agar would indicate that it was not the identical species and the products of its fermentation confirm this conclusion.* The special experiments with this culture may now be described.

(i) The contents of the 9th sub culture of tube No. IV were inoculated into 250 cc sterile mash after 4 months. It was incubated at 37°C until the fermentation was complete. The fermented liquor emitted a strong smell of acetone.

(ii) A large quantity of rice mash was made as follows:—

A glazed earthenware jar (capacity 11 litres) was thoroughly cleaned and compltetely sterilised.

Then 300 gms of rice powder, 8 litres of distilled water, and 8 gms. of ammonium phosphate were mixed together and added to the above mentioned sterile jar provided with a sterile rubber cork and a delivery tube. The whole was then autoclaved at 10 lbs pressure for 5 hours. When cooled the big jar with the mash was kept in a box incubator heated by electric bulbs maintaining the temperature at nearly 40°C.

Now 200 cc. of the fermented liquor of (i) were inoculated into the mash (ii) and the whole kept as above at nearly a constant temperature of 40°C.

The fermenting vessel was about two-third full of mash, the space above the latter being occupied by air. The following results were obtained :—

1. Fermentation started —22 hours after inoculation.
2. Duration of fermentation —72 hours.
3. Volume of gas evolved —13 litres.
4. Acidity of 100 cc. in terms of N. alkali when the fermentation was complete —2·856 cc. — 0·139% acid.

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*cf Fowler. Wad and Gokhale *loc cit p. 7.*

The acidity coincides fairly with the critical acidity observed in previous work on the acetone fermentation (*loc cit*).

The percentage of the acid contents of the fermented liquids was so small that they could not be conveniently isolated but they were qualitatively tested before distillation and were found to contain butyric acid and acetic acid.

The composition of the gas evolved was as follows :—

Hydrogen	...	...	...	45·6%
Carbon dioxide	...	...	...	28·1%
Marsh gas	...	...	...	Nil.
Air	...	...	...	27·5%

The whole of the fermented liquor was taken and 10% of its volume distilled off. The distillate had a strong odour of acetone and butyl alcohol. It was then fractionated over fused calcium chloride at various temperatures ranging from 56°C to 118°C. Four products were isolated. One fraction distilled at a range of 56°C to 61°C, the other at a range of 81°C to 85°C; the third at a temperature range of 106°C to 108°C and the fourth at about 118°C.

They were refractionated over fused calcium chloride and the following products were isolated :—

(a) Acetone ... ... —24·57 gms.
i e., 8·8% on the weight of rice taken.

(b) Iso-butyl alcohol ... ... —1·634 gms.

(c) n-butyl alcohol ... ... —21·06 gms.

(d) The distillate at a temperature range of 81°C to 85°C was so small that its composition could not be ascertained.

The presence of the acetone and butyl alcohol producing bacillus in the Indian paddy is therefore conclusively proved from the above experimental evidence.

Further sub-culture would probably result in a higher percentage yield of acetone and butyl alcohol and the subject is one for careful investigation from several points of view. The question suggested itself whether the acetone producing bacillus was the result of a selective process from germs already present, or whether a series of sub-cultures at high temperature gave rise to a modification of species to the extent at any rate of a change in the enzyme content of the cell. The answer to the question has its bearing on general theories of heredity.

The fact that direct heating of rice under pressure as described in Part III resulted in the elimination of all organisms

save those producing a pleasant acetone smell rather indicates that sub-culturing is merely a means of selection and not of modification.

The following experiments on what may be termed the direct method were designed to throw further light on this question.

(a) 80 gms of Bangalore polished rice powder were mixed with 800 cc of sterile water and 1 gm of ammonium phosphate and kept in a sterile flask. The whole was boiled for 30 minutes at 10 lbs. pressure. when cooled it was incubated at 40°C for self fermentation. The following results were obtained:—

(1)	Fermentation started	—48 hrs. after incubation.
(2)	Duration of fermentation	—96 hrs.
(3)	Acidity of 100 cc. in terms of N alkali when the fermentation was complete	—3·6 cc. — 0·176% acid.
(4)	Gas evolved	—4500 cc.

Composition of gas.

Hydrogen	...	...	40%
Carbon dioxide	...	...	23·3%
Marsh gas	...	...	Nil.
Air	...	...	Remaining portion.

The fermented liquor had a pleasant smell of acetone and butyl alcohol. Ten per cent of the volume of the liquor was distilled off. The distillate was shaken up with ether for five minutes. The etherial layer was then separated. The ether was distilled off over water bath at about 35°C. The residual liquid was then fractionated. Nearly one cc of acetone and one cc of isobutyl alcohol were isolated.

(b) A similar fermentation was started with the same sample of rice for comparison.

Here also a mash was made with 80 gms. of rice powder and one gm. of ammonium phosphate and 800 cc of sterile water. The whole was kept in a sterile flask (capacity 2 litres) and autoclaved at a pressure of 10 lbs for 30 minutes. After cooling it was incubated at 40°C. The flask was provided with sterile rubber cork and delivery tube. The results are given below :—

(1)	Fermentation started	...	20 hours after incubation.
(2)	Duration of fermentation	...	72shrs.
(3)	Acidity of 100 cc in terms of N. alkali	...	3·8 cc. — 0·186% acid.
	Gas evolved	...	5100 cc.

The fermented liquor contained butyric acid and emitted a smell of acetone and butyl alcohol similar to (a). It was then distilled and fractionated as aforesaid in (a). Here also nearly one cc of acetone and one cc of isobutyl alcohol and a few drops n-butyl alcohol (the latter was ascertained by its boiling point) were obtained.

(c) As the above sample of rice was exhausted, a quite different sample was purchased from the market for further investigation. A large "direct" fermentation was started with this sample of rice:—

300 gms. of rice powder and 8 gms. of ammonium phosphate and 8 litres of sterile water were mixed together and kept in a sterile Chinaware jar (capacity 11 litres) provided with a sterile stopper and with a delivery and a siphon tube. The whole thing was autoclaved for 30 minutes at 10 lbs pressure. When cooled it was kept in a box incubator heated by electric lamps maintaining a temperature range from 40 to 41°C. In this case, the rate of evolution of gas and the increase of acidity were recorded just after the fermentation was started. The latter was recorded by tapping a few cc of the fermented liquid by the syphon tube. Fermentation started after 8 hours incubation.

Duration of fermentation—82 hours.

The following figures give an idea of the rate of gas evolution and the corresponding acidity.

Time.	Volume of gas.	Acidity of 10 cc in terms of N. alkali.
21-8-1920.		
6 a. m.	2000 cc	0·8 cc
9 ,,	3500 cc	1·2 cc
12 ,,	3000 cc	2·1 cc
3 p. m.	3000 cc.	3·2 cc
6 ,,	2500 cc	3·6 cc
9 ,,	2000 cc	4·1 cc
22-8-1920.		
6-30 a. m.	4000 cc	5·8 cc
9-30 ,,	1600 cc	6·1 cc
12-30 ,,	1200 cc	6·4 cc
3-30 p. m.	500 cc	7·0 cc
6-30 ,,	200 cc	7·8 cc
9-30 ,,	nil	do

Time.	Volume of gas.	Accidity of 10 cc in terms of H. alkali.
23-8-1920.		
6-30 a. m.	nil	7.8 cc
9-30 „	nil	do
12-30 „	nil	do

Total gas evolved ... 32·5 litres.

Acidity of 100 cc. of the completion of fermentation, in terms of N. alkali 7 8 cc.

or the fermented liquor contained ... 0·48% of acid·

The fermented mash was opened on the 23rd August. It smelt strongly of butyric acid. One litre of the mash was distilled 10% of its own volume. It gave only 1·1% of acetone. The acids of the mash contsisted mostly of butyric acid and traces of acetic acid. The whole quantity of the gas was kept in a large cylinder, and analysed with the following results :—

Hydrogen	...	...	44%
CO_2	..	...	30·1%
Marsh gas	...	...	nil
Remaining	...	...	air

In this fermentation, the yield of acetone is very low in comparison with those obtained from different samples of rice as in (a) and (b). This unusual result may be due to the fact that the number of acetone bacilli present in different samples of rice varies.

(d) The same direct method was tried with paddy. 80 gms of Bangalore paddy powder and 800 cc of sterile water were mixed together in a sterile flax and boiled at 10 lbs pressure for 30 minutes. After cooling it was incubated at 37°C.

Fermentation started after 36 hours of incubation.

Duration of fermentation ... 72 hours.

Acidity in terms of N. alkali ... 3·4 cc.

Owing to some leakage in the collecting cylinder, the gas evolution could not be recorded. Ten per cent was distilled away from the whole volume of liquor. Two layers were obtained and they were fractionated over fused calcium chloride and found to contain 1 cc of acetone and 1·5 cc of butyl alcohol (normal).

The experiments recorded in Part IV clearly indicate that bacteria occur on ordinary Indian paddy which are capable of resisting very severe temperature conditions and which can ferment starch to acetone and butyl alcohol. Similar bacteria

occur on unpolished rice, and together with many other species on polished rice.

They may be isolated either by a series of sub-cultures into paddy mash at a temperature just below boiling or by the direct heating of paddy to a temperature and pressure just short of that required to produce complete sterilisation.

Both processes of isolation are therefore selective, the original spores being present in the material, at the outset. Further continuance of the process results in a further selection from organisms of varying fermentative power of those specially adapted to effect the particular fermentation required. There is no evidence of actual modification of species.

PART V.—Biochemical observations on polished and unpolished rice.

In Part III it was shown that polished and unpolished rice showed marked differences in the number of bacteria present on the grain and in the character of the fermentation when the two kinds of grain were incubated after partial sterilisation.

Careful examination into the reason for this difference revealed a number of interesting facts.

In the first place the earlier observations on the bacterial content of the washings from polished and unpolished rice were confirmed by taking 5 gms of each material and shaking in sterile flasks with sterile water for 5 minutes. 2 cc from each dilution were plated out and it was found that:—

1 gm unpolished rice carried 3,700 bacteria.
1 ,, polished do. 11,600 ,,

Such a result does not accord with that would be expected from the general external appearance of polished and unpolished rice, the former looking much cleaner and more attractive.

On examining sections of the two materials under the microscope it was found that the grains of unpolished rice are surrounded by a smooth outer skin, serving as protection to the contents of the grain. In the case of the polished rice however this protective skin was removed and the edge of the section was rough and irregular the broken cell walls having a matted appearance and evidently affording an excellent nidus for bacteria.

The physical difference is seen when equal quantities of sound grains of polished and unpolished rice are each allowed to soak in a given volume of 0·5 % copper sulphate solution. In the case of the unpolished rice more copper was withdrawn

from solution than in the case of polished rice, apparently owin to the skin of the unpolished rice acting as a semipermeable mem brane and causing a concentration of copper within the grain.*

Chemical examination revealed other important differ ences.

For this purpose a quantity both of paddy husk and ric polishings was obtained and carefully analysed, as the significan substances are likely to be concentrated in these constituents o of the raw grain.

The paddy husk was found to contain about 1 per cen of reducing sugars which would form pabulum for bacteria.

The rice polishings contained two important substances an oil and a nitrogenous substance of an alkaloidal nature.

The analytical details are as follows:—

115 gms of rice polishing were extracted with ether and a green solution was obtained. The ethereal solution was decolourised by animal charcoal and filtered three times in order completely to remove the latter. The oil thus obtained was heated to 105°C to remove any trace of moisture.

The oil is solid at ordinary temperature and melts at 40°C when it becomes yellow in colour.

The polishings contain 2·7 per cent of oil.

It gave the following constants:—

Refractive index	1·461 at a temperature of 42°C.
Iodine value	88·1

The alkaloidal substance was extracted in the following way:—

100 gms of rice polishings were extracted three times with 80% alcohol, the alcoholic extract filtered and the alcohol distilled off under reduced pressure.

*[*Note*:—This observation is apparently contrary to that of Adrian Brown (Roy. Soc. Proc. Series B. Vol. 81, 1908) who found that when sundry cereal grains were exposed to solutions of copper sulphate (of gram molecular strength) the grain absorbed water and rejected the copper sulphate. On repeating Adrian Brown's experiment with wheat and barley his results were confirmed, the grain becoming quite soft as a result of water entering the grain, and the copper solution becoming more concentrated. No copper was found in the grain.

Precisely the opposite effect was observed with paddy, the grain remained quite hard, the copper strength of the solution was slightly reduced and traces of copper were found in the grain.

This apparently abnormal behaviour is obviously connected with the natural conditions of growth of paddy, which necessitate that the grain should be impervious to external moisture.]

The viscous residue left in the distilling flask was then dissolved in water and again filtered to remove coagulated matter.

3 per cent by volume of pure concentrated hydrochloric acid was added to the aqueous solution and the mixture kept for four days whereby remaining colloidal matter and traces of fat separated. The solution was again filtered.

One drop of this concentrated hydrochloride evaporated on a micro cover glass gave a beautiful crop of dagger-shaped crystals.

It forms a double salt with platinum chloride and a precipitate with phosphotungstic acid.

The substance would appear to be identical with the alkaloid isolated from rice polishings and recently described by F. Hoffmeister (Bio-Chem Zeitsch 1920, 218—225).

A compound of a similar character has been found in the seeds of "*Cassia tora*" in the course of the researches (shortly to be published by one of us and M. Srinivasiah) into the biochemistry of the indigenous indigo dye vat, its function in this case being quite clearly to act as an antiseptic to all but certain specific organisms.

The behaviour of polished and unpolished rice on fermentation and the much larger number of bacteria present on polished rice would lead to the conclusion that a similar selective antiseptic action was exercised by the alkaloidal substance present in rice polishings.

The question then occurs as to the reason for this selective antiseptic action which must obviously serve some useful purpose in connection with the life of the seed.

Certain observers have already ventured the opinion that the symbiosis of bacteria is necessary to the germination of the seed. Thos. A. Wilson (J. Am. C. S. *26*, 289, 1904) holds that the real starting agents of germination are the lactic acid bacteria which by the development of acid dissolve the albumen and liberate the enzyme.

He brings forward a certain amount of evidence that antiseptics fatal to bacteria but harmless to enzymes will inhibit germination.

This is contradicted by Windisch and K. Schonewold (Woch Brau 1905, 22, 200) who state that barley can be completely sterilised with mercuric chloride solution in alcohol and yet the power of germination is unaffected.

The whole question is now the subject of careful research, the results of which will be published in due course.

It may be stated that there is evidence to show that the presence of specific bacteria, if not absolutely necessary to germination, does certainly facilitate it.

SUMMARY.

The results of the experimental work described in the foregoing pages may be shortly summarised as follows :—

1. The chief source of bacterial infection in a starch factory is the grain used as raw material.

2. The simplest method of sterilisation when the grain is not required for seed is by means of sulphur dioxide.

3. Ordinary paddy carries certain resistant germs some of which are allied to the acetone producing bacillus of Weizmann.

4. The ordinary methods of cooking rice do not produce sterility, the surviving bacteria being those which produce acetone.

5. The results, so far as they go, of isolating these bacteria by direct heating of paddy mash or by continuous sub-culture into hot sterile paddy mash, do not indicate that any modification of species is brought about by the latter method. Both methods are simply means of selecting varieties already present.

6. Polished rice carries more bacteria than unpolished rice. This would appear to be due to the removal in the process of polishing of an antiseptic of an alkaloidal nature together with the protective epidermis of the grain.

7. The observed selective effect of a natural antiseptic secreted by the grain on the bacteria present confirms similar observations (shortly to be published by one of us (G. J. F.) and M. Srinivasiah) in the case of the seed of *Cassia tora* which is used as a fermenting agent in the indigenous indigo dye vat.

8. Experiments are in progress to determine the relation, if any, between the presence of selected bacteria on the grain and the phenomena of germination.

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