Research Notes on the Acetone Fermentation Process in India.

By Gilbert J. Fowler, Y. D. Wad and A. G. Gokhale.

The fermentation process of producing acetone from starch or other carbohydrate came under the consideration of the senior author of these notes in connection with a request in the autumn of 1915 from the Engineering Agents to the Government of Hyderabad. He was asked to obtain for them a culture of "*Mycoderma aceti*" with the object of producing acetic acid and thence acetone from the large quantities of alcohol produced in Hyderabad by the fermentation of the sugar contained in the mahua flower "*Bassia latifolia*".

Arising out of the work on this matter the suggestion was made to the writer by Prof. Dixon of the University of Manchester that the direct fermentation process for producing acetone might well be introduced into India and that the writer's recently accepted appointment at Bangalore would enable him to conduct any necessary experiments.

After meeting Mr. G. E. C. Wakefield of Hyderabad, then on leave in England, an interview on the subject took place at the India Office, where at Mr Wakefield's suggestion the possibility of using mahua was discussed. It was arranged that a small consignment of mahua should be sent to London for investigation by Dr. Weizmann.

Very little time was available before the writer sailed for India but preliminary trials showed that the Weizmann bacillus did ferment an infusion of mahua flower. A visit was paid to the experimental plant at the Royal Naval Cordite Factory, where maize was being used, the chief characteristics of the bacillus employed by Dr. Weizmann were explained by his assistant at the Lister Institute and a number of cultures and spore tubes were supplied and safely conveyed to Bangalore where the writer arrived in the middle of February 1916.

A consultation was then held in Delhi at which it was decided that a factory for the production of acetone should be started and that the experimental scientific work in connection therewith should be done under the writer's direction at Bangalore.

An experimental plant was afterwards set up in the Applied Chemistry Department at the Indian Institute of Science in which 800 gallons of mash could be fermented and distilled.

Messrs. Wad and Gokhale were appointed first assistant bacteriologist and first assistant chemist respectively and Messrs N. R. Gurjar and V. L. Chandratreya, junior assistants.

Major E. Moore Mumford, who was Superintendent of the Government Acetone Factory during the early stages of the project, was mainly responsible for the erection of the plant and the general control of the technical operations.

During the war the work both scientific and technical was of course confidential. In view of the publication in the Journal of the Society of Chemical Industry and elsewhere of several papers (see p. 14) dealing with the large scale production of acetone by the fermentation process it would appear to be of interest to place on record some of the results of the Bangalore experiments noting *e. g.* where they confirm or differ from the results obtained under different climatic conditions and with different raw materials in England, Canada and India. Permission has been kindly granted by the Indian Munitions Board for this to be done.

Description of Experimental Plant.

The experimental plant consisted of the following :—

Fermentation plant.

i. Two inoculating vessels, of 1 gallon capacity.
ii. 25 gallon seed vessel.
iii. 800 gallon fermentation tank.

Distillation plant.

i. 100 gallon preliminary distillation still.
ii. 10 gallon still and rectifying column.

Effluent purification plant.

i. Settling tanks.
ii. Aeration tank.
iii. Filter press.

The Fermentation Plant.

This plant was got out from England and was indeed part of the equipment to be finally used at the Government Acetone Factory at Nasik. All the vessels were of aluminium, both the 25 and 800 gallon vessels being provided with stirring gear and all necessary steam and cooling coils, manholes, inlet and outlet valves &c.

This plant was capable of producing 6 gallons of acetone and 12 gallons of butyl alcohol per operation. The stirring gear was driven from shafting actuated by a 15 H. P. A. C. Motor belonging to the Applied Chemistry Department.

The Distillation Plant.

For the purpose of distilling the fermented mash, plant already in the possession of the Applied Chemistry Department was utilised. In the first place the fermented mash was pumped by hand from a small iron tank placed below the exit valve of the fermentation vat into a 100 gallon still, in portions of 100 gallons at a time and 10 per cent distilled off and fractionated by means of a copper rectifying column fixed over a steam heated pan.

Effluent Purification Plant.

The purification of the effluent from the process was important to study for two reasons, the prevention of nuisance and the possibility of utilising it for fertilising purposes, particularly in relation to the fixation of nitrogen, as will be explained later.

For this purpose the liquor after distillation was run off from the still and after screening lifted by compressed air into one of two tanks of cement concrete constructed above ground. After treatment with lime and settling the liquor was run into an aeration tank for final purification if possible by means of aeration in presence of activated sludge.

The compressed air was furnished by a gas-engine and blower, the property of the Institute. The tanks were specially constructed for the purpose.

A filter press was purchased for handling the sludge and has since been sent to the Government Acetone Factory at Nasik.

The main experimental work done may be summarised under the following heads :—

1. Raw material.
2. Behaviour of Organism.
3. Practical control of process.
4. Purification of effluent.
5. Sundry subsidiary investigations.

1. Raw Material.

Mahua. (Flowers of *Bassia latifolia*) Mahua flowers from various sources were examined more particularly for their carbohydrate and nitrogen content.

The nitrogen content was found to vary from 0·65 to 1·1% being apparently higher in the younger than in the well developed flowers.

The total sugars varied from about 40% in Hyderabad mahua to 60% in a sample from Kaira in North Gujarat.

On the other hand the percentage of disaccharides was higher in Hyderabad mahua varying from 11·0 to 21·7% compared with 2·4 to 11·4 in the Gujerat sample.

The composition of course varies with conditions of gathering, transport and storage. The whole question of the development and composition of the carbohydrate content of mahua flowers has been the subject of subsequent researches in the Applied Chemistry Department of the Indian Institute of Science*.

The ash content of the flowers varied from 3·6 to 5% and was found to contain appreciable amounts of potash and phosphates.

The mahua flower therefore contains little or no starch and the nitrogen content is not high.

Possibly for these reasons the fermentation experiments with mahua were not very encouraging. At first a filtered extract of the flowers was sterilised and inoculated with a culture developed in maize extract. A certain amount of fermentation

* Studies in the Bio-chemistry of the Mahua Flower—G. J. Fowler with Messrs. Edal-Behram, Bhate, Habib Hassan, Mahdi Hassan and Inuganti, Journal of the Indian Institute of Science, Vol. 3, Part VI, p. 81.

took place, but it did not proceed to completion and a sub-culture into a second portion of mahua extract was even less active.

Further experiments with an unfiltered mahua mash were not much more successful. It was found always that a greater proportion of reducing sugars than of disaccharides was always fermented and so a number of experiments were carried out on the best method of inversion of the disaccharides. The most satisfactory method was decided to be the addition of yeast cells deprived of their vitality by acetone. In this way inversion took place and some additional nitrogenous food was provided. No great improvement in fermentability was attained.

Considerable development of acid took place in the mahua fermentation, so chalk was added to neutralise any possible ill effects due to this cause, but the result was still unsatisfactory.

It would appear either that there is some substance in mahua which is toxic to the acetone bacillus or that the latter will only act on starch-containing material. The question deserves further study, as it is quite likely that an organism kept active in maize might be able to ferment the less favourable mahua for one generation at any rate and so obviate the necessity of using a food grain as the main raw material.

Incidentally it was found that the acids or other consitituents of the fermenting mash rather rapidly corroded iron. Such corrosion however can readily be obviated by the use of glass, enamel or even of cheaper bitumenous covering materials.

Materials containing starch.

Owing to the urgency of the case instructions were received from Government to carry on with starch-containing raw material, rice being specified as having been successfully used in England. A search among other sources of starch fortunately revealed a much cheaper source of starch than rice, and one giving equally satisfactory results *viz.*, *jawari* or *cholam*. This grain gives the following typical analysis :—

Nitrogen	1·2
Starch	52·9
Ash	1·6

As this at the time of the investigation could be obtained as cheaply as mahua, there was no financial advantage in using the latter, and so furthur investigation of its possibilities was postponed.

The following record of a laboratory trial fermentation of jawari will indicate the character of the results obtained.

1200 grams of jawari flour were taken, cooked for half an hour with half the required quantity of water added as steam, then autoclaved for one hour, diluted to 7·5% strength and finally sterilised in steam.

500 cc of a vigorous maize culture, were added. The results of the fermentation are given in the following table :—

	Grams per 100 cc.	
	Before Fermentation.	After Fermentation.
Nitrogen	0·09	0·08
Starch	4·10	0·07
Reducing sugars	0·26	0·07
Acidity cc of N alkali per 100 cc	0·16	3·61

70 cc of acetone were obtained being 11 % on the starch originally present, in addition 128 cc of butyl alcohol were produced.

Good fermentations were obtained with rice dust, bajri, roots of *Mirabilis jalappa*, and with tapioca starch.

Jawari combined the advantages of low price and ready availability and was therefore chosen as the raw material for larger scale work.

2. Behaviour of the organism.

The organism was brought from England in one or two maize tubes simply plugged with cotton wool and in a number of specially prepared sealed tubes containing spores. An attempt was made to prepare sub-cultures from the maize tubes en route without much success, but the original maize tubes successfully fermented maize mash prepared in Bangalore. Good fermentation was always obtained from the spore tubes up to 18 months at any rate.

As already stated the organism would only ferment mahua at all satisfactorily when inoculated direct from a maize culture and on the other hand would ferment starch-containing materials of various descriptions quite readily. It would thus seem that it is essentially a starch fermenting organism though the subject is one needing further study. Whether it actually needs starch or only dextrin is not yet certain, nor whether it can

be acclimatised to a purely sugar-containing medium like mahua extract.

The observation of A. Gill (*loc cit*) that actual portions of maise proteid need to be present in the mash explains the fact that it was often difficult to inoculate tubes with a platinum loop. Pipettes with the constricted points cut off to enable portions of insoluble mash to be drawn up were found to be most suitable for inoculation purposes, the bacteria probably adhering to the suspended matter.

It was found that the most vigorous fermentation took place with cultures only two or three generations from spores. It was important therefore to ensure that the cultures did not lose their power to sporulate, as they tended to do after continued sub-culture in simple maize mash.

By inoculating into maize or jawari mash containing meat extract, peptone or gelatine, cultures were obtained which on sub-culturing into ordinary maize mash produced spores. In certain cases however sporing cultures were obtained by sub-culturing in maize mash only. The presence or absence of sufficient maize proteid may account for this.

A number of attempts were made to obtain a suitable solid medium on which plate cultures of the organism could be prepared. In all cases the experimental plates were incubated either in pyrogallol chambers or in chambers evacuated to 25 inches of mercury. In general smear cultures were made in order to avoid any possibility of too great heating of the organism by the melted agar.

The only moderately successful result however was obtained with maize wort agar containing 0·5% of peptone. A hazy profuse plate growth was obtained which fermented maize broth successfully for two sub-cultures. Further inoculations in maize broth produced no fermentation, although the colonies actually could be made to grow on the solid medium for seven sub-cultures.

The results of the culture medium trials described by Gill would indicate that probably the acidity in this case was not exactly suited to the organism. Encouraging results were obtained from a wort gelatine broth but such a medium could not remain solid at the temperature of a South Indian laboratory.

An attempt was made by means of the iodoform method of estimating acetone, roughly to ascertain the acetone producing

power of various cultures, even in volumes of mash of not more than 20 cc. Quite useful though naturally only approximate results were obtained.

3. Practical Control of Process.

The chief difficulty in large scale work is undoubtedly the maintenance of sterility.

Very few failures occured in laboratory trials or even up to the 25 gallon vessel, but they were more frequent in the 800 gallon plant.

The main source of infection would seem to be the grain itself, as it harbours large quantities of sporing bacteria, the spores being very resistant to high temperatures. Any lodgment of masses of semi-solid 'porridge' as it might be termed, generally if examined was found not to be sterile throughout.*

Other channels of infection are leaky valves, through which air may enter when there is a decrease of internal pressure on cooling, or faulty flanges on cooling coils allowing infiltration of unsterilised water.

Incomplete sterilisation nearly always showed itself by excessive production of acidity and it appeared difficult if not impossible to recover a brew which had once gone wrong, either by neutralisation, resterilisation and reinoculation or by a combination of these processes.

The difficulty of obtaining complete sterility was experienced at the Royal Naval Cordite Factory and a letter was received from the Engineers Messrs Sir Douglas Fox and Partner dealing with the subject and suggesting a 7 hour sterilisation period.

Samples were consequently taken hourly from the vat under aseptic conditions by means of a cock half way up the side and examined for sterility, acidity and ratio of soluble and insoluble carbohydrate.

The examination of the samples indicated that 4 hours heating under 15 lbs steam pressure was necessary to secure sterility.

The conditions of sterility would seem to require an absence of undisintegrated masses of " porridge" *e. g.* immediately

*Recent unpublished work by Mr D. L. Sen (Department of Applied Chemistry, Indian Institute of Science) has confirmed the great difficulty of sterilising masses of boiled rice.

round the bearings of the stirring gear, the maintenance of all valves and pipes in good condition bacteriologically and mechanically and the continuance of 15 lbs steam pressure for at least 4 hours.

On more than one occasion the need for recording thermometers (as used in Toronto) was evident, when the carelessness of a vat watcher during the night had evidently allowed too much steam to enter the coils and so to raise the temperature of the brew above the vital point of the adult bacteria with consequent checking of the fermentation.

It is possible that fewer failures would have occurred had it not been the custom after inoculation immediately to mix the inoculant with the whole bulk of the liquid. A suggestion from A. Appleyard, (now Superintendent of the Government Acetone Factory at Nasik) to allow the inoculant to diffuse gradually through the mash economises power in stirring and allows time for a vigorous fermentation to take place at one point and spread gradually throughout the mass. A fermentation started in this way in the 800 gallon vat was one of the best on record. On the other hand in the Toronto operations the inoculant is described as being injected into the cooled and cooked mash on its way to the fermentation vats.

In general the Bangalore experiments fully confirm the conclusions arrived at by the Toronto workers as to the untrustworthiness from a bacteriological point of view of all valves and flanges and the necessity for designing and operating the plant in such a way that thorough sterilisation of the mash is ensured up to the point of inoculation and that subsequently no foreign organism enters the fermenting vat.

4. Purification of Effluent.

For every ton of acetone produced by the fermentation process nearly 50,000 gallons of effluent has to be disposed of, which is capable on putrefaction of creating considerable nuisance.

The effluent contains the husk of the grain in suspension, oily and proteid matter, residual unfermented carbohydrate material and sundry organic acids.

The husk can be removed by fine screening and may be fed to cattle advantageously.

An even better food product is obtained if the husk is pressed along with the sludge which results from plain

sedimentation without the assistance of lime. The pressed cake on drying has then the following approximate percentage composition, the figure giving a maximum value in each case.

Moisture	5·8
Fat	23·9
Woody fibre	11·3
Digestible carbohydrates	37·4
Albuminoids[1]	21·6
Ash[2]	3·0
Starch	9·0

[1] Containing nitrogen	3·4
[2] „ sand	1·6

After plain sedimentation a turbid liquid is left containing finely colloidal or flocculent organic matter. Addition of alum produced very little clarification, but an adequate amount of lime, especially if added to the warm liquid produced immediate clotting and had a distinct antiseptic value. For this result 500 parts of lime per 100,000 parts by weight of effluent was required, or more than one ton per 50,000 gallons and the volume of sludge to be dealt with every day was about 40% of the volume of the effluent. Less than one fifth of this amount of lime was required for simple neutralisation, and clarification took place after half an hour's settlement, but the volume of sludge to be dealt with was about the same and little or no antiseptic effect was produced. The sludge was also of a slimy character and difficult to press.

The effluent produced by the smaller quantity of lime is adapted for further treatment on land or by suitable bacterial methods, but the sludge would require a further addition of lime before it could be quickly pressed into cake.

The effluent after lime treatment still contains much oxidisable and nitrogenous matter, giving the following figures on analysis.

Oxygen absorbed	Parts per 100,000.
4 hours test	420·0
Albuminoid nitrogen	84·0

It was thought that such an effluent would be of considerable manurial value if the nitrogen could be properly utilised

It was also considered that the carbohydrate residues still present might be favourable to nitrogen fixation.

With these ideas in mind some preliminary experiments were made to purify by means of activated sludge the effluent obtained after neutralising with lime.

It was not possible in the time available to carry these trials very far, but the results showed that it would be necessary to dilute the effluent in the initial stages at any rate before rapid purification could be effected. In the case of a factory employing numerous hands and where the water carriage system of sewage disposal was in force, the domestic sewage would partly serve to dilute the effluent and furnish the original activated sludge. Further dilution can also be obtained by means of the effluent itself. In this way by gradually building up a sludge suited to the special liquid to be purified there is no reason to doubt that an effluent and also a sludge could be produced suitable for agricultural purposes, particularly *e. g.* the growth of sugar cane.

By such careful treatment of the various waste products the unlimed sludge being used for cattle food, the limed sludge for transportable manure and the final effluent and surplus activated sludge for crop raising in situ, the costs of treatment should be met and serious nuisance avoided.

5. Subsidiary Investigations.

Analytical methods. In researches of this kind it is generally found that current analytical methods are capable of advantageous modification or that certain factors must be given special attention if successful results are to be obtained.

Hence the following descriptions of methods used for determining sugar in mahua flowers and starch in cereals may be usefully recorded.

To determine sugars in mahua flowers about 5 grams of the flowers were boiled for about 10 minutes with 100 cc of distilled water. It was found to be immaterial whether the flowers were pulped or not. The extract together with the flowers was then transferred to a 500 cc graduated cylinder, diluted slightly with cold water and if necessary cooled to room temperature (between 24° to 29° C), 10 cc of basic lead acetate added and the mixture well shaken. A characteristic change in appearance follows immediately. After 2 or 3 minutes the excess of lead is precipitated by sodium phosphate solution and the whole made up to 500 cc. It is then filtered and titrated with Fehling's solution in the

usual way before and after inversion and after neutralisation by sodium bicarbonate. The volume of the solids was not considered when making up to 500cc.

For the determination of starch in grain, *e.g.* jawari, the method of Sachse was used which may be shortly described as follows:—

The grain is ground very fine and a quantity between 2·5 and 3 grams is weighed out. 200 cc of distilled water are then added together with 20 cc of HCl (s. gr. 1·125) and the mixture kept on a boiling water bath, with an air condenser to prevent change in concentration, for exactly three hours. It is then carefully neutralised by alkali and made slightly acid by the addition of HCl. It is then clarified by 10 cc. basic acetate of lead and Na_2HPO_4, made up to 500 cc and filtered, the sugar contents are then determined by Fehling's solution and calculated as glucose. Multiplying the glucose figure by 0·9 gives the carbohydrate expressed as starch.

Any variation from the above conditions especially as regards (1) the fineness of flour, (2) the weight of sample taken (3) the concentration of acid used (4) delay in neutralisation and (5) clarification has been found to yield widely varying results.

The following table shows the results of duplicate experiments done under the specified conditions. It will be seen that the results vary at the most by 1% when the same sample of sterilised grain was used.

Results.

Experiment No.	% as glucose.		Difference.	% as starch.		Difference
	I	II		I	II	
I.	79·71	79·79	0·08	71·832	71·811	0·021
II.	80·02	79·12	0·90	72·018	71·208	0·810
III.	80·07	79·70	0·37	72·063	71·730	0·333

Flashing and explosive concentrations of mixtures of acetone vapour and air.

A knowledge of the limits of danger of possible escapes of acetone vapour under factory conditions is obviously of great importance for reasons of safety.

The experiments in this direction were undertaken without the knowledge of the work of Wheeler and Whittaker (J. Chem. Soc. April 1917) shortly afterwards published, but the

results agree very closely in spite of the somewhat rough methods employed.

The following is a description of the method of experiment and the results obtained.

A kerosine tin ($33{\cdot}5 \times 22{\cdot}5^2$)c. cm. with a cap having a small hole that could be corked was used. The capacity of the tin was 16959·4 cc and held in consequence 13·9247 litres of air at N. T. P., the temperature at the time of experiment being 25°C and pessure 677·02 mm. A measured quantity of liquid acetone was introduced into the tin through the small hole by means of a graduated pipette and then the hole was corked. The acetone was spread over the whole of the inner surface by slowly rolling the tin, thus converting the acetone into vapour. It was kept for a time to allow complete mixture with air and then was ignited by a match or petrol torch by removing the cap, and in the case of explosive mixtures by removing the cork. In case the mixture was explosive the cap went off and gave an idea of the violence of the explosion. Care was taken to perform all experiments under the same conditions. Everytime the tin was well aerated after explosion.

The following table gives the results of the observations. Many of them were repeated and confirmed.

22·4 litres of acetone vapour=58 grs..

1 cc liquid acetone at 25°C=0·2858 litres at N. T. P.

No.	Quantity of acetone	% of vapour by weight in air at N. T. P.	*Ignition effect.
1	0·25 cc	0·51%	No flash.
2	0·50	1·02	„
3	0·75	1·53	„
4	1.00	2·04	„
5	1·10	2·244	„
6	1·15	2·346	„
7	1·20	2·448	Slight flash.
8	1·25	2·55	Good flash
9	1·75	3·57	Flashes with force.
10	2·25	4·59	Explodes moderately.
11	2·50	5·10	Forcible explosion.
12	2·75	5·61	Strongest do.
13	3·00	6·12	Explosion equal to No. 11.
14	4·00	8·16	Forcible flash.
15	5·00	10.20	Burns quietly.

The nature of the process adopted makes the results only approximate though they are sufficiently accurate for the purpose in hand.

It appears that a concentration of acetone vapour in air up to 2·3 % is safe. There will be a flash above this varying in force as the concentration increases and reaching a maximum violence at a concentration of 5·61 %. Then the force of explosion diminishes until it settles down to a quiet flame at a concentration of 10·2 %. It is quite obvious that no concentration above 2·3 % can be safely allowed.

Specific gravity of mixtures of butyl alcohol and water.

In the distillation of butyl alcohol from fermented mash a constant boiling mixture of butyl alcohol and aqueous vapour comes over at 91°C. On cooling it separates into two layers, the top layer butyl alcohol with about 10 % water and the lower layer water with 10 to 12 % butyl alcohol. Pure butyl alcohol is obtained by careful fractionation and dehydration by potassium carbonate.

For factory purposes it is obviously necessary to be able quickly to determine the strength of a sample of butyl alcohol and for this purpose a set of specific gravity determinations was made of various mixtures of butyl alcohol and water at 20°C and at25°C.

A full account of this work is given in a separate paper by two of the present authors.

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