I. STUDIES RELATING TO THE ACETONE-PRODUCING ORGANISMS.

By Gilbert J. Fowler and V. Subramanyan.

On account of their technical, and more recently, possibly pathological importance, the chemical changes accompanying the production of acetone by the fermentation of starch from various raw materials have been studied by numerous workers.[1]

Problems awaiting solution are: (1) the longevity of the spores of the organisms, (2) the exact method of their nutrition, e.g., whether they attack the starch molecule as such or only after partial hydrolysis, (3) whether they are capable of directly fermenting sugars under any conditions, and (4) the discovery, if possible, of the function of the numerous acetone-producing organisms in nature, as they appear to be widely distributed; although differing among themselves, they belong to the same general type and are all obtainable from cereal grains by suitable methods of sub-culture.

EXPERIMENTAL.

ORGANISM USED.

Difficulty having been experienced in obtaining a satisfactory pure strain of the organism from certain available laboratory cultures, recourse was had to one of several spore tubes of *Bacillus granulobacter pectinovorum*, still remaining out of the stock originally brought from England in 1916. The tube was carefully opened and a portion of the culture at once transferred to a sterile mash of jawari (*Andropsorum sorghum*). Vigorous fermentation began after some days. By repeated sub-culturing, a healthy, actively fermenting strain could be obtained within a month. It is evident, then, that the spores of the acetone bacillus, developed on maize mash, retain their vitality for at least seven years.

The best culture medium was found to be jawari mash, containing the insoluble residue of the grain. This medium, as other workers have noted, is very difficult to sterilise. A convenient method of

[1] For references see Fowler, Wad and Gokhale, *This Journal*, 1921, 4, 14; also Speakman, *J. Biol. Chem.*, 1920, 41, 319.

preparation is to mix powdered jawari with a small quantity of water to form a cream, and to cook this under four atmospheres pressure for one hour. The pasty mass thus obtained is mixed with an excess of water, made up to the necessary volume and finally sterilised by autoclaving at fifteen pounds pressure for one hour. The mash thus obtained was found to be quite sterile.

ESTIMATION OF ACETONE.

The following modification of Van Slyke's method [1] for the estimation of acetone in urine was used throughout.

An aliquot portion of the fermented mash is first clarified with cupric hydroxide, a treatment which also removes reducing substances which might otherwise interfere with the estimation. The clarified liquid containing acetone in great dilution, and therefore not easily lost by evaporation, is then filtered, mixed with excess of acid mercuric sulphate, dissolved in sulphuric acid (1 : 4), and boiled under reflux. After an hour's vigorous boiling, the flask is allowed slightly to cool, and its contents filtered through a prepared Gooch crucible. The precipitate thus obtained is dried in the steam oven and estimated as basic mercuric-acetonyl-sulphate, represented by the formula :

$$3HgSO_4, 5HgO, (CH_3)_2 CO.$$

Control determinations with pure acetone showed the method to be more accurate than the customary iodine titration method. In all cases the yield of acetone is given as the percentage weight calculated on the weight of solid raw material.

For the detection of small quantities of acetone, the following colour reactions given with sodium nitroprusside were found useful :—

(*a*) A deep red colour, quickly fading to deep yellow, on adding a concentrated solution of sodium nitroprusside and a few drops of caustic soda ; and change of the yellow to carmine by subsequent addition of glacial acetic acid.

(*b*) A deep purple on adding concentrated sodium nitroprusside solution and a few drops of strong ammonia solution (s.g. 0·88).

Acidity.—In common with previous workers, we have found the determination of post-fermentation acidity very valuable in judging the purity of the culture, and the efficiency of the fermentation. The acidity is expressed in c.c. of N alkali per 100 c.c. of medium, and was determined by titration with phenol-phthalein as indicator.

[1] *J. Biol. Chem.*, 1917, **32**, 455.

FOOD FACTORS.

As already stated, one of the main objects of the work was to examine in further detail the nutritive requirements of the organism, with respect to carbohydrate, protein and mineral food supply.

Carbohydrate.—In order to study the value of various carbohydrate foods, a two per cent. jawari mash, inoculated in bulk with the organism, was divided into portions of 100 c.c., and 100 c.c. of two per cent. solutions of the following carbohydrates added :—Starch, glucose, dextrin, maltose and sucrose. In the case of starch only was there any appreciable increase in percentage yield of acetone.

Experience being thus confirmed that starch is the main starting point of the carbohydrate metabolism of the organism, an attempt was made to follow the progressive stages in the breaking down of starch during the acetone fermentation. Attempts to determine the amount of unattacked starch by arresting the fermentation at various stages with toluene, adding a known quantity of diastase extract, and determining the resultant maltodextrin by conversion into glucose, did not give concordant results. Direct determination of the glucose and dextrin-content of the mash showed a steady increase in glucose-content from 0·1 to 1·7 per cent., up to the period of gas production, i.e., for the first 20 hours, after which concordant results could not be obtained.

Corresponding polarimetric observations revealed at no time more than traces of dextrin. Qualitative tests also indicated no sugars other than glucose during the early stages. During the later stages it was impossible to identify the precipitates formed with phenyl-hydrazine.

An attempt was made to study the changes in other sugars during fermentation. Maltose and dextrin, though not yielding appreciable quantities of acetone, do break down as far as glucose, when left in contact with the organism.

Practically no change in the percentages of pentoses originally present in the cereal mash could be observed, using Ling and Nanji's method of determination,[1] nor could any change be remarked when an additional source of pentose in the form of gum-arabic was added to the medium.

In order to see whether the residue after jawari fermentation was actually a source of pentose, it was digested with 1 per cent. sulphuric

[1] *Biochem. J.*, 1921, 466

acid at two atmospheres pressure, neutralised with calcium carbonate and filtered. The filtrate, which should contain all the xylose of the mash, was sterilised and inoculated with strains of *B. aceto-ethylicus*,[1],[2] and *Lacto-bacillus pento-aceticus*,[3] and the percentage yields of acetone and alcohol determined. These were as follows :—

TABLE I.

Organism	Acetone	Alcohol	Acidity
B. aceto-ethylicus	1·7	1·9	1·4
Lacto-bacillus	...	0·8	1·3

The recent work of Ling and Nanji [4] suggested the possibility that further light might be thrown on the metabolism of the organism if its action upon the various preliminary disintegration poducts of the starch molecule could be ascertained.

Accordingly, a sample of 'amylopectin' was obtained by the dialysis of rice-starch, and isomaltose was prepared from it by Ling and Nanji's method. Comparative fermentation experiments were then made with two per cent. jawari mash, alone, and mixed with an additional two per cent. of (*a*) rice-starch, (*b*) amylopectin and (*c*) isomaltose. The results, given in Table II show that the organism prefers the more complex molecule of amylopectin to the less complex isomaltose.

TABLE II.

Carbohydrate added	Acetone	Acidity
Nil (original mash)	9·5	1·8
Rice-starch	9·6	1·8
Amylopectin	10·9	1·9
Isomaltose	9·2	1·7

[1] *J. Biol. Chem.*, 1919, **39**, 1. Northrop, Asche and Senior.
[2] *Ibid.*, 1920, **44**, 465, Arzberger, Peterson and Fred.
[3] *Ibid.*, 1920, **44**, 40, Arzberger, Peterson and Fred.
[4] *J. Chem. Soc.*, 1923, **123**, 2666.

PROTEIN.

The protein-requirement of the organism appears to be fairly well defined. Repeated experiments showed that it could not be made to thrive well in presence of degradation products, such as amino-acids, but that it required insoluble protein, preferably of vegetable origin. Casein is to some extent assimilable, but only in presence of starch. Yeast-water, whether obtained from brewery yeast or from yeast built up in a medium devoid of organic nitrogen, was ineffective in supporting fermentation activity.

In order to define more exactly the character of the protein required by the organism, determinations were made of (1) the water-soluble, (2) the salt-soluble, (3) the alcohol-soluble, and (4) the insoluble protein-content of (*a*) the dry cereal, (*b*) the cooked mash, (*c*) the mash immediately after the main fermentation and (*d*) the mash one month after completion of the main fermentation.

The methods of analysis followed were those described by Osborne ; the results are given in Table III.

TABLE III.

Character of protein	Dry cereal	Percentage in		
		Mash before fermentation	Mash immediately after fermentation	Mash 1 month after fermentation
Water-soluble... ...	0·46	0·52	0·55	0·61
Salt-soluble	0·57	0·73	0·72	0·70
Alcohol-soluble ...	0·43	0·52	0·50	0·49
Insoluble	5·41	5·10	4·90	4·86

It is clear that the organism thrives mainly at the expense of the less soluble proteins, which show a perceptible decrease after fermentation, with a slight total increase in the water-soluble percentages.

That the organism really needs insoluble protein in order to function, and that the insoluble portion of the mash does not act merely as a mechanical support for the organism, as e.g., in the case of *B. Macerans*, and certain strains of thermophylic bacteria, is clear from Table IV, where the effect of adding various materials to ordinary wort is shown.

TABLE IV.

Medium used	Result
Clear wort	No fermentation.
Wort plus paper pulp	Do.
Wort plus mahua waste from yeast fermentation	Do.
Unfiltered wort, with mash	Fermentation : Acetone yield 6·5 per cent. Acidity 1·9.

It would thus appear that the pasty residue from the malt mash, which plays a practically negligible part in alcoholic fermentation with yeast, enables the acetone-organism to thrive, and to develop the power of attacking carbohydrates other than starch.

A quantity of barley-malt, freed from starch in the malting process, was therefore prepared, and freed also from sugars and dextrin by repeated washing with water. Weighed quantities of different carbohydrates were mixed with a defined amount of this mash and the yield of acetone determined in each case. The results are given in Table V.

TABLE V.

50 c. c. of mash were mixed in each case with 200 c.c. of a two per cent. solution of the carbohydrate to be tested, except in the case of mahua extract, when three per cent. of mahua was used.

Carbohydrate added	Acetone yield per cent.
Mash alone	0·15
Starch	6·50
Glucose	5·8
Lactose	3·5
Dextrin	3·7
Cane sugar	3·4
Maltose	4·7
Mahua extract	...

Mineral Salts.

Experiments were also made to determine the part played by various mineral salts in the nutrition of the organism. To a two per cent. jawari mash, 0·05 per cent. of the following salts was added, each salt being tested separately in regard to its effect on the fermentation, viz., ammonium phosphate, magnesium sulphate, ammonium chloride, calcium carbonate and potassium nitrate. Only with ammonium phosphate was there any perceptible enhancement of the yield of acetone. Ammonium chloride and calcium carbonate appeared slightly to reduce the yield. A further set of trials with calcium carbonate in larger proportions showed a very marked inhibitory effect. As the quantity of calcium carbonate added was increased from 0·05 per cent. to one per cent., the yield of acetone decreased correspondingly from 9·3 per cent. to 4·5 per cent., intermediate quantities showing a proportionate effect.

Having regard to the well-defined inhibitory action of calcium acetate on the activity of yeast and of acetifying bacteria, it would seem likely that in this case also, calcium acetate, formed as an intermediate product, exerts a similar effect.

Enzymatic Action of Organism.

It has been found in practice[1], that better fermentation takes place in absence of stirring in the initial stages, owing apparently to enzymatic concentration. In order to make certain that this was the case, and that the injurious effect of early stirring was not simply due to the aeration of the mash caused thereby, an experiment was made in sealed flasks of about 100 c.c. capacity, almost filled with the inoculated mash. One of the flasks was stirred by rotating it vigorously every half-hour, the other was allowed to remain quiescent. The fermentation was found to begin six hours earlier under quiescent conditions. A repetition of the experiment with rather larger volumes gave the same result showing clearly that the presence or absence of air is not the governing factor in this difference in activity.

The chief enzymes which are active would seem to be a proteolytic enzyme, as the organism readily liquifies gelatine, and an amylolytic enzyme. The latter functions apparently only in presence of insoluble protein. Careful examination of the liquified starch revealed the presence of glucose, but not maltose. No evidence of the presence of invertase could be obtained.

[1] Cf. Fowler, Wad and Gokhale, *loc. cit.*, p. 9.

The observation of Witzemann [1] that hydrogen peroxide converts butyric acid into acetone suggested the idea of adding small quantities of neutral hydrogen peroxide (three per cent.) to the mash at the peak of fermentation activity, and to note if the yield of acetone is affected by its presence. The yield obtained was, in fact, lower than the normal, being only 8·7 per cent., and the final acidity 1·4, showing that the fermentation had not proceeded to its normal conclusion. There is thus no evidence of the presence of a peroxidase.

POST-FERMENTATION CHANGES.

It has been frequently noticed in large-scale operations that the quantity of acetone in the fermented mash rapidly disappears if the distillation is delayed for one or two days. Our experiments clearly confirm this observation. Flasks containing fermented mash were kept for a fortnight or more, the amount of acetone and the corresponding degree of acidity being determined day by day. The results are given in Table VI.

TABLE VI.

Age of mash at the time of distillation	Percentage	
	Acetone	Acidity
Original unfermented	Nil.	0·05
Immediately after fermentation ...	9·5	1·84
4 days ,, ,, ...	9·2	1·89
5 ,, ,, ,, ...	9·0	1·94
7 ,, ,, ,, ...	...	2·0
10 ,, ,, ,, ...	8·7	2·25
12 ,, ,, ,, ...	8·5	2·4
15 ,, ,, ,, ...	8·3	2·5
30 ,, ,, ,, ...	7·8	3·4
90 ,, ,, ,, ...	5·6	3·9

Control experiments showed that ordinary sterile unfermented mash does not, apparently, increase in acidity on keeping whatever the pressure used in sterilisation (from one to ten atmospheres).

[1] *J. Biol. Chem.*, 1918, 35, 83

In order to prove that the disappearance of acetone and increased acidity are not caused by evaporation or by purely chemical reactions among the various products of fermentation, the following experiments were made :—

(1) Fermented mash was heated under a reflux condenser and allowed to stand for some days.

(2) A mixture of acetone and butyl alcohol in the calculated fermentation proportions was made up with water and acidified with mineral acid to the degree usual after fermentation.

(3) A similar mixture was acidified by acid concentrates left after fermentation.

(4) Insoluble mash-residues were added to a mixture similar to (3).

(5) 10 c.c. of a vigorous strain of the organism were added to a mixture similar to (4).

In all cases the acetone-content and the degree of acidity were determined at regular intervals. In no case did the acidity vary more than 0·03, except in the case of the inoculated mixture, which gave the figure 3·4 after standing for a month. The acetone also remained practically constant at 0·94 per cent. in the first four, but had sunk to 0·58 per cent. in (5).

It is clear, therefore, that post-fermentation changes are practically entirely due to bacterial action, and that in absence of additional raw material the organisms continue to subsist on the products of the original fermentation, much as the acetic bacteria continue to oxidise acetic acid to carbon dioxide in absence of further additions of alcohol. During the post-fermentation changes the amount of water-soluble protein is also increased, as is seen from Table III.

The Fermentation of Mahua Flowers to Acetone.

Earlier workers[1] have experienced difficulty in obtaining commercial yields of acetone from mahua, although actual fermentation and formation of butyl alcohol did take place on inoculating small samples of mahua mash with a vigorous culture from maize. The exact cause of this difficulty was then left undetermined, and consequently the question was re-examined during the present inquiry. A sample of mahua flowers was obtained from Hyderabad (Deccan), which, on analysis, gave only some 23 per cent. of total sugar, and had obviously been stored for some time.

[1] Cf. Fowler, Wad and Gokhale, *loc. cit.*, pp. 4–5.

In order to discover the inhibitory agent present in the mahua flower, fermentation was attempted with products prepared in five different ways, the results being given in Table VII.

TABLE VII.

Medium	Acetone yield	Acidity
Extract alone	...	1·7
Extract freed from tannins by gelatin	0·5	1·72
Do. do. hide-powder ...	...	1·7
Sugar-free mash	3·4	1·4
Do. after boiling off essential oil ...	3·4	1·4

It is evident that the acetone is derived from the insoluble hemicellulose of the flower and not from the sugars. The essential oil does not seem to have any inhibitory effect. Consequently some starch was added to a quantity of this insoluble mash-residue and the mixture inoculated. The fermentation was, however, not vigorous and only 2·5 per cent. acetone was obtained after a fortnight's incubation, the acidity being 1·6.[1]

Symbiotic Fermentations.

In view of the powerful amylolytic action of the organism, it was thought that it could render possible the direct fermentation of starch to alcohol, if allowed to function in symbiosis with yeast. An experiment on these lines with a two per cent. jawari mash gave the following yields :—Acetone 4·5, alcohol 5·6, and acidity 2·8. In the same way alcohol was produced by the symbiotic activity of the acetone organism and *aspergillus oryzae*, the proportions being as follows :—Acetone 3·5, alcohol 3·2, acidity 1·5.

A similar symbiotic fermentation was attempted in a saccharine medium, viz., mahua mash, *Saccharomyces ellipsoideus* and mahua yeast being the organisms chosen to work with the acetone-organism; a greater proportion of alcohol was naturally obtained, the yields being as follows :—

Organisms used	Acetone	Alcohol	Acidity
Mahua yeast and acetone-organism	1·8	6·9	1·5
Saccharomyces ellipsoideus and acetone-organism ...	2·1	5·8	1·6

[1] Cf. This vol., p. 86.

That the acetone is derived probably more from the hemi-cellulose of the residual mahua flowers than from the sugar, is confirmed by an experiment where the mahua was fermented with yeast; the resultant alcohol and volatile acids were removed by distillation, and the residue fermented with the acetone-organism. A 2·3 per cent. yield of acetone was obtained, with an acidity of 1·3.

The present investigation confirms earlier experiments in showing that mahua is not at any rate a trustworthy raw material for the commercial production of acetone. For the preparation of power-alcohol, containing a small proportion of acetone to facilitate ignition, the fermentation of mahua in presence both of yeast and the acetone-organism, would, however, seem to offer possibilities.

Occurrence of Acetone-producing Organisms in Nature.

In order to discover the most general habitat of these organisms, and thence, to deduce their function in nature, several types of soils, fresh and dry vegetation, and sewage matter were examined and inoculated into jawari mash. The acetone-producing organisms were sub-cultured in jawari mash by the Weizmann method,[1] up to the sixth sub-culture and the strain thus obtained, though still not certainly pure, submitted to a fermentation test on a scale large enough to permit detection or isolation of products.

The following criteria were taken as indicative of the presence of acetone-producing organisms of the Fitz type:—(*a*) Gas production. (*b*) Acetone production from cereal mashes. (*c*) Acidity range not exceeding 3 at the termination of fermentation. (*d*) Resistance of spores to a sub-cultural temperature of 90° (approximately). (*e*) Morphological characteristics.

The results are shortly stated in the following paragraph:—

(1) *Paddy field soil.* (*a*) *Wet.*—Organism smaller than Weizmann bacillus, produces ethyl alcohol instead of butyl alcohol. Requires calcium carbonate for good fermentation. Acetone yield 9·3 per cent. Final acidity 1·89. (*b*) *Dry.*—Up to the fourth sub-culture the acidity was uniformly high, ranging between 4·8 and 6·0, with practically no production of acetone. The sixth sub-culture gave acetone 4·3 per cent., final acidity 1·35.

[1] *E. P.*, 4845 [1919].

(2) *Garden soil.* (*a*) *Dry.*—Acetone-production after sixth sub-culture 4·3 per cent., acidity 1·9. (*b*) *Wet.*—Containing some farmyard manure, acetone even at sixth sub-culture, nil, acidity 4·5–5·5.

(3) *Dry road-soil.*—Acetone after sixth sub-culture, traces only. Acidity 0·75.

(4) *Ant-hill earth.*—No gas production, acidity 3·4, acetone nil.

(5) *Dry leaves.*—Acetone traces, acidity 2·1.

(6) *Fresh leaves of Casuarina.*—Acetone two per cent. after sixth sub-culture, acidity 1·9.

(7) *Lantana stem.* (*a*) *Dry.*—Acetone after fifth sub-culture, 2·1 per cent., acidity 1·9. (*b*) *Green.*—Acetone after fifth sub-culture, 2·3 per cent., acidity 1·87.

(8) *Potatoes* (fresh and ripe).—Pure strain isolated after eighth sub-culture. Acetone 8·9 per cent., final acidity 1·7.

(9) *Activated sludge.* (*a*)—Fresh sludge allowed to incubate for two days and then used as inoculant :

First sub-culture	Acetone nil,	acidity,	4·1.
Fourth sub-culture....	,,	1·1,	,,	3·2.
Sixth ,, ...	,,	3·9,	,,	2·3.

(*b*)—After four days' aeration of the sludge it yielded no acetone even after the sixth sub-culture ; the final acidity was 2·08. In the first sub-culture there was a considerable growth of mould.

The foregoing results give fairly clear evidence that the organism is to be found in greatest abundance in presence of starch, whether in the seeds of cereals, in potato tubers or in leaves. Its presence in sewage is natural owing to its occurrence in staple foods and the resistance of the spores to heat. Anaerobic conditions evidently favour its development. The failure to detect it in soil containing farmyard manure, may be due to the increased competition from other organisms.

Large-scale Experiments.

In order to test the efficiency of one of the most active in acetone-production of the organisms thus isolated, viz., No. 1 from wet paddy soil, a few large-scale experiments were carried out in a 25-gallon fermentation vessel.

The strain was first developed in jawari-peptone-calcium carbonate mash, and two litres of vigorous seed-culture prepared. The fermentation medium was prepared in the vessel itself by maintaining the mixed meal (15 lbs. of jawari powder, 1½ lbs. of calcium carbonate and 25 gallons water), at 15–20 lbs. steam pressure for 6–7 hours. The mash was then cooled by allowing cold water to pass through the surrounding jacket while the mash was vigorously stirred. The seed-culture was then added with aseptic precautions.

The fermentation began in 12 hours and was allowed to continue for 3, 11 and 6 days respectively in the three experiments. The contents of the fermentation vessel were then discharged, distilled to 1/15 of the original volume and the distillate fractionated for acetone. The following yields were obtained :—

Weight of jawari	Calcium carbonate added	No. of days fermenting	Acetone yield
14 lbs.	2 lbs.	3	250 grms.
15 lbs.	1½ lbs.	11	400 grms.
10 lbs.	1 lb.	6	330 grms.

CONCLUSIONS.

The main conclusions from the foregoing studies may be stated shortly as follows.

1. The spores of the Weizmann bacillus developed in maize culture, and preserved in sealed tubes, retain their vitality for upwards of seven years.

2. The organism grows best on cereal mashes, the insoluble vegetable protein of the grain being essential to its healthy metabolism.

3. The post-fermentation changes in acidity and acetone percentage are mainly due to bacterial activity.

4. An extract of mahua flowers, if these have been stored for a prolonged period, appears to be unfermentable by the acetone-organism under investigation. The residual mash is, to some extent, fermentable.

5. Organisms akin to the Weizmann bacillus are shown to be widespread in nature, associated especially with the presence of starch in seeds, tubers or leaves.

II. MAHUA FLOWERS AS RAW MATERIAL FOR THE ACETONE-FERMENTATION PROCESS.

By A. G. Gokhale.

As stated on p. 81 difficulties were experienced in the early experiments on the use of mahua as a raw material for the production of acetone by fermentation, and the matter was left over for further investigation. Most of the experiments now to be described were made prior to 1920, but after discussing the recent observations, it was decided to make some further trials to elucidate certain critical points.

The experiments may be grouped under the following heads :—

1. Experiments with fresh undried flowers.
2. ,, with fresh sun-dried flowers.
3. ,, with flowers from various sources.
4. ,, on effect of additions to the fermentation medium.

The general method of procedure was similar in all cases. A quantity of flowers sufficient to give 2–5 per cent. concentration calculated on the dry flowers was mashed with boiling water, and the volume made up to 4 litres in a flask. The flask and its contents were steamed for 4 hours, and then autoclaved at 5 lbs. pressure for two hours on each of two successive days. It was then cooled down to 37° and inoculated with 100 c.c. of a vigorous culture of the acetone micro-organism.

1. Fresh Undried Flowers.

For these experiments fresh ripe flowers were collected as they fell from a tree near the laboratory. The flowers were a pale cream-colour, and gave the following percentage results on analysis :—

Moisture, 84·4 : Total sugars, 9·8 : Reducing sugars, 6·8 : Nitrogen, 0·13.

These figures expressed on dry flowers give :—

Total sugars	....	62·75
Reducing sugars	....	43·70
Nitrogen	....	0·82

The fermentation results are summarised in the following statement :—

No. of experiment	Duration of fermentation, hours	Final acidity	Yield of acetone percentage on dry Mahua
1	40	...	5·2
	60	1·6	7·1
2	60	2·3	7·3

2. Fresh Sun-dried Flowers.

1	90	4·5	2·1
2	67	1·9	5·6
3 *	137	2·0	5·3

A number of fermentations were made using different concentrations of sun-dried flowers, from two to twelve per cent. The acetone was not separately determined in these cases, but the yield of mixed oil (acetone and butyl alcohol) in c.c. per unit gives a measure of the fermentation activity. The results are summarised in the following statement :—

Concentration per cent.	Duration, hours	Final acidity	Yield of oil
2	88	1·8	2·8
4	88	2·0	8·7
6	120	2·3	9·0
8	120	2·6	9·1
10	144	5·3	Trace
12	69	5·3	...

It was noticed that the proportion of involution forms of the organism present in the medium increased with the duration of the fermentation.

* In the previous experiments the Mahua mash was sterilised under pressure. This caramelised the mash to a slight extent. To prevent this a flask was prepared and sterilised in the steamer for one hour on each of two successive days. The colour of the resulting mash was much lighter than that sterilised under pressure. The results of fermenting this mash are given as Experiment No. 3 * in the above table.

3. Experiments with Flowers from Various Sources.

The results may be summarised as follows :—

Source of flowers	Moisture-content	Duration of fermentation, hours	Final acidity	Yield of acetone. Percentage on dried flowers
Peint Range, Nasik District ...	7·90	70	...	5·3
Katni Murwara, C. P.	9·35	91	3·8	5·4
Cawnpore	12·60	91	3·2	0·2

4. Experiments on Effects of Additions to the Fermentation Medium.

These experiments were undertaken with the object of seeing whether the speed of the fermentation could be accelerated by the addition of mineral nutrients, such as ammonium phosphate, by neutralisation of a portion of the acidity with lime, and by the addition of a certain proportion of jawari flour. The effect of the presence or absence of the mashed flowers in the medium, of the essential oil of mahua, and of the preliminary inversion of non-reducing sugars, was also studied. The results are summarised in the following statement :—

Character of fermenting medium	Substance added	Duration of fermentation, hours	Final acidity	Yield of acetone. Percentage on dried flowers
Mahua mash of fresh flowers, 1·55 per cent. concentration.	Jawari flour 3 per cent. concentration ...	60	2·3	7·3
Mahua mash of fresh undried flowers, 4·5 per cent. concentration.	Jawari flour 1·5 per cent. concentration ...	36	1·8	6·6
Fresh sun-dried flowers, mash 5 per cent. concentration.	Jawari flour 1·5 per cent. concentration ...	67	1·8	6·2
Mash of sun-dried flowers, 6 per cent. concentration.	Sodium-ammonium phosphate, 0·1 per cent.	144	2·0	52 c.c. mixed oil
Mash of sun-dried flowers, 6 per cent. concentration.	Calcium oxide to reduce acidity to 0·1 ...	1 week	2·0	38 c.c. mixed oil
Non-reducing sugars in mash inverted with sulphuric acid and acidity neutralised with lime.	...	162	2·0	4·2
Mahua flower extract without residual mash.	...	More than a week	2·9	38 c.c. mixed oil
Mash of flowers, steamed to remove essential oil.	(*a*) Nil.	64	2·6	12 c.c. mixed oil
	(*b*) Lime	64	2·6	Nil.

CONCLUSIONS.

From the foregoing experiments the following conclusions are drawn :—

(1) Fresh undried flowers give the greatest yield of acetone, and the completion-period of fermentation is comparatively short.

(2) Dried flowers give a yield of about 5 per cent. acetone, but the time of complete fermentation is very long, viz., 90 hours or more.

(3) The reduction of initial acidity, the addition of phosphate, increase in the proportion of inoculant, removal of essential oil by steaming, and the presence or absence of the mash-residue in the fermenting medium, have no effect on the time of fermentation.

(4) Concentration of the mash to 10 or 12 per cent. inhibits fermentation, though within certain limits there is an increased yield with increased concentration.

(5) Addition of starchy material does not make any appreciable difference in the fermentation.

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