THE BIO-CHEMISTRY OF THE INDIGENOUS INDIGO DYE VAT.

By Gilbert J. Fowler, and M. Srinivasiah.

In the course of experiments conducted by one of us in conjunction with Mr. Padmanabha Pillay on indigenous dyes referred to in the Appendix to the Eighth Annual Report of the Indian Institute of Science, the subject of the indigenous indigo dye vat came under consideration.

Some laboratory dyeing trials were made and certain local dye vats were inspected. It was evident from this inspection and from ordinary text book information that the indigenous method of indigo dyeing was a complex and somewhat unsavoury operation.

It was determined that as opportunity offered a careful study should be made of the subject with a view to a better scientific understanding of the process and therefore to its more efficient control. Mr. Pillay received a scholarship from the Travancore Government to proceed to England for the general study of dyeing and textiles. Mr. Srinivasiah joined the Department with a scholarship from the Mysore Government given for the special purpose of studying indigenous dyes, and the work was therefore placed in his hands.

Mr. Srinivasiah was able to obtain interesting material and information from indigo dyers in the locality which furnished the starting point for the present researches.

The process of indigo dyeing comprises as is well known, three stages; the reduction of indigo blue to indigo white by suitable reducing agents, the steeping of the fabric in the bath of reduced indigo thus obtained and the subsequent dyeing of the fabric by exposure to air when the indigo white spontaneously oxidises to indigo blue.

The various dye vats differ mainly in the different reducing agents employed.

Among purely chemical agents may be mentioned:—

(a) Lime and copperas
(b) Lime and zinc
(c) Sodium bisulphite and zinc
(d) Stannous chloride
(e) Sodium "hydrosulphite" ($Na_2S_2O_4$).

Peter Maguire (J. Soc. Dyers and Col. 1907, p. 36) working with different vats has shown that the hydrosulphite vat is by far the most economical of chemical vats. He tabulates the losses of indigo in the various vats thus :—

	% loss.
Bisulphite zinc lime vat ...	... 30
Copperas vat ...	... 20
Zinc lime vat ...	... 10
Hydrosulphite vat ...	... 1—2

The main loss occurs through absorption of indigo by the sludge produced in the first three processes. No sludge is produced in the hydrosulphite vat and this is its main advantage.

Stannous chloride is more costly than the above mentioned materials and can be used only for a short time.

Among organic reducing agents carbohydrate material capable of liberating nascent hydrogen on fermentation is employed such *e. g.* as "sharps," bran, starch, dates, &c. Fermentation sets in spontaneously or may be started by the addition of a little putrid urine or guano. Attempts have been made (Collin and Benoist Journ. Soc. Chem. Ind. 1885 p. 493; Binz. Ber. 1906. 39 1627, 1631) to maintain a pure fermentation by utilising a single species of organism, but so far such a method has not established itself in Europe.

In the dye vats employed in Bangalore and generally in the south of India a species of seed is the fermenting agent, about 9 lbs of seed being added to the vat per 1 lb of indigo of about 50% indigotin content.

The object of the researches to be described in the following pages was to determine the nature of the fermentation set up by the seeds as a preliminary to the scientific control of the process.

The main directions of the work are indicated under the following heads :—

1. Chemical examination of the seed.
2. Study of the bacteria occurring in the seed.
3. Study of the fermentation under special laboratory conditions.

1. Chemical examination of the seed.

General. The plant which produces the seed in question is known as *Cassia tora* and belongs to the order Leguminosae. The seeds are hard, greyish in colour, and about 1/16 inch in diameter, and their ends appear to be cut off obliquely. The seeds are covered with a white coating which can be removed by rubbing. It swells on being soaked in water and has the appearance and properties of a mucilage, being, in all likelihood an exudation from within the seed of the mucilage described in greater detail later.

The crushed seeds have a bitter taste due to a substance also referred to in more detail later.

The seeds swell up in water and begin to germinate on the second day after immersion. Under ordinary circumstances fermentation sets in apparently spontaneously, hydrogen and carbon dioxide being evolved.

The endosperm is large and consists of a spongy tissue which swells up in water yielding a mucilaginous mash. It appears therefore that this mucilage is the reserve food material of the plant.

The germ and the cotyledons are yellow in colour, the germ occupying a large proportion of the bulk of the seed. This may account for the high nitrogen content of the seed, the nitrogen apparently occurring as a sulphur protein. The colouring matter is fairly easily soluble in water or alcohol to an orange yellow solution.

Microscopical examination.

Sections of the seed were prepared for microscopical examination as follows :—

Well developed seeds were selected, washed with copper sulphate solution (20%) and then with water and afterwards soaked in 10% formalin solution for a week. They were then

softened in glycerine for a week and cut into two halves. These were transferred first to 95% alcohol and finally into absolute alcohol. The seeds thus dehydrated, were embedded in paraffin by the following process. They were placed in a small 50 cc. beaker and just covered with xylol. The beaker was placed in an incubator at 45°C and solid paraffin added at intervals. Finally the whole was kept in a water bath at 60°C for a week, when the seeds were satisfactorily embedded.

Sections, 20 μ in thickness, were cut by a Minot microtome, and permanent preparations made as follows :—

The paraffin was dissolved out in xylol, the section cleared in clove-oil (or in the absence of clove oil, cinnamon oil or oil from *Hardwickia pinnata* may be employed) and again immersed in xylol and finally in alcohol, after which they could be examined by staining and finally fixed in Canada balsam.

The general appearance of the seed and of a section is given in the accompanying drawings.

Staining with iodine revealed very little starch, picric blue showed the presence of cellulose (blue) and ligno-cellulose (yellow) lac dye was taken up only by the nitrogenous germ.

Chemical analysis.

General. A preliminary systematic analysis of the seeds gave the following results.

	Per cent.
Moisture (dried at 93·5°C)	10·3
Alcohol extract (95 % alcohol)	16·3
Petrol „	6·7
Carbon disulphide extract (does not dissolve colouring matter)	6·0
Nitrogen	1·4 to 1·9
Proteins (calculated N x 6·3)	12·0
Sugars on acid hydrolysis	20·2
Ash	3·9

Analysis of the ash showed the presence of phosphorus and sulphur in the following percentages calculated on the weight of the dry seed.

Phosphorus	·005
Sulphur	·013

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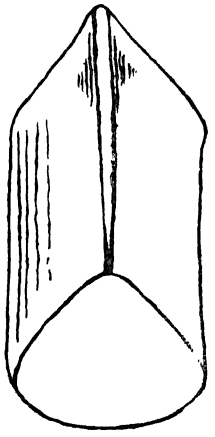

Figure 1. Drawing showing general appearance of Seed.

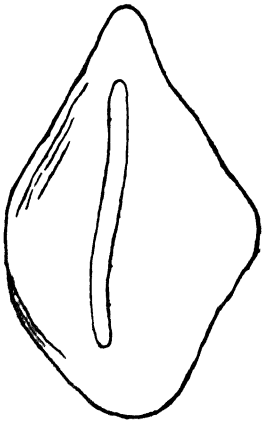

Figure 2. Drawing showing cotyledon of embryo.

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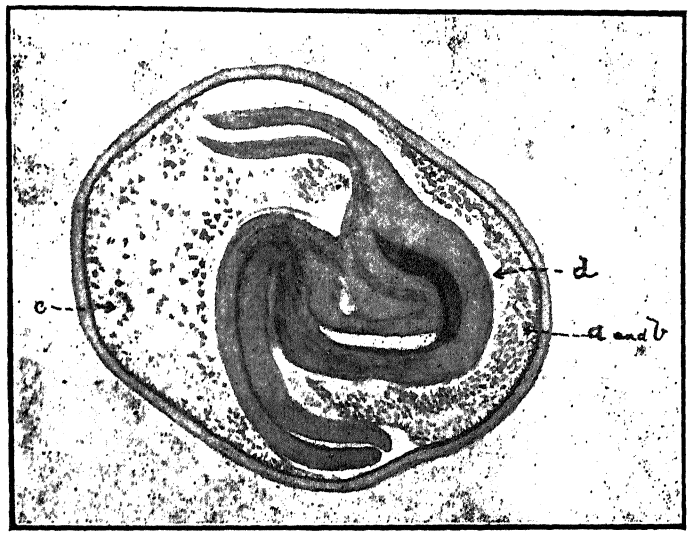

Figure 3. Drawing showing transverse section of seed.
a & b testa and tegmen.
c spongy tissue containing mucilage.
d Embryo.

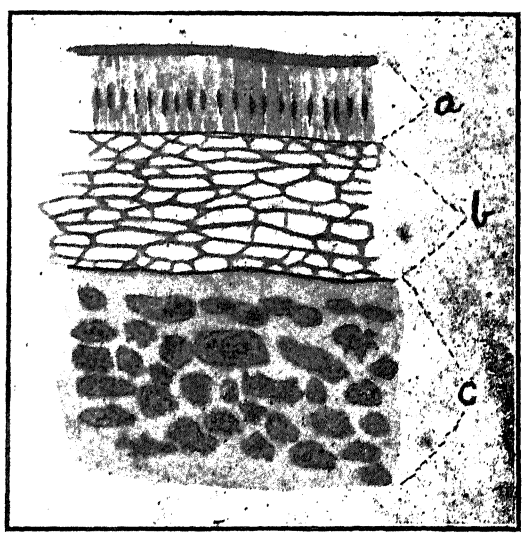

Figure 4. Drawing showing a portion of transverse section enlarged to show :—
a testa
b tegmen
c the structure of the spongy tissue.

During acid fermentation of the seed notable quantities of hydrogen sulphide are given off. It evidently is formed from in a sulphur protein.

Examination of the mucilage.

Method of preparation. The seeds were soaked in water, a little copper sulphate being added to prevent fermentation, and allowed to soften over night. On gently crushing, the germ could be readily separated from the endosperm, which was then boiled with water and the resulting mucilage filtered through a fine cloth. A clear transparent solution was obtained, a final clarification with animal charcoal being given where necessary

The mucilage has the following *physical* properties:—

1. It is a colloid and does not pass through parchment paper.

2. It holds gases in solution which are given off if the mucilage is placed under a vacuum.

3. It holds finely ground indigo in suspension indefinitely.

The foregoing characteristics clearly are of importance in providing conditions favourable to indigo reductions.

4. The mucilage is optically inactive.

The following are among its chief *chemical* characteristics.

1. It gives a reddish brown colour with iodine.

2. It does not reduce Fehling's solution.

3. It is precipitated by alcohol, salts of heavy metals such as copper sulphate or lead acetate and by phosphotungstic acid.

4. It gives no reaction for inulin.

It gives no red colouration when boiled with resorcinol and strong hydrochloric acid.

The foregoing reactions show that it is of the nature of a gum mucilage and that starch and also pentoses are absent.

5. The mucilage is converted into sugars by acid hydrolysis, by enzyme action and by bacterial fermentation.

Acid hydrolysis yields glucose and galactose, the details of the method of examination being as follows :—

The water extract of the seeds, consisting mainly of mucilage was hydrolysed with dilute sulphuric acid on a water bath under a reflex condenser for three hours. The hydrolysed liquor had then completely lost its mucilaginous character. It was neutralised with barium carbonate, filtered, and the filtrate treated with lead acetate and excess of lead removed by precipitation with sodium phosphate and filtration.

The filtrate containing the sugars was evaporated down to a small bulk and then treated with phenylhydrazine in acetic acid solution and kept on a water bath for half an hour.

Yellow crystals of osazones were formed, which were crystallised from hot alcohol. Two kinds of crystals were obtained which were identified as glucosazone and galactosazone, a sample of the latter being specially prepared from galactose for comparison.

The mucilage can also be saccharified by malted seeds of *Cassia tora*. The malted seeds do not, in fact, yield the mucilage on being soaked or boiled in water, the spongy reserve material being broken down to sugars by the action of the enzymes developed during germination.

On exposure to air the mucilage undergoes fermentation as well as when inoculated with special organisms isolated from the indigo vat.

Isolation of bitter principle of seeds.

As already stated the seeds have a bitter taste. Experiments to be described later, indicated that the substance giving this taste might have important antiseptic properties. Consequently it was examined in some detail as follows :—

500 gms of the crushed seeds were extracted with alcohol (95%). The alcoholic extract was diluted with twice its own volume of water, to precipitate all the resins, fats and chloroplasts which had been extracted by means of alcohol. After filtration the liquid was clarified by basic lead acetate and the colour removed by animal charcoal. The clear extract was evaporated on a water bath to a small volume and extracted with chloroform. The product was not obtained crystalline, but is readily soluble in water, ether. acetone, alcohol and chloroform It tastes both bitter and sweet. It therefore would appear likely to be a glucoside but the quantity available was insufficient for further chemical investigation.

2. Study of the bacteria occurring on the seed.

The following experiments were carried out with the object of ascertaining the general character of the bacteria present in the seeds and whether the fermentation was due to bacterial action or to an enzyme secreted by the seed.

Experiment 1. 5 gms of the seeds were washed in 100 c. c. of sterile water and one c. c. of the washing used for inoculation on to nutrient agar plates which were incubated at 37°C ; a number of colonies appeared after 24 hours.

The bacteria were found to be short, actively motile bacilli.

On replating on to nutrient agar a good growth was obtained. On inoculation of the seed mucilage with the bacteria a good fermentation and gas evolution was obtained.

The bacteria ferment glucose broth and potato broth.

Experiment 2. The seeds were soaked in 2% copper sulphate solution for an hour washed free from copper sulphate with sterile water, transferred to a sterile flask, which was filled with water and plugged with cotton wool.

A control with unsterilised seed and water was kept in a similar flask under similar conditions. After 20 hours, it was found that fermentation had set in in the control, while the other flask did not ferment.

Experiment 3. In case the copper sulphate might be supposed to have inhibited enzyme action a further experiment was tried.

Two flasks were set up with 100 c. c. of a two per cent mash of fresh seeds. To one was added thymol while the other was left without an antiseptic. After 24 hours no action had taken place in the flask containing thymol while the contents of the control flask had undergone complete fermentation. It appears evident then that the fermentation produced by the seeds is due to bacteria residing on the surface of the seeds and fermenting the mucilaginous contents of the endosperm.

3. Studies of the fermentation under special laboratory conditions.

Having discovered that the fermentation was due to bacteria acting on the mucilage of the seeds, the process was examined more in detail.

Two flasks were set up and the fermentation conditions noted day by day as follows:—

Flask A. 5 gms of crushed seeds were taken with 600 cc. of tap water, an arrangement being made to collect the gas over brine.

Flask B. The same except that alkali was added from time to time as acids were formed in the course of fermentation.

The following table shows the results obtained:—

TABLE I.

Date.	Flask A. (no alkali)	Flask B. (with alkali)	Remarks.
7th October 11 a.m.	Start.	Start.	
8th do.	Fermentation begun. 17 cc gas.	Fermentation begun. 15 cc. gas.	
9th do.	65 do. Acidity=12 cc of alkali per 20 cc of fermented liquor.	62 do. Acidity=12 cc of alkali per 20 cc of fermented liquor.	Flask B was neutralised with alkali.
10th do.	99 cc gas. 18 cc alkali per 20 cc.	132 cc gas.	Flask B again neutralised with alkali.
11th do.	do.	207 cc gas. 28 cc alkali for 20 cc.	Both flasks were neutralised.
12th do.	Gas evolution began.	...	...
14th do.	28 cc of alkali per 20 cc.	...	Note that the final acidity reached is the same in both cases after neutralisation.

N. B. Strength of the alkali used was N/100.
Indicator — Phenolphthalein.

The difference in the behaviour of the two flasks would appear to be due to the evolution of hydrogen sulphide in the early stages of the fermentation which acts as a poison and stops fermentation in flask A. On neutralisation with alkali fermentation recommences and finally the acidity reaches the same point in both flasks.

The gas evolved was analysed after transference to a Lunge nitrometer, by treatment with potash and pyrogallol successively. The residual gas was transferred by means of a capillary tube filled with mercury to an eudiometer and exploded with excess of oxygen. The gas remaining after explosion was treated with potash and pyrogallol in succession.

The result of the analysis was as follows:—

Gas taken	...	...	41·4 cc
Carbon dioxide	...	...	20·1 „
Hydrogen	...	...	20·0 „
Nitrogen	...	...	1·0 „
Oxygen	...	...	0·2 „

Evidently the fermentation gases consist of hydrogen and carbon dioxide in equal proportion and nascent hydrogen is the reducing agent in the indigo vat.

Examination of the contents of the flasks.

Bacteria. The bacteria were plated out on nutrient agar and after 24 hours growth, the colonies were examined under the microscope.

A practically pure culture was obtained of bacteria similar to those described in section 2 page 211. This observation is significant having regard to the fact that no special precautions were taken to sterilise the contents of the flask before fermentation.

The following table shows the fermenting properties of the bacteria thus isolated:—

TABLE NO. II.

Bacteria from.	Starch peptone.	Potatoe broth.	Glucose broth.	Glycerine broth.	Decoction of the seeds.
Flask A.	—	+	+	+	+
„ B.	—	+	+	+	+

Chemical products of fermentation.

The acid fermented mash was distilled and the distillate neutralised with baryta and evaporated on a water bath. The residue was transferred to a small distilling flask and treated with dilute sulphuric acid. An acid was given off having the characteristic smell of butyric acid. Hydrogen sulphide was also recognised by its smell and by the blackening of lead acetate paper.

The fermentation is evidently a butyric fermentation of the carbohydrate of the mucilage, protein at the same time being decomposed with evolution of hydrogen sulphide.

Such a fermentation must it would seem, be necessarily of an offensive character, except in so far as the production of free acid is checked by periodical addition of alkali.

Reduction of indigo by seed bacteria.

It was of interest to determine under what conditions indigo could be reduced by the nascent hydrogen produced in the fermentations just described. It was evidently necessary that the indigo should be in a fine state of division and be held in suspension during the progress of the reaction.

The following media were therefore prepared :—

I.	Ammonium phosphate	... 1·0 gm
	Glucose	... 4·0 „
	Water	... 200 cc
II.	2% seed extract.	
III.	2% potato extract.	
IV.	Glucose	... 2%
	Gum arabic	... 2%
V.	Glucose	... 2%
	Peptone	... 1%
	Lemco	... 1%
	Sodium chloride	... ·5%
	Ammonium formate	... ·01%

All these media were rendered slightly alkaline coloured blue with finally ground indigo put into sterile tubes and sterilised. They were inoculated with plated out bacteria and the tubes after inoculation were kept in a vacuum desiccator over pyrogallate of soda to prevent oxidation of reduced indigo. The whole was incubated at 37°C. The results are set out in the following table :—

TABLE No. III.

Seed and indigo No. I.	Glucose, ammonium phosphate and indigo. No. II.	Potato extract and indigo. No. III.	Glucose, gum arabic and indigo. No. IV.	Glucose broth, ammonium formate and indigo. No. V.
Indigo is gradually reduced, when shaken in air it turns blue and is again reduced next day.	Vigorous fermentation, indigo precipitated but not reduced.	Vigorous fermentation, indigo precipitated but not reduced.	Gum helps to hold indigo in suspension, but fermentation was slow and indigo was not reduced.	Good fermentation and some reduction of indigo but most of it precipitated.

It is evident from the above results that the seed mucilage possesses all the properties necessary to bring about the reduction of indigo. It holds the indigo in suspension, is itself

fermentable and occludes the reducing gas. Gum arabic is not fermentable by the bacteria employed and so does not give the same results in spite of its similar physical properties. On the other hand ammonium formate appears to hasten reduction and the experiment was repeated with larger quantities of material 250 cc flasks being used. The following results were obtained :—

Seed mucilage	Good reduction.
Glucose broth	Good fermentation. Small reduction.
Glucose broth and ammonium formate.	Good reduction.

The effect of ammonium formate is of interest and deserves further study. From the researches of E. C. Grey (Proc. Roy. Soc. B. Vol. 91, p 294) it would appear likely that it increases bacterial activity.

Formates are used to increase the relative concentration of hydrogen producing bacteria.

Effect of temperature on the reduction process.

Reduction trials were made in litre flasks with seeds and indigo and the necessary amount of alkali, the flasks being kept at different temperatures thus :—

Flask.	Temperature.	Time taken for reduction.
A	20°C	very slow.
B	37°C	8 days.
C	43-45°C	7 ,,

A reduction was carried out in two 6 litre cylinders, one kept at room temperature, the other at 45°C.

At the high temperature 6 days were required for completion while at the lower temperature 9 days were required for an equivalent amount of reduction to take place.

Whether the same amount of carbohydrate is fermented or whether the fermentation follows the same course in the two cases has not been determined, but the other things being equal, it is evident that rate of reduction increases up to a certain point with the temperature.

Reduction with bacteria from various sources.

It has been shown that the bacteria occurring on the seeds are mainly of one kind and that they are identical with

those which produce the fermentation. It was of interest to find out whether the same bacteria occurred in vat liquors from different localities.

A sample of liquor was therefore obtained from Bangalore and two from Madura and compared with the bacteria used in the foregoing experiments.

The details of manipulation were as follows :—

One cc of the vat liquor was taken in a sterile pipette and diluted in a sterile flask with a litre of sterile water. From the dilute solution a loop was taken, inoculated into nutrient agar and plated. In 24 hours the plates were examined microscopically and it was found that in all four cases the predominating organism was identical with the one invariably accompanying the seed and which is responsibe for the reduction of indigo.

This striking similarity in the bacterial content of the different vats and the persistent occurrence of the same organism, leads to the conclusion that some specific relation exists between the organism and the seed. It appeared likely that the bitter principle occurring in the seed the extraction of which is described on p. 210 might exercise a selective antiseptic effect.

The following experiments were made to determine this point. A number of tubes of glucose nutrient broth were prepared and to half of them were added a few drops of an aqueous solution of the bitter principle. The tubes were then inoculated with various types of bacteria which were available in the laboratory. The results are given in the following statement.

\+ denotes fermentation.

— ,, no fermentation.

	Source of organism.	With bitter principle.	Without bitter principle.
1.	Butyric fermentation	—	+
2.	Nitrogen fixing medium	+	+
3.	Sewage	—	+
4.	Acetone fermentation	—	+
5.	Indigo vat	+	+

Instead of the glucose broth a sterile extract of the seeds was used, prepared in one case from the seeds without special

treatment and in the other case from seeds which had been extracted with alcohol with consequent removal of the bitter principle.

The result were as follows :—

	Character of organism.	Extract of untreated seeds.	Extract of seeds after treatment with alcohol.
1.	Butyric fermentation.	—	+
2.	Acetone.	—	+
3.	Sewage.	+	+
4.	Madura Indigo vat.	+	+
5.	Yeasts.	—	—

The above experiments taken together show clearly the selective antiseptic properties of the bitter principle. Such an effect must have some relation to the life conditions of the seed and it would appear likely to be a general phenomenon. The observation led to the investigation of the alkaloid present in unpolished rice which was found to have a similar function.*

The antiseptic effect of the bitter principle of the seeds is shown further in an experiment which had for its object to discover whether indigo itself was attacked by bacteria in absence of other source of nitrogen.

It is well known that under certain circumstances cyclic compounds can be broken down by bacteria† and it appeared likely that absence of sufficient nitrogenous pabulum in the indigo vat, other than indigo itself, might result in the destruction of the latter.

Three vats were therefore set up each of 2 litre capacity and containing one gram of indigo.

The following additions were made :—

Vat I. Unsterilised seed.

„ II. Potato starch.

„ III. Potato starch and peptone.

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* See Fowler and Sen "Studies relating to the bacteria associated with rice and other cereals". Journ Ind. Ins. Sc. Vol. 4 p. 120.

† *Cf e. g.* Fowler, Ardern & Lockett. Roy. Soc. Proc. Series B, Vol 83 1914, p 149.

Beesley J. Chem. Soc. Trans, 1914, Vol, 105, p 1014.

To each vat 5 gms of unsterilised seeds were added to start the fermentation.

On the second day 10 cc of 20% caustic soda was added to each vat and on the fourth day the same amount was again added together with the following further proportions of fermentable matters.

Vat. I. 2 gms seeds.
„ II. 2 „ potato starch.
„ III. 2 „ potato starch and peptone.

5 cc alkali was added to vat I on the 5th day and on the 6th day the vat liquors were examined.

The indigo content was determined as follows:—

2 cc of the liquor were taken, diluted to 50 cc and air blown through. The colours in the three vats were then compared. Vat I and vat III were practically identical and vat II showed a distinct deficiency in comparison with the others.

In the absence of the seed mucilage, both vats II and III did not show such good reduction as vat I.

The fact that vat II contains less added nitrogen than either vat I or vat III would indicate that the deficiency observed in its indigo content after fermentation was due to the utilisation of the indigo by the bacteria as a source of nitrogen, but further confirmatory work is necessary on this point.

The *bacteriological* examination of the liquors showed distinct differences between the vats.

The cultures obtained by plating out on nutrient agar showed that vat I contained practically a pure culture of the organism observed throughout as associated with the seeds, vat II was swarming with cocci and vat III was also infected though to a less extent.

The selective antiseptic action of the seeds is again evident.

Dyeing trials with and without special culture of bacteria.

The experiments so far described would indicate that the presence of the seeds in the vat ensures a reasonably pure fermentation. It remains to be seen whether any difference in the

dyeing effect of the liquor can be observed when the vat is inoculated with the seeds only or with a special culture prepared from them.

Two laboratory vats of six litres volume were made up in the tall cylinders used for the purpose. The composition and behaviour of the vats can be seen from the table.

TABLE No. IV.

	NATURAL VAT.	PURE CULTURE VAT.
First day ...	Seeds—30 gms. water—6 litres. indigo—5 gms. alkali 10 cc of 20% strength added in the evening when fermentation had set in.	Boiled seeds—30 gms. indigo—5 gms. water—6 litres. Inoculated with the pure culture grown on seed mash 250 cc. Alkali—10 cc of 20% strength added in the evening when fermentation had set in.
Second day ...	Added 20cc alkali, fermenting vigorously.	Added 20 cc alkali, fermenting quite well.
Third day ...	Added 10 cc alkali and stirred up the liquor.	Added 10 cc alkali and stirred up the vat.
Fourth day ...	10 gms. of seeds were added with 250 cc of water and 10 cc of alkali. Flurry had appeared at the top. The liquor was greenish blue.	10 gms of seeds *boiled* with 250 cc water added to the vat. 10 cc of alkali. Flurry had begun to appear, the liquor was greenish blue.
Fifth day ...	The liquor was yellowish green, 5 gms. of seeds were added and the liquor was stirred up.	Added 5 gms of *boiled* seeds and the liquor stirred up. Greenish liquor.
Sixth day ...	Liquor had been completely reduced and was golden yellow in colour. The vat was quite fit for dyeing.	The liquor was greenish yellow.
Seventh day ...	...	Complete reduction had taken place and was fit for dyeing.

N. B.—The indigotin content of the indigo used was 57 % estimated by the permanganate method.

The vats were examined daily at 10 a. m.

It will be been that the "pure culture" vat took seven days for complete reduction as against six days for the 'natural vat'. This delay is due to the fact that the boiling of the seeds before their addition to the "pure culture" vat destroyed the bacteria and consequently more time was required for the necessary growth to take place from the smaller quantity of inoculant.

Dyeing trials with each vat were made with cotton yarn, boiled out with soda, three dips of five minutes each being given. No difference between the shades could be observed.

It is evidently therefore of no advantage artificially to inoculate an indigo vat with separately prepared pure cultures. of bacteria. So long as an adequate supply of '*cassia tora*' seeds can be obtained they ensure a practically pure fermentation owing to

the selective antiseptic action of the bitter substance which they contain.

A number of experiments on the practical control of the indigo dye vat have been made, under varying conditions, in the light of the results described in the foregoing pages.

Thus the effects of acidity, alkalinity, temperature, light, the addition of various carbohydrates and also of excessive addition of seeds are being examined.

It is hoped to publish an account of this work in a further communication.

Conclusions.

The conclusions to be drawn from the experiments described in the present paper may be briefly summarised as follows:

The seeds of *Cassia tora* carry bacteria which ferment the mucilaginous content of the seeds with evolution of hydrogen and carbon dioxide. This hydrogen in the nascent state is the effective agent in reducing the indigo.

The reduction is assisted by the fact that the indigo is held in suspension by the mucilage while this is undergoing fermentation.

The seeds contain a bitter principle which exercises a selective antiseptic effect on the bacteria occurring in the seeds, so that practically only one species is present.

So long therefore as there is a sufficient supply of *Cassia tora* seeds, a reasonably pure culture is assured and a special technique to obtain such a result is unnecessary.

This selective antiseptic effect appears to be a general phenomenon and has been observed in the case of paddy*.

It is evidently of importance to the life of the seed and extended research work on the subject is in contemplation.

In the case of *Cassia tora* it is worthy of remark that the plant grows in barren soils and yet the seed has a high percentage of nitrogen. Further it may be noted that organisms from a medium in which nitrogen fixation had occurred were not inhibited by the bitter principle (see p. 216). These facts would lead to the conclusion that in the case of *Cassia tora* at any rate

* Fowler and Sen *loc cit* p 145.

the organisms occurring on the seeds may play some part in nitrogen fixation.

In conclusion we wish to thank Dr. Marsden for furnishing us material from Madura and for many helpful suggestions.

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