# SOME ENZYMES OF THE LEAF OF LANTANA CAMARA.

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One of the greatest industries of India and the East is the cultivation and manufacture of tea. The tea plant thrives however only under rather special natural conditions and the tea leaf must therefore always be somewhat expensive as the raw material for a popular beverage.

Under these circumstances it is of interest that according to Dalzell, an infusion of Lantana pseudo-thea (Syn. Lippia pseudo-thea) is used in the Brazils as a substitute for tea.

Some preliminary investigations have been made in this connection by Kanga (private communication) and it was at his suggestion that the work was taken up. The subject appeared to be of sufficient importance to warrant careful investigation, especially with a view of identifying and studying the enzymes of the lantana leaf and comparing them with the enzymes present in the tea leaf.

Lantana, as is well known, is generally looked upon as a rather noxious weed, suppressing all other growth on land on which it gets a hold. This view is not universally held however, some authorities contending that growth of lantana fosters the development of trees in otherwise barren tracts.

However this may be, it is certain that lantana grows freely under almost all conditions and if some definite use could be discovered for it, it would appear to offer considerable industrial possibilities.

The investigations described in this paper are in three directions :—

I. The identification of specific enzymes present in the leaf.

II. The examination of the collective behaviour of the enzymes present.

III. The preparation of a tea substitute from the leaves.

## I. The identification of specific enzymes present in the leaf.

*Oxidase.* In the manufacture of tea, an oxidase is the principal enzyme that brings about chemical changes in the leaf and determines the quality of the tea according as the fermentation process is controlled. The oxidase in lantana leaves was studied by a slight modification of Bunzell's method* of determining the oxidase content of plant juices. The method consists in allowing a known quantity of the enzyme extract to mix with a fixed volume of 1% pyrogallol solution in a Schrotter's apparatus at 37°C. Soda lime is placed in the absorption limb of the apparatus, to the neck of which is attached a manometer. The contraction in volume as shown by the manometer is due to the activity of the enzyme resulting in the absorption of oxygen and formation of carbon dioxide which is absorbed by the soda lime. Schrotter's apparatus (in duplicate, marked I and II) was suspended by a wire in an electric incubator and an arrangement made to gently rock the apparatus about 15 times a minute by means of a water turbine (see sketch). Before the shaking was commenced the apparatus was kept in the incubator for an hour so as to allow it to attain the experimental temperature. After the apparatus acquired the incubation temperature the pyrogallol and enzyme extract were allowed to mix, making sure that the height of the mercury in both the limbs of the manometer was the same. The shaking was now commenced by starting the water turbine and periodical readings taken by means of a cathetometer.

*Preparation of the enzyme extract.* 10 gms of the freshly plucked leaves of Lantana Camara were ground to a pulp in a wedgewood mortar and 5 gms of hide powder (pure for analysis) were added to remove tannins and the mass again ground thoroughly together. 50 ccs of water were now added to the mixture and the whole well stirred and allowed to stand 2 hours. The mass was then filtered through fine muslin cloth with pressure and the extract thus obtained was immediately utilised for investigating the activity of the enzyme.

2 ccs of the extract obtained as detailed above were placed in the Schrotter's apparatus and 8 ccs of 1% pyrogallol allowed to mix as before described and the shaking of the apparatus started.

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* The measurement of the oxidase content of plant juices by H. H. Bunzell—Bulletin No. 238, U. S. A. Department of Agriculture as modified by V. A. Tamhane.

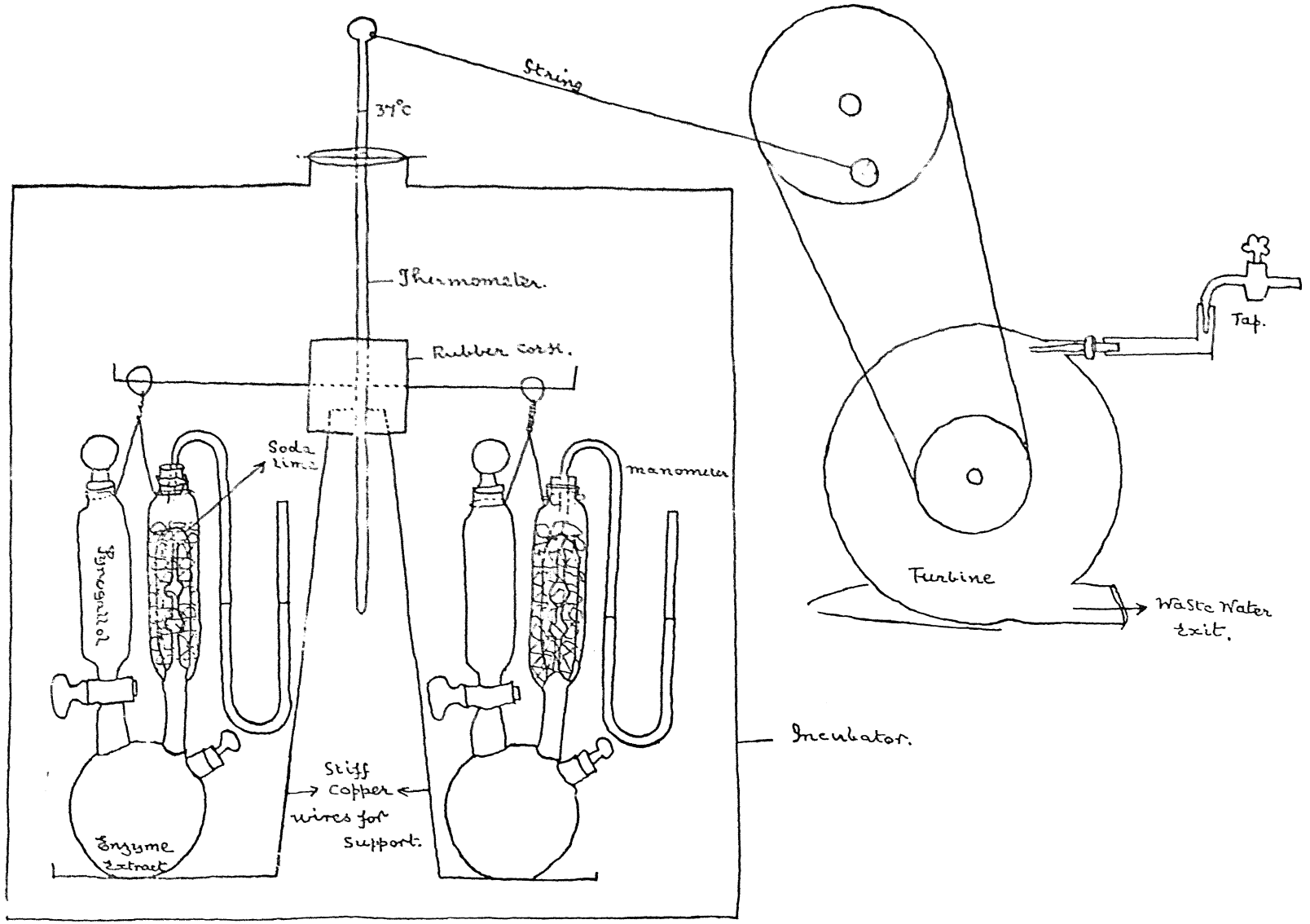


Diagram. Showing the oxidase apparatus.

The extract prepared from the growing tip leaves gave the following results :—

Quantity of extract employed ... 2 ccs
Temperature of experiment... ... 37°C

| Duration in hours. | Contraction in volume. | |
|---|---|---|
| | I | II |
| 3 | not observed | |
| 18 | ·7 | ·68 |
| 21 | ·8 | ·79 |
| 24 | ·87 | ·86 |
| 36 | 1·88 | 1·87 |
| 39 | 1·9 | 1·89 |
| 42 | 1·9 | 1·89 |

In another experiment 4 ccs of the freshly prepared extract were employed instead of 2, other experimental conditions being the same. The results obtained were as under :—

| Duration in hours. | Contraction in volume. | |
|---|---|---|
| | I | II |
| 3 | ·24 | ·25 |
| 15 | 1·36 | 1·37 |
| 24 | 1·8 | 1·82 |
| 30 | 2·45 | 2·46 |
| 39 | 3·75 | 3·76 |
| 42 | 3·75 | 3·76 |

With a view to see whether the enzyme content varies from leaf to leaf as in the case of the tea leaf, the extract was now prepared from leaves of an elder growth below the tip leaves and examined with the following results :—

Quantity of the extract employed ... 2 ccs.

| Duration in hours. | Contraction in volume. | |
|---|---|---|
| | I | II |
| 3 | ·13 | ·14 |
| 15 | ·66 | ·68 |
| 24 | ·87 | ·88 |
| 30 | 1·25 | 1·26 |
| 39 | 1·3 | 1·3 |
| 42 | 1·3 | 1·3 |

Other experimental conditions being the same, with 4 ccs of the extract the following results were obtained :—

| Duration in hours. | Contraction in volume. | |
|---|---|---|
| | I. | II. |
| 3 | ·25 | ·25 |
| 12 | ·96 | ·95 |
| 18 | 1·31 | 1·30 |
| 24 | 1·75 | 1·74 |
| 30 | 2 25 | 2·24 |
| 39 | 2·62 | 2·61 |
| 42 | 2·62 | 2·61 |

Both sets of experiment show that the activity of the enzyme is apparent after 3 hours and is over in 39 hours. The period of experiment when the enzyme showed the greatest activity cannot be definitely stated as successive readings for the periods of 3 hours are not available. The experiments also show that the enzyme content varies from leaf to leaf, the enzyme content in the tip leaf being greater than that in the leaves of an older growth. A similar fact has been pointed out by Mann* in the case of the tea leaf.

*Catalase.* The next enzyme investigated was catalase. The tip-leaves, as employed for the oxidase experiment were taken. 10 gms of these were thoroughly ground to a pulp, 5 gms hide powder added and 50 ccs of distilled water thoroughly incorporated with the mixture. The whole was left aside for 4 hours, strained through cloth and centrifuged for 10 minutes. The semi-transparent extract thus obtained was immediately utilised for experimental purposes, 2 ccs. of the extract being used with 25ccs of a three-volume solution of hydrogen peroxide. The oxygen evolved was collected in an incubator at 35°C. The apparatus was kept in the incubator to attain the experimental temperature and the enzyme extract allowed to run in by means of the tap funnel and mixed by shaking.

| Time. | (Oxygen evolved in ccs.) Without extract | With boiled extract. | With living extract. |
|---|---|---|---|
| After 15 minutes | 0 | 0 | 0 |
| „ 30 „ | 0 | 0 | 0 |
| „ 45 „ | 0 | 0 | 0 |
| „ 1 hour | 0 | 0 | 0 |
| „ 18 hours | 0 | 0 | 0 |

*The enzymes of the tea leaf—Part I—Mann Indian Tea Association.

Thinking that the enzyme may not have been soluble in water, 50 ccs of a 2·5% sodium chloride solution were employed for extracting the enzyme instead of distilled water.

| Time. | (Oxygen evolved in ccs.) Without extract | With 5 ccs living extract. |
|---|---|---|
| 15 minutes | 0 | 5 ccs |
| 30 ,, | 0 | 8 ,, |
| 45 ,, | 0 | 9 ,, |
| 1 hour | 0 | 9 ,, |

In this experiment 5 ccs of the enzyme extract were employed with 25 ccs of a three-volume solution of hydrogen peroxide. After 18 hours no further gas was evolvoed and the liquid in the flask was tested for oxygen with potassium permanganate and sulphuric acid. A copious evolution of oxygen and decolourisation of the permanganate solution showed that there was oxygen enough for further evolution but the enzyme activity had stopped.

To determine whether greater quantities of enzyme would go into solution with a stronger solution of salt, 50 ccs of a 5% sodium chloride solution were used for extraction and the results obtained were as under :—

| Time. | (Oxygen evolved in ccs.) Without extract. | With 5 ccs. extract. | With 10 ccs. extract. |
|---|---|---|---|
| After 15 minutes | 0 | 3 | 6 |
| ,, 30 ,, | 0 | 5 | 10 |
| ,, 45 ,, | 0 | 5·5 | 11 |
| ,, 60 ,, | 0 | 6 | 12 |
| ,, 75 ,, | 0 | 6·2 | 12·4 |
| ,, 90 ,, | 0 | 6 2 | 12·4 |

the reaction completely stopping in 75 minutes.

It is evident that in excess of a certain amount, increased concentration of salt solution does not tend to extract more enzyme. The enzyme thus identified may be the same as the β catalase of * Dr. Loew.

* U. S. Dept. of Agri Bulle. No. 68, Catalase by Oscar Loew.

*Amylase.* A 0·5% soluble starch paste was prepared and 25 ccs of this paste were put into 5 large test tubes and the enzyme extract added as follows :—

To (1) 5 ccs of the extract prepared as before described were added.

„ (2) 10 ccs of the extract were added.

„ (3) 5 ccs of the boiled extract were added.

„ (4) 5 ccs of distilled water were added.

The test tubes were kept in a constant temperature bath at 40°C for one hour. ½ cc was drawn from each of the four tubes and to each 9·5 ccs of distilled water added and shaken, and finally one drop of N/10 iodine solution carefully added from a burette and the colouration given by the starch iodine reaction was compared.

The colouration given by (3) & (4) was absolutely identical, an intense blue ; (2) gave a faint blue reaction and (1) gave a fairly deep blue reaction.

This shows that amylase was present in the extract and that a comparatively greater hydrolysis had taken place in (2) than in (1) owing to the presence of a greater quantity of amylase.

*Lipase.* The leaves of lantana on analysis showed the presence of higher fatty acids. 20 gms of freshly plucked tip leaves were extracted with 100 ccs of distilled water without hide powder and allowed to stand 4 hours. The extract was strained through cloth and the strained extract passed through a filter candle. The now clear extract was tested with litmus and did not show an acid reaction.

10 ccs of neutral cocoanut oil prepared by steaming and addition of lime water were put into 4 test tubes :—

(1) was kept by itself,

to (2) one cc of boiled enzyme extract,

to (3) one cc of the living enzyme extract,

to (4) two ccs of living enzyme extract,

were added and all the tubes were vigourously shaken from time to time and kept at room temperature 22-25°C for 30 hours, using 2% toluene as antiseptic.

1 cc out of each of the above tubes was pipetted off separately into 4 test tubes. 9 ccs of distilled water added to each

and a drop of litmus solution carefully added from a burette. (1) and (2) showed a distinct blue colour whereas (3) and (4) showed a well marked acid reaction. That in (4) was much more red than in (3) practically double in intensity. It would thus appear that this acidity was certainly due to lipase.

*Invertase.* A 5% solution of pure cane sugar was prepared and 25 ccs put into 4 test tubes and the enzyme extract as prepared above was utilised after filtering through a filter candle. (1) was made up with 5 ccs of boiled extract and 25 ccs cane sugar solution plus 5 ccs distilled water. (2) was made up exactly as (1) but with 5 ccs living extract. (3) was made up with 10 ccs living extract and 25 ccs cane sugar solution.

Thus in all the tubes there were 35 ccs of the combined extract and cane sugar solution. The tubes were kept in a thermostat at 45°C for 4 hours and then titrated with Fehling's solution. The tubes were taken out all at the same time and their activity stopped with a drop of strong alkali

| | | |
|---|---|---|
| 2 ccs of Fehling's required | 15·5 ccs of | (1) |
| do | 12·1 „ | (2) |
| do | 10 „ | (3) |

showing that inversion had taken place. Reduction shown by tube (1) seems to be due to the reducing compounds present in the enzyme solution itself.

A lesser quantity of (2) was required owing to the presence of reducing sugar formed by enzyme activity and a still less quantity of (3) was required because of the greater concentration of reducing sugars formed by a proportionately greater quantity of the enzyme.

The difference was also apparent in the appearance of the solutions after boiling with Fehling's solution.

*Tannase.* The enzyme extract was prepared as before described and filtered through a filter candle. A 1% solution of pure tannic acid was prepared and 4 sterile stoppered bottles were filled with 25 ccs solution.

To (1) 10 ccs of distilled water were added,
„ (2) 5 ccs of boiled enzyme extract and 5 ccs distilled water,
„ (3) 5 ccs of enzyme extract plus 5 ccs distilled water and
„ (4) 10 ccs of enzyme extract.

2% toluene was added as antiseptic. After 48 hours incubation at 37°C the tubes were examined to see if any gallic acid was produced. No evidence as to the presence of gallic acid was forthcoming by the usual methods of estimating gallic and tannic acids. Very probably in the preparation of the enzyme extract the use of hide powder may have precipitated the enzyme along with the tannins. Further examination of a water extract of leaves without the addition of hide powder is required, but tannase is an enzyme not very readily identified.

*Enzymes connected with glucosides.* Kanga summarises in his paper on the constituents of Lantana "that the aqueous liquid was found to contain a large quantity of tannins and sugar, the solution in which sugar was found was glucosidic in character. The ether extract of the resin was found to contain a crystalline substance, which is a glucoside; the formula of this substance may very probably be $C_{27}H_{42}O_4$*".

It is natural to suppose that some enzymes connected with glucosides may be present. The glucoside would break up into glucose under the enzyme influence during the process of the fermentation of the leaf after rolling. 10 gms of rolled lantana leaf (rolled for ½ an hour and allowed to ferment 4 hours) were used for extracting the enzyme as already described and the enzyme extract so obtained was used for experimental purposes.

20 ccs of a. 0·5% solution of salicin were put into 4 different flasks:—

(1) was kept as control

To (2) 5 ccs of the boiled extract were added plus 5 ccs distilled water

„ (3) 5 ccs of the living extract plus 5 ccs distilled water

„ (4) 10 ccs of living extract.

These were incubated for 24 hours at 37°C. 5 ccs out of each flask were pipetted into test tubes containing 1 cc Fehling's solution and the test tubes kept in a boiling water bath for ½ an hour.

(1) no reduction apparent, liquid totally blue in colour
(2) liquid dark green, little reduction
(3) light green, reduction well marked
(4) very little green, reduction to a greater degree than in (3)

* Kanga, unpublished communication

This shows that the glucosides in the leaf had an enzyme corresponding to them which was breaking them up in the process of fermentation as was shown by a greater reduction in (4) than in (3). (2)showed some reduction because of the property of the extract itself, whether in the living or boiled condition, to reduce Fehling's solution.

*Examination of the enzyme content after rolling and fermentation:* 5 gms of the rolled and fermented leaf were ground in a mortar, 5 gms hide powder added and 75 ccs of distilled water thoroughly incorporated with the mixture and allowed to stand 3 hours. The extract obtained was strained through cloth, it was brown in colour and then filtered through a filter candle. This extract was used for testing the presence of enzymes.

Oxidase was tested qualitatively with guaiacum tincture and 2—3 minutes elapsed before the characteristic blue reaction became apparent. It was not so intense as obtained with the living extract, thus showing roughly the comparative decrease in the quantity of the oxidising enzyme after the fermentation process is over.

*Lipase.* In the same extract lipase was looked for exactly as before in the case of fresh leaves. No acidity was produced in the cocoanut oil even after a lapse of 30 hours.

*Amylase.* Amylase was looked for under the same experimental conditions as with a fresh leaf extract and found to be present in equally large amount.

The remaining enzymes were not looked for.

## II. Collective behaviour of enzymes.

Throughout this investigation, it was a matter of frequent occurrence that the enzyme extract when allowed to stand exposed to the air turned spontaneously blueblack even though it was prepared after the precipitation of tannin with hide powder and filtered through a filter candle. With a view to study the changes taking place in the extract 20 ccs of this extract were placed in a beaker under vacuum, the extract turning darker on standing 24 hours. Another lot was kept exposed to the air in a flask plugged with antiseptic cotton and the colour of the liquid went on gradually darkening. A drop of the liquid was examined under the microscope and found to contain bacteria. To a second lot of extract contained in a test tube a drop of concentrated sulphuric acid was added so as to kill the enzyme. The colour of the extract immediately became intense green which did not change on 20 hours standing. To a third lot of extract a drop of strong KOH was added, the original colour of the extract

which was yellowish green became at once yellow. The yellow kept the same as the previous day even after 20 hours.

The extracts when allowed to stand even in solution showed the well marked characteristic reaction with the guaiacum tincture after a period of full six days, the extract simply darkened in colour although kept in a vacuum desiccator. It is thus evident that the changes are either brought about by the enzymes or bacteria both in presence and absence of air.

It was next a question whether the extract passed through a filter candle and that strained through cloth showed any difference in activity. During the process of filtration the extract colourless to start with became yellow, then yellowish green and finally gradually went on darkening to a deep blue. One cc each of the 'strained and candle filtered' extract and 'strained only' extract was taken in two separate test tubes, and ½ cc of 2% guaiacum tincture added to each. The strained extract gave immediately an intense bluish green colouration whereas the candle filtered extract only gradually developed a blue colour. The latter did not acquire the same intensity of colour and the blue did not change to the characteristic green. It would thus appear that the strained extract in passing through the candle may be leaving behind the enzyme or a substance which may be playing its role in imparting the green colouration. The strained extract gradually went on giving up its intensity and became yellowish green. The candle filtered extract however did not give up its intensity and maintained it. Within about 10 minutes the strained extract became very pale yellow at the same time the green colouring matter precipitated at the bottom. The filtered extract however continued to hold its colour and was of the same intensity.

*Effect of boiling on the extracts.* 20 ccs of the enzyme extract were boiled in a steam steriliser for 15 minutes and 5 ccs of this boiled extract were pipetted out in a test tube A and 5 ccs of the same extract unboiled into another tube B and ½ cc of 2% guaiacum tincture added from a burette to each. B showed an intense bluish green colouration at once whereas not the slightest trace of colour appeared to have developed in A. After 24 hours the boiled and unboiled extracts were again examined as above but with the same results. The flask containing filtered and unfiltered extracts kept with 2% toluene as antiseptic gave distinct colouration with guaiacum even after 48 hours but the boiled flasks containing the strained and candle filtered extracts gave negative results with guaiacum. It is thus evident that at least in this case the oxidase does not regenerate itself even after a period of 48 hours.

Precautions were taken in the above experiments to exclude any possibilities of purely bacterial action, but as certain of the solutions were found not to be entirely sterile, the results require further examination.

The foregoing results may be briefly summarised as under :—

(1) That the leaf of Lantana contains the following enzymes.

(a) A powerful oxidase whose activity is evidenced with pyrogallol in about 3 hours and ends in 39 hours. It gives an intense bluish green colouration with guaiacum tincture.

(b) A catalase soluble in salt solution probably similar to the β catalase of Dr. Loew.

(c) An amylase which persists even after the fermentation process is over.

(d) An invertase.

(e) A lipase which is not ordinarily met with in the leaves of plants.

(f) Perhaps a tannase whose absence is not conclusively proved.

(g) An enzyme breaking up the glucosides into glucose which is perhaps developed after the cell walls are ruptured during rolling and may not be present in the fresh leaf to start with.

(2) That owing probably to the collective action of several at any rate of the enzymes present, striking colour changes take place in a decoction of lantana leaves in presence of air. These changes vary according as the solution has been filtered through a filter candle or merely strained through filter cloth.

## III. The preparation of a tea substitute.

Mann gives the following interesting short account of how tea is made in actual practice*. " Having obtained the leaf it is allowed to wither—to lose its turgescence—by exposure in very thin layers to air as cool as possible until the whole has got such a condition that on rubbing in the hand the leaves no longer break, but are sufficiently pliable to roll up. At this stage it is rolled, a process whose effect is to break up the cells of the leaf, allow the sap to spread itself over the surface, and so come

* Enzymes of the tea leaf Part I. Indian Tea Association.

in contact with air during the process of fermentation. This latter merely consists in exposing, for a time varying from 2 to 6 hours, the rolled leaf in thin layers in as cool and airy a room as possible; marked changes here take place; the green leaf takes on a brown coppery colour and acquires an aroma totally different from that of the fresh leaf. When sufficiently fermented the whole mass of tea is dried usually by a powerful current of hot air, sorted and put on the market. It is evident that the changes imparted from our point of view principally takes place during withering and fermentation".

The following experiments were made to prepare a tea substitute from Lantana leaves:—

Withering operation was done in shade at room temperature on tiles.

Rolling on a four legged iron plate with an iron roller weighing some 12 lbs.

Firing in a hot air or steam oven at temperatures noted against the experiments.

Comparison of the colour of infusion—2 gms of Lipton's yellow label tea were allowed to infuse with 100 ccs of boiling water for five miuntes and strained through muslin. An infusion from tea prepared from the Lantana leaf was similarly made and both transferred into separate Nessler glasses and their colours compared.

The results are summarised in the following table :—

Table showing experiments done with a view to prepare tea from Lantana.

| Time withered. | Time rolled. | Firing. | Colour of tea. | Infusion. |
|---|---|---|---|---|
| 1. 24 hours at 77°F. | ¾ hour | 3 hours in steam oven at 95°C. | Greenish, approaching black. | Coloured very dark brown with green tinge. |
| 2. 4 hours | ½ „ | ½ hr. at 98°C in air bath. | Greenish. | Slightly greenish dark brown in colour, deeper than Lipton's yellow label. |
| 3. 5 hours at 74°F. | Rolled by hand at 77°F. | Kept 19 hrs. before firing ½ hour. | Colour like that of tea leaves. | Approaching to tea, slight smell of Lantana oil. |
| 4. 24 hours at 69-73°F. | Hand rolled. | 2 hours. | Black tinged with green. | Greenish brown. |
| 5. 6 hours at 79-81°F. | 20 minutes. | Not fired. | Light black. | Light green without green tinge. |
| 6 3 hours at 75-77°F. | ½ hour with increasing pressure. | ... | Light black. green tinge absent. | Brown with slight green tinge. |
| 7. 4 hours at 75-77°F. | do | Allowed to dry at room temperature. | Black. | Thin body free from green tinge |
| 8 Not withered. | do | ½ hour in air bath at 80°C. (28 hours after rolling). | Black slightly greenish. | Thin body, brown but greenish |
| 9. 5 hours at 75-77°F. | ¾ hour. | ½ hour at 75°C. (after allowing to ferment 18 hours). | Black with faint yellow tinge. | Very much like Lipton's yellow label. |

These experiments are not by any means exhaustive but the investigation would lead to the conclusion that although from the leaves of the Lantana a product cannot be obtained equal to ordinary tea, yet a tea substitute could in fact be made at an exceedingly cheap price. As regards the question of flavour, of first importance from the tea consumer's point of view, it appears that the major portion of the unpleasant essential oil escapes during the process of manufacture especially in firing, leaving in the final product only a slight smell which is not at all unpleasant.

The author is unable at present to devote further time to the research and it is probable that the ideal conditions for making black tea from Lantana have not been hit upon, but further study of the subject may easily reveal a method of profitably utilising a plant now generally considered to be an agricultural nuisance.

The work described in the foregoing pages was carried out in the Department of Applied Chemistry. The thanks of the author are due to Dr. Fowler for his interest in the work and assistance in the preparation of this paper.

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I. I. S: No. 54—R. P. B.—4-1919.—500.