BIOCHEMISTRY OF TAN-LIQUOR FERMENTATION.

By P. D. Dalvi.

That vegetable tan-liquors undergo fermentation resulting in the destruction of their tanning properties, has been known from almost prehistoric times: but until recent years no systematic attempt was made to study the nature of the fermentation and seek means to stop it.

Van Tieghem (*Compt. rend.*, 1867, **65**, 1091) first observed that tan-liquors contain filamentous fungi, chief among which are *Aspergillus niger* and *Penicillium glaucum*, which hydrolyse tannic acid to gallic acid and glucose which have no tanning properties. Fernbach (*Compt. rend.*, 1901, **131**, 1214) observed that *Aspergillus niger* growing in Raulin's medium containing tannic acid in place of sugar develops an extra-cellular ferment, tannase, which brings about the hydrolysis of tannic acid in the same way as the living fungus. Knudson (*J. Biol. Chem.*, 1913, **14**, 159, 185) made a systematic study of the action of *Aspergillus niger* and *Penicillium glaucum* in tan-liquors and biological media, observing that the fungi (*a*) normally preferred sucrose to gallic acid and (*b*) did not develop tannase except in presence of tannic or gallic acid.

Rhind and Smith (*Biochem. J.*, 1922, **16**, 1) and later, Nicholson and Rhind (*Analyst*, 1924, **49**, 505) studied the application of tannase to the estimation of tannins. Nierenstein (*Biochem. J.*, 1922, **16**, 514) observed that the tannase developed by *Aspergillus luchuensis* hydrolysed tannic acid, but was without any action on other tannins. Freudenberg (*Z. physiol. Chem.*, 1926, **164**, 262) standardised conditions for the preparation of tannase and studied the action of this enzyme on methyl gallate. No systematic work has, however, been so far carried out on the action of tannase on tannic acid which is the chief constituent of tan-liquors. All previous attempts to check the growth of fungi without impairing the quality of tan-liquor proved unsuccessful.

The objects of the present investigation were to (*a*) isolate and identify the commoner microflora of indigenous tan-liquors, (*b*) prepare the enzyme tannase in an active form and study the factors influencing its action on tannic acid, and (*c*) study the efficacy of different preservatives in preventing the loss of tannic acid by fermentation.

Microflora of Tan-Liquors.

The organisms present in tan-liquors are probably those introduced by dust and by the hides and skins soaked in them. Very little is however known regarding their precise nature and mode of distribution beyond the observations of Asai (*J. Coll. Sci., Imp. Univ. Tokio*, 1918, **39**, 1) who noted the presence of growing yeasts and fungi. It was therefore considered desirable to determine the flora characteristic of tan-liquors from different sources.

Tan-liquors.—Specimens prepared from (1) Avaram bark (*Cassia auriculata*), (2) Divi-divi (*Cæsalpinia coriaria*) and (3) Myrobalans (*Terminalia chebula*) were obtained directly from the vegetable tan-vats in which hides and skins were soaking for several days. The liquors were yellowish brown and, except in a few rare cases, had superficial growths of fungi; they contained also fragments of hides and skins and large amounts of suspended matter which settled on standing and occupied about 1/16th of the total volume. Their barkometer strength (Sp. Gr.) varied between 1·02 and 1·06. The hydrogen-ion concentration of the liquors could not be determined by colorimetric methods as they were strongly coloured; it was therefore determined electrometrically and found to vary between P_H 3·0 and P_H 4·0.

The liquors were occasionally found to be ropy owing to profuse growth of certain types of bacteria. Such specimens were about 10 per cent. more viscous than water and generally free from surface growths of fungi; their tannic acid contents were invariably low.

Isolation of different organisms.—This was effected by three methods, (1) *dilution*, (2) *direct* and (3) *direct microscopic*. 1. Platings were made with the liquors diluted to 1 in 100 to 1 in 1,000 for bacteria, and 1 in 1,000 to 1 in 10,000 for fungi and yeasts. Incubations were at 25° for fungi and yeasts and at 37° for bacteria, the time being 3 days in all the cases. The platings were repeated to isolate the different organisms.

2. The method of Waksman (*Soil Sci.*, 1917, **3**, 565) was adopted chiefly to isolate and identify the fungi occurring vegetatively. Incubations were for 24 hours at 25°. 3. Following Conn (*N. Y. Agric. Exp. Sta. Tech. Bull.*, **64**, 1917 ref.) the liquors, after suitable dilution, were examined directly under the microscope. This method, though suitable for bacteria and yeasts, was not easily adaptable to fungal hyphæ which were abundant in tan-liquors.

Media used for isolation.—Czapeck's synthetic medium and wort-agar were found to be most suitable for the isolation of fungi and yeasts and nutrient agar for bacteria. The numbers of fungi varied from 180,000 to 400,000 per c.c. of liquor, invariably increasing with the age of the liquor. Counts of yeasts varied between 300,000 and 500,000 per c.c. and were generally greater in specimens collected on hot days (25–30°) than in cooler weather (20–25°). The bacterial numbers at no time were so large as those of fungi and yeasts, varying from 500 to 2,000 per c.c. The counts tended to increase with the progress of the fermentation.

IDENTIFICATION.

Seventeen different strains of fungi, three of yeasts and ten of bacteria were isolated, and most of them identified. The few that remained unidentified were found to be without any action on tannic acid and hence of no interest to the present investigation.

Fungi.—(1) *Aspergillus niger*, (2) *A. flavus*, (3) *A. spinulosum*, (4) *A. oryzæ*, (5) *A. candidus*, (6) *A. nidulans*, (7) *Penicillium glaucum*, (8) *P. breviçaule*, (9) *P. oxalicum*, (10) *P. camemberti*, (11) *P. claviforme*, (12) *P. granulatum*, (13) *P. X* (unidentified), (14) *Mucor racemosus*, (15) *M. mucedo*, (16) *M. rouxii*, (17) *Rhizopus nigricans*.

Bacteria.—Of the ten isolated, three were identified with (1) *B. acidi lactici*, (2) *B. aceti* and (3) *B. coli*. The cultural characteristics of the remaining seven organisms (Nos. 4–10) were studied.

Yeasts.—One was identified with *Saccharomyces apiculatus*. The physiological activities of the other two (Nos. 2 and 3) were studied in some detail. None of the yeasts was, however, found to have any action on tannic acid though Biddle and Kelley (*J. Amer. Chem. Soc.*, 1912, **34**, 914) observed that a certain yeast brought about a change in the optical rotation of tannic acid without hydrolysing it to gallic acid.

Cultural characteristics of organisms.—In order to study the response of the more prominent fungi to tannic acid, they were inoculated into media containing varying percentages, and the results tabulated below:—

Growths, /-/-/-/- = excellent; /-/-/- = very good; /-/- = good; /- = slight; — = no growth. Of the four that withstood tannic acid, at all concentrations, the most satisfactory growth was made by *Aspergillus niger*.

TABLE I.

Organism	Percentages of tannic acid			
	0·25	2	5	10
Aspergillus niger ...	/-/-/-/-	/-/-/-	/-/-	/-
,, *flavus* ...	/-/-/-/-	/-/-/-	/-	/-
Penicillium glaucum ...	/-/-/-/-	/-/-/-	/-	/-
,, *X* ...	/-/-/-/-	/-/-/-	/-	/-
,, *camemberti* ...	/-/-/-	/-/-	/-	—
,, *granulatum* ...	/-/-/-	/-	/-	—
,, *claviforme* ...	/-/-/-	/-	/-	—
Mucor rouxii ...	/-/-	/-	—	—

Some of the unidentified bacteria (Nos. 4–8) were compared for their ability to produce acid and gas in nutrient broth containing sugars, mannitol and dulcitol. The observations (Table II) were made after incubation for 48 hours at 37°.

TABLE II.

Number	Sucrose	Galactose	Mannitol	Dulcitol	Lactose	Dextrose	Laevulose
4	/-/-	—	/-/-	—	/-	/-/-	/-/-
5	/-/-	—	/-/-	—	/-	/-/-	/-/-
6	/-/-	/-	/-/-	—	/-	/-/-	/-/-
7	/-/-	/-/-	—	—	/ /-	/-/-	/
8	G	G	G	—	G	G	G

/- = slightly acid ; /-/- = acid ; G = acid and gas ;— = no acid and no gas.

Other morphological and physiological characteristics of the different organisms have been presented in Tables III and IV.

TABLE III.

Bacterium number	Ammonia production	Liquefaction of gelatin at 20°		Indol formation	Potato culture growth	Size in microns	
		24 hrs.	96 hrs.			Bacterium	Spore
4	/-	...	...	...	Good	1·0–1·1 by 4·5–4·7	1·0 to 1·1
5	/-	...	...	...	,,	0·6–0·7 by 2·0–2·1	0·6 to 0·7
6	/-	...	...	...	,,	0·6–0·7 by 1·6–1·8	0·6 to 0·8
7	—	/-	/-	—	good-bluish tint	1·0–1·2 by 2·0–2·2	0·4 to 0·5
8	/-	—	—	/-	Very good	0·4–0·5 (cocci).	...
9	—	—	—	—	,,	...	0·5 to 0·6
10	/-	—	—	/-	,,	...	Non-sporing

/— = reaction positive; — = reaction negative.

TABLE IV.

Action on Litmus Milk.

Reaction after	Bacterium number						
	4	5	6	7	8	9	10
24 Hours ...	—	—	—	—	/-	—	/-
48 Hours ...	—	—	—	—	/-/-	—	/-/-
...	...	slightly yellow	...	decoloration of litmus	...	...	...
72 Hours ...	—	—	—	—	/-/-	—	/-/-
...	slightly coagulated	slightly coagulated	slightly yellow	...	...	...	...

— = no change; /— = acid; /—/— = acid and gas.

The unidentified yeasts (2 and 3) were inoculated into media containing peptone (1 per cent.) and sodium chloride (0·5 per cent.)

together with one of the sugars or hexitols (2 per cent.) indicated in Table V.

TABLE V.

Number	Sucrose	Mannitol	Dulcitol	Galactose	Lactose	Dextrose	Lævulose
2	—	—	—	—	—	/-	/-
3	—	—	—	—	—	/-	/-

/- = fermentation ; — = no fermentation.

It may be seen from the foregoing tables that *Aspergillus niger*, *Penicillium glaucum* and *Penicillium X* developed well even in presence of 10 per cent. tannic acid. *Mucor Rouxii* grew fairly well in a solution containing 2·5 per cent. of tannic acid, but could not withstand higher concentrations. The remaining fungi grew only in solutions containing less than 2·5 per cent. of tannic acid. The yeasts isolated from tan-liquors fermented dextrose and lævulose, but did not ferment other sugars tried. Among the bacteria, some were nitrate reducers; one liquefied gelatin and four produced acid and gas from nutrient media containing sugars.

Preparation of Tannase.

Source of the enzyme.—It was noticed previously that *Aspergillus niger* flourished most vigorously even in presence of high concentrations of tannic acid. In view of the above and the observations of several previous workers that *Aspergillus niger* was the most common and the most active of the organisms found in tan-liquors, the enzyme tannase was prepared from that fungus for further investigation.

Choice of a medium for growing the fungus.—Several synthetic nutrient media including those of Czapeck and Raulin in which sugar had been replaced by tannic acid were tried. It was observed that although the fungus grew fairly well on most of them, the yields of the enzyme from such sources were not encouraging. Freudenberg's medium, in a slightly modified form, was then tried and found to be highly satisfactory.

Myrobalan powder (600 g.) was stirred with 4 litres of distilled water in a pail, the suspension boiled for 15 minutes, cooled and poured with the solid residue into a Winchester bottle. The supernatant

liquid containing most of the soluble matter was syphoned and the sediment repeatedly extracted with boiling water until almost all the tanning material was removed. The extracts were combined, made up to 12 litres and the following added:— Ammonium sulphate, 300 g.; di-potassium phosphate, 9 g.; magnesium sulphate, 3 g.; and sodium chloride, 1 g. The reaction was adjusted to P_H 3.2–3.4 and the mixture sterilised.

Growth of the fungus.— The medium was placed in several 750 c. c. Erlenmeyer or flat-bottomed Pasteur flasks to the depth of about 3 cm. The flasks were plugged and steam-sterilised, the medium then being temporarily somewhat turbid owing to precipitation of magnesium phosphate. *Aspergillus niger* began growing in the medium, having been inoculated in the form of spores obtained from a previous growth on the same medium and incubated at 32–34°.

White specks of the fungus mycelium began forming on the surface of the medium. On the third day, when the growth was nearly complete, the fungus 'felt' was removed by means of a nickel wire. The growth was not allowed to proceed any further since it was observed that the fungus then began sporulating, with consequent loss of enzyme.

Preparation of enzyme powder.—The mass of mycelia was immersed for a few minutes in running water to remove adhering culture solution, squeezed free of water, pressed between thick folds of filter paper and then ground up with four times its weight of acetone. After 24 hours the extract was drained, the residue washed with a fresh quantity of acetone, and then extracted with ether, after which it was spread out in a thin layer for about two hours until the odour of solvent was no longer perceptible; the coarse powder remaining was dried over phosphorus pentoxide in a desiccator.

The preparation contained the enzyme tannase together with such constituents of the cells as were not soluble in acetone or ether; it contained 5·2 per cent. of nitrogen. The aqueous extract (*a*) was faintly opalescent and gave a feeble biuret reaction, and (*b*) did not give either Millon's or Hopkins and Cole reaction.

Extraction and concentration of the enzyme.—The dry mould powder (10 g.) prepared as above was agitated with a litre of distilled water and 10 c. c. of pure toluene in a glass-stoppered bottle maintained at 22° in an ice incubator for 24 hours, and shaken from time to time. The extract was then filtered through washed kieselguhr and concentrated by distillation under reduced pressure at a temperature not exceeding 40°.

It was not possible to concentrate the enzyme by freezing out the water since a large part of the enzyme was retained in the ice.

Purification.—The enzyme was precipitated from a concentrated aqueous solution with absolute alcohol free from gaseous impurities like acetylene. It was then redissolved in water, dialysed for 48 hours in collodion membranes prepared according to Farmer (*J. Biol. Chem.*, 1917, **32**, 447), and passed through a Berkefeld filter to free it from suspended impurities.

The purified enzyme did not give any of the commoner protein reactions. It was associated with a small amount of diastase, but no attempt was made to remove the latter by repeated precipitation with alcohol since it was observed that (*a*) such treatment tended to reduce the activity of tannase, and (*b*) the diastase did not interfere with the action of tannase on tannic acid.

Preservation.—The mould powder and the precipitated dry enzyme retained their activity almost indefinitely. The solution was not stable at high temperatures but maintained its activity for quite long periods at 0° in presence of toluene.

Factors Influencing the Action of Tannase.

To determine whether the enzyme was similar in behaviour to those obtained by Freudenberg and other workers, some preliminary trials were carried out.

Hydrolysis of methyl gallate.— The ester was prepared by the method of Homburg (*Monatsh.*, 1898, **19**, 594). A solution of the ester (0·271 g.) in 45 c. c. of conductivity water was treated with 5 c.c. of an aqueous extract of the enzyme and 1 c. c. of toluene, and the mixture placed in a thermostat at 33°. Aliquot parts of the reaction mixture were removed at intervals and titrated electrometrically against N/200 sodium hydroxide until P_H 7·16 — the reaction of a mixture of equivalent amounts of sodium hydroxide and gallic acid — was attained. From the quantities of alkali required at different stages, the corresponding amounts of gallic acid present in the medium were calculated. The results have been presented in Table VI.

The observations show that (*a*) the reaction became more and more acid as the concentration of gallic acid increased in the medium and (*b*) the hydrolysis did not follow the course of a monomolecular reaction. Similar results were obtained by Freudenberg and Vollbrecht (*Z. Physiol. Chem.*, 1922, **116**, 277).

TABLE VI.

Time in hours	P_H	Percentage hydrolysis	Free gallic acid, mg.
0	5·05	...	...
4	4·52	1·84	4·60
8	4·30	2·70	6·80
16	4·10	4·12	10·30
32	3·82	6·40	16·00
48	3·70	7·80	19·50
72	3·55	10·70	26·50

Influence of salts on the hydrolysis of methyl gallate.—The reaction mixture was composed of 40 c.c. of an aqueous solution containing 0·271 g. of the ester, 5 c.c. of an aqueous extract of the enzyme, 0·25 c.c. of a 0·1 *M* solution of a salt, 4·75 c.c. of conductivity water to make up the volume to 50 c.c. and 1 c.c. of toluene. The temperature of hydrolysis and the method of titration were the same as those described in the previous experiment. The results have been given in Table VII.

TABLE VII.

Time in hours	Sodium chloride		Potassium acid phosphate (KH_2PO_4)		Di-potassium phosphate (K_2HPO_4)	
	P_H	Hydrolysis per cent.	P_H	Hydrolysis per cent.	P_H	Hydrolysis per cent.
0	5·05	...	5·08	...	5·92	...
4	4·55	1·5	4·52	1·9	5·76	1·4
8	4·30	2·6	4·35	3·1	5·40	3·1
16	4·15	4·0	4·12	4·6	4·84	5·9
32	3·85	6·3	3·85	6·5	4·25	9·3
48	3·72	7·6	3·72	8·1	4·05	11·6
72	3·60	10·2	3·55	10·9	3·96	14·1

The hydrolysis was hastened by adding di-potassium phosphate (K_2HPO_4), while the other salts were without any effect. Variation of

the concentration of sodium chloride was also without any appreciable effect.

The chemical mechanism of the hydrolysis of tannic acid into gallic acid is still not properly understood. In view of the above and the possibility of at least a part of the tannic acid undergoing change to products other than gallic acid, the action of tannase was followed, in the subsequent series of trials, by studying both decrease in tannic acid and corresponding increase in gallic acid.

Effect of substrate concentration.—Aqueous solutions of tannic acid of different concentrations (5·0, 2·0, 1·0 and 0·5 per cent.) were prepared. To 25 c. c. of each, an equal volume of enzyme solution was added and the mixtures maintained at 50° in a thermostat. Aliquot parts were examined at intervals for their tannic and gallic acid contents by the method of Gardner and Hodgson (*J.C.S.*, 1909, **95**, 1819). The results have been presented in Table VIII.

TABLE VIII.

Time in hours	Concentration of tannic acid in the reaction mixture							
	2·5 per cent.		1·0 per cent.		0·5 per cent.		0·25 per cent.	
	A	B	A	B	A	B	A	B
6	9·5	8·0	23·8	18·0	28·5	25·5	23·3	19·5
12	12·5	10·5	39·0	32·8	45·8	39·9	41·3	36·8
24	27·5	23·0	66·0	56·5	69·3	60·8	60·9	59·5
48	50·0	42·5	103·5	93·0	101·0	86·3	72·8	64·8
72	52·0	43·5	129·0	115·3	125·0	110·3	87·8	77·9
96	53·0	43·5	133·5	120·0	128·4	114·0	88·0	78·0

A = Tannic acid hydrolysed in mg. B = Gallic acid formed in mg.

The observations show that the activity of the enzyme increased with decrease in substrate concentration.

Influence of temperature on hydrolysis.—The reaction mixture which was composed of 25 c.c. of a 1·0 per cent. solution of tannic acid and an equal volume of enzyme solution was maintained at different temperatures and the progress of the hydrolysis followed (Table IX).

TABLE IX.

Time in hours	Temperature					
	30°		50°		60°	
	A	B	A	B	A	B
6	10·6	8·3	28·5	25·5	25·8	22·5
12	18·2	15·8	45·8	39·9	70·8	47·8
24	28·5	26·8	69·3	60·8	115·6	102·1
48	42·1	39·3	106·0	86·3	138·8	123·0
72	60·4	54·1	124·8	110·3	152·0	135·5
96	73·2	64·6	128·4	114·0	155·3	140·4

A = Tannic acid hydrolysed in mg. B = Gallic acid formed in mg.

The foregoing observations were checked by a further series of experiments in which tannic acid was estimated by the method of Lowenthal (*J. pr. Chem.*, 1863, **3**, 150). The results are given in Table X.

TABLE X.

Temperature	Percentage of tannic acid hydrolysed after	
	20 hours	48 hours
21·5°	8·4	12·6
25·5	12·0	13·5
50·0	13·5	15·4
59·0	15·1	19·8
65·0	10·9	14·8

It may be inferred from the above tables that the optimum temperature for the hydrolysis is about 60°.

Concentration of enzyme and rate of hydrolysis.—To three flasks, each containing 0·25 g. of tannic acid dissolved in 45 c.c. of water, 2·0,

2·5 and 4·0 c.c. of the enzyme solution were added, the volume increased with water to 50 c.c., maintained at 50° and the quantities of tannic and gallic acids present at different stages determined (Table XI).

TABLE XI.

Time in hours	Volume of enzyme solution					
	2 c.c.		2·5 c.c.		4 c.c.	
	A	B	A	B	A	B
6	16·4	12·9	19·5	16·6	25·5	21·0
12	28·5	25·2	34·8	28·8	47·8	40·8
24	52·8	44·3	66·2	55·7	97·5	82·8
48	92·7	81.0	105·8	96·8	126·3	120·0
72	118·3	104 3				

A = Tannic acid hydrolysed in mg. B = Gallic acid formed in mg.

The rate of hydrolysis increases with the concentration of the enzyme, but is not directly proportional to it.

Effect of concentrating the enzyme.—This experiment was carried out to determine whether concentration of the enzyme, by distilling an aqueous extract under reduced pressure, tended to reduce its activity. One litre of enzyme solution was concentrated to 50 c. c. by distillation at 40°. To 25 c.c. lots of a solution (1 per cent.) of tannic acid, 25 c.c. of the dilute and 1·25 c.c. of the concentrated extracts respectively were added. After making up the volume, in the latter case to 50 c.c. by addition of water, the two sets of mixtures were maintained at 50° and the progress of hydrolysis studied (Table XII).

It may be inferred that the activity of the enzyme is not affected during the process of concentration.

Dialysis of the enzyme.—To determine whether the enzyme can be purified by dialysis, an aqueous extract was dialysed for 72 hours in collodion membranes against distilled water out of direct contact with air. The dialysate was collected and concentrated to about 20 c.c. by distilling under reduced pressure. The activities of the dialysed enzyme, and the concentrated dialysate as compared with that

TABLE XII.

Time in hours	Percentage of hydrolysis	
	Condition of enzyme	
	Dilute	Concentrated
8	2·5	3·2
16	5·5	5·7
24	8·1	9·3
48	16·2	17·3
72	23·7	25·7
96	32·1	34 0

of an equivalent amount of a fresh aqueous extract of the enzyme were determined in the manner described in the previous experiment (Table XIII).

TABLE XIII.

Time in hours	Percentage of hydrolysis		
	Condition of enzyme		
	Not dialysed	Dialysed	Concentrated dialysate
15	6·4	4·5	2·9
41	20·4	9·6	10·6

The observations show that the enzyme passed partially through the collodion membrane; it may therefore be inferred that it is not possible to purify the enzyme by continued dialysis.

Influence of salts.—The reaction mixtures were made up as follows:—45 c.c. of a 0·56 per cent. solution of tannic acid, 2 c.c. of enzyme solution, 1 c.c. of conductivity water and 2 c.c. of a 0·1 *M* solution of the salt; those tried were sodium chloride, diacid potassium phosphate (KH_2PO_4) and di-potassium phosphate (K_2HPO_4). The

reaction mixtures were maintained at 50°; progress of the hydrolysis was as follows.

TABLE XIV.

Time in hours	No salt (control)		Di-potassium phosphate (K_2HPO_4)	
	A	B	A	B
6	15·1	12·3	19 5	16·1
12	25·5	23·3	30 8	27·0
24	42·3	38·3	48·5	43·6
48	65·4	57·8	85·6	66·6
72	80·7	72·0		
96	83·3	73·8		

A = Tannic acid hydrolysed in mg. B = Gallic acid formed in mg.

The results obtained for mixtures treated with sodium chloride and di-acid potassium phosphate have not been recorded as they were identical with those for the control.

The reaction was, however, accelerated by addition of di-potassium phosphate. The effect of the latter could not have been due to the presence of any active ion since the same ions were present in the solution of the di-acid phosphate, which was without any influence on the course of the reaction: it was probably due to a change in the H-ion concentration (*Vide* Table XVII).

The foregoing observations do not lend support to the theory of Euler and Josephson (*Z. physiol. Chem.*, 1927, **166**, 294) who state that addition of salts either hastens or retards the action of enzymes. A similar observation has also been made by Karmarkar and Patwardhan (*J. Indian Inst. Sci.*, 1930, **13A**, 163) who find that salts are without any action on the activity of vegetable amylases.

Influence of gallic acid concentration.—This experiment was conducted to determine whether the accumulation of gallic acid affected the progress of hydrolysis. To mixtures containing 40 c.c. of 1·25 per cent. tannic acid and 10 c.c. of enzyme solution, 0·05 and 0·1 g. respectively of gallic acid was added and the progress of the hydrolysis followed in the manner described already (Table XV).

TABLE XV.

Time in hours	Quantities of gallic acid added					
	None (control)		0·05 g.		0·1 g.	
	A	B	A	B	A	B
6			16·3	12·2	27·0	22·5
12	45·6	36·0	33·2	27·0	48·2	39·0
24	90·0	72·0	64·5	56·5	73·5	58·5
48	141·8	120·9	120·0	105·0	108·9	90·0
72	178·0	154·5	134·5	120·5	130·5	114·5
96	211·5	183·9	136·0	121·0	133·0	117·6

A = Tannic acid hydrolysed in mg. B = Gallic acid formed in mg.

The results show that gallic acid retards the hydrolysis of tannic acid, particularly in the later stages.

In some later trials it was observed that 0·25 and 0·5 g. of gallic acid proved so inhibitive that hydrolysis proceeded no further than 7·4 and 2·2 per cent., respectively, at the end of 72 hours.

Effect of light on the action of tannase.—The reaction mixture in each flask consisted of 25 c.c. of 1 per cent. tannic acid and 25 c.c. of enzyme solution. One set of flasks was exposed to diffused light and the other placed in darkness. The rates of hydrolysis in the two sets of cases were followed (Table XVI).

TABLE XVI.

Time in hours	Percentage of hydrolysis	
	In light	In darkness
4	18·0	18·20
8	32·5	23·80
16	47·2	47·50
28	65·5	65·40

The observations show that conditions of light or shade have no effect on the activity of the enzyme.

Influence of H-ion concentration.—Most of the commoner buffer mixtures were found to be unsuitable for the present series of trials because they either turned the media turbid or interfered with the estimation of tannic and gallic acids. McIlvaine's phosphate and citric acid buffer was, however, found to be free from these defects up to P_H 5·15 and was used in the following series of trials. Preliminary experiments having shown that the buffer mixture had no direct action on either tannic acid or the enzyme, reaction mixtures containing, in each case, 10 c.c. of 2·75 per cent. tannic acid, 40 c.c. of buffer mixture of a particular reaction, 2·5 c.c. of enzyme solution and 2·5 c.c. of water were placed in a thermostat at 50° and the progress of hydrolysis followed in the usual way (Table XVII).

TABLE XVII.

Time in hours	Reactions											
	Control, unbuffered P_H 3·58		P_H 2·3		P_H 2·9		P_H 3·64		P_H 4·25		P_H 5·5	
	A	B	A	B	A	B	A	B	A	B	A	B
6	9·5	8·2	4·7	4·0	10·0	8·7	10·0	8·6	12.5	10·8	22·0	19·5
12	18·5	16·4	10·0	9·1	18·0	15·8	18·0	15·8	24·5	23·1	46·0	43·3
24	36·5	32·4	30·4	27·2	41·4	36·6	37·4	33·4	65·0	58·3	80·0	70·9
48	68·5	60·7	30·4	27·3	52·5	46·3	64·5	57·8	109·0	96·7	142·0	131·8
72	100·0	88·6	...	...	53·0	46·4	92·4	87·1	124·0	109·9	216·0	192·0
96	130·5	115·6	...	...	...	...	117 8	100·2	182·0	161·4	250·5	221·5
120	160·5	142·3	...	...	...	...	142·0	122·6	217.5	194·5	...	...

A = Tannic acid hydrolysed in mg. B = Gallic acid formed in mg.

It may be inferred from the above that a highly acid reaction inhibits the action of the enzyme. A nearly neutral reaction, on the other hand, favours hydrolysis to such an extent that at the end of 96 hours, nearly all the tannic acid is destroyed.

PRESERVATION OF TAN-LIQUORS.

Under tropical conditions, vegetable tan-liquors lose considerable amounts of tannic acid by fermentation, and consequently deteriorate. In some tanneries the loss may amount to as much as 30 per cent. of the total tannic acid in the liquor. So there is great need to devise means to check or arrest the progress of the fermentation.

Most of the commoner fungicides such as salts of heavy metals, phenols and phenolic acids are unsuitable for the preservation of tan-liquors because some of them precipitate tannic acid, many by staining affect the quality of the leather produced, and all prove deadly to the bacteria and the yeasts which are useful to the tanning process. Only substances like the volatile antiseptics which will keep down the growth of fungi without becoming completely miscible with the tan-liquor can be safely tried as preservatives.

In the present series of trials, the efficacy of minute quantities of kerosene, thymol, camphor, carbon disulphide and formaldehyde in checking the development of tannase by *A. niger*, *A. luchuensis* and *P. glaucum* in solutions of tannic acid were studied. A number of flasks, each containing 25 c.c. of tannic acid of a particular concentration were sterilised in the autoclave. After cooling, each flask was first treated with a minute quantity of one of the antiseptics and then sown with the spores of one of the fungi. The flasks were incubated at 30° and their contents examined, at two stages, for growth of fungi, tannic acid lost and gallic acid formed.

TABLE XVIII.

Organism	Preservative	Final growth	Time in days			
			6		16	
			A	B	A	B
At the start, tannic acid = 447 mg. ; gallic acid = 52 mg.						
P. glaucum	None	/-/-	Not determined	Not determined	224	Not determined
,, ,,	Camphor, 0·1 g.	/-	,,	,,	7	nil
At the start, tannic acid = 466 mg. ; gallic acid = 67 mg.						

A. niger	None	/-/-	Not determined	Not determined	319	263
,, ,,	Camphor, 0·1 g.	/-	,,	,,	44	Not determined
,, ,,	,, 0·2 g.	/-	nil	nil	nil	nil
,, ,,	Carbon disulphide 0·1 c.c.	/-/-	Not determined	Not determined	291	278
,, ,,	Formalin, 0·05 c.c.	/-/-	274	173	312	254
,, ,,	,, 0·1 c.c.	/-/-	248	163	285	209
,, ,,	,, 0·15 c.c.	/-/-	131	60	259	211
A. luchuensis	None	/-/-	Not determined	Not determined	115	81
,, ,,	Carbon disulphide 0·1 c.c.	/-/-	,,	,,	126	93

At the start, tannic acid = 606 mg. ; gallic acid = 5 mg.

A. niger	Camphor, 0·05 g.	/-	340	283	447	391
,, ,,	,, 0·1 g.	/-	nil	nil	53	nil
,, ,,	,, 0·2 g.	nil	nil	nil	21	14
,, ,,	,, 0·4 g.	nil	nil	nil	16	15
,, ,,	Thymol, 0·05 g.	/-	nil	nil	54	42
,, ,,	,, 0·1 g.	/-	21	8	46	33
,, ,,	,, 0 15 g.	nil	16	8	32	23
,, ,,	,, 0·2 g.	nil	11	5	26	19
,, ,,	Kerosene, 0·05 c.c.	/-/-	254	217	452	396
,, ,,	,, 0·1 ,,	/-/-	144	132	446	400
,, ,,	,, 0·2 ,,	/-/-	69	46	441	391
,, ,,	,, 0·4 ,,	/-/-	16	Not determined	414	364

A = Tannic acid lost in mg. ; B = Gallic acid formed in mg.
/- = slight growth , /-/- = Good growth.

The foregoing observations show that carbon disulphide, formalin and kerosene were not effective in checking either the growth of fungi or the loss of tannic acid. Thymol and camphor proved highly efficient even in minute quantities.

In addition to the above, the study of the factors influencing the action of tannase has already shown that hydrolysis of tannic acid is

checked by either adding gallic acid, which is a product of the enzyme action, or adjusting the reaction to about P_H 2.0. It may not, perhaps, be practicable to collect and concentrate gallic acid though considerable amounts of it pass into tannery waste: but it should be possible to develop an easy method of adjusting the reaction of the liquors so that the action of tannase can always be held in check.

Investigations carried out by workers in this laboratory and elsewhere have shown that sulphur, even in minute quantities, is highly efficacious in checking the growth of fungi and maintaining, through the agency of the sulphur bacteria, a reaction corresponding to about P_H 2.0. The sulphur-oxidising organisms are present in the soil and are doubtless carried into tan-liquors by dust or soil adhering to tan barks. It therefore appears possible that addition of minute quantities of sulphur to the tan-liquors will help not only to check the growth of fungi but also lead to spontaneous development of the reaction required to retard the action of tannase.

Further work should be carried out under conditions prevailing in the tanneries, and with liquors from various sources before any one particular method of preservation can be considered to be efficient. Careful study of the direct and the indirect effects of the proposed treatment on the quality of leather produced should also be made before recommending its use by tanners.

SUMMARY.

The more important micro-organisms present in vegetable tan-liquors were isolated and identified. Fungi were found in great abundance both vegetatively and in the form of spores. Several of the organisms were tested for their action on tannic acid and it was observed that *Aspergillus niger*, *Penicillium glaucum*, and an unidentified fungus, *P. X* grew even on 10 per cent. tannic acid.

A preparation of tannase was obtained from the mycelia of *A. niger* growing on myrobalan decoction and its properties studied.

The optimum temperature for the hydrolysis of tannic acid by tannase was about 60°. A reaction corresponding to P_H 2.3 retarded the action of the enzyme, while one which was less acid (P_H 5.15) was highly favourable. The rate of hydrolysis (*a*) increased with the concentration of the enzyme, but decreased with that of the substrate, (*b*) was retarded by increasing amounts of gallic acid, (*c*) was not influenced by either neutral or acid salts and (*d*) was not affected by conditions of light and shade.

A preliminary study showed that minute quantities of thymol or camphor helped to check the growth of fungi on tannic acid.

A few of the possible methods of preventing the fermentation of tan-liquors have been discussed.

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