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Control of Sulphide Formation in Coconut Toddy

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Abstract : Toddy, fermented with wild yeast, contains volatile sulphide.[3] The total sulphide content of toddy fermented under different conditions was quantitatively estimated spectrophotometrically (after aspiration from acid medium and trapping H_2S as an insoluble sulphide) by a method modified from Budd and Bewick[1] which is specific for inorganic sulphide. Results showed that 0.01 % NH_4^+ was, in most cases, sufficient to eliminate sulphide formed during wild fermentation. In a few instances, however, much higher concentrations were necessary. Urea did not inhibit at the same order of nitrogen concentration. Sulphide formation could also be controlled by choice of strain of yeast ; several strains isolated from toddy gave ferments nearly free of sulphide. Strains capable of forming high levels of sulphide do not produce this compound in the presence of 0.05 %NH_4^+.

Volatile sulphide in ferments can be trapped as CuS by addition of Cu turnings. Addition of Fe or steel to the ferments increased sulphide formation several fold ; NH_4^+ (0.1 %) only partly inhibited this.

Studies have shown that SO_4^{2-} does not increase sulphide formation, but that cysteine and (to a lesser extent) methionine appear to provide the sulphur for sulphide formation.

1. Introduction

Coconut toddy, the fermentation product of coconut sap (an exudate of the tender floral spathe[2]) is used directly as an alcoholic beverage[4] and also as the starting material for coconut arrack (a distilled product). Both products have been exploited extensively on a commercial scale for a number of years. More recently the unfermented material (sweet toddy) has been introduced in the bottled form.[7] Under some conditions all these products contain volatile sulphide[3,8,10] which contribute to the objectionable odour of toddy.

This paper describes the method by which inorganic sulphide formation in fermenting toddy can be prevented. The paper also describes in detail a method of determining the sulphide content of toddy.

2. Experimental

2.1. Materials

2.1.1. *Toddy*

Sweet toddy was obtained by collecting the sap after the normal tapping procedure[2] in clean vessels (free of microorganisms). Tapping commenced at 6 p.m. and samples were collected at 6 a.m. the following morning. Analysed at 8 a.m. the

sweet toddy contained 14 to 16% sugar. Samples were used directly for studies of fermentation with wild yeast and generally contained 10^6 to 10^7 cells/ml. For studies with pure cultures, toddy was either sterilized by autoclaving or by flash heating as in the sterilization procedure for bottled coconut water[5]; the toddy was used immediately in the first case and after bottling for future use when the second procedure was adopted. Toddy samples stored in this way gave only traces ($< 4\ \mu$ moles/l) of sulphide when tested after a period of weeks, showing that there was negligible formation of sulphide by non-microbiological pathways.

2.1.2. *Yeast cultures*

Strains of yeast isolated from toddy and other sources, as well as commercial yeast strains were used. Yeast cultures were single cell isolates, free of bacteria and were maintained on glucose-peptone-yeast extract-agar slants. Cells for experiments were grown aerobically on a shaker on standard glucose-peptone-yeast extract medium and used when physiologically active (showed a high rate of fermentation).

2.1.3. *Synthetic medium for sulphide formation*

The basal synthetic medium used for H_2S studies consisted of glucose, 12%; KCl, 0.05%; KH_2PO_4, 0.1%; $MgSO_4.7H_2O$, 0.5%and $FeSO_4$ trace, 0.001%. To this medium was added NH_4Cl, methionine and cysteine as given in section **2.5** The final pH of the medium adjusted to 4.5.

2.2. The fermentation process

Sweet toddy (200 to 250 ml) was introduced into a sterilized gas washing bottle (500 ml) and the required variables added. Studies with wild yeast were performed at a cell density of about 10^6 to 10^7 cells/ml while in studies with pure yeast strains, a cell density of 2×10^7 to 5×10^7 cells/ml was used. In the latter case cells were washed once with sterilized water before use. CO_2 evolved was led into a trap of cadmium acetate (200 ml of 0.3% w/v) in another similar gas washing bottle. The completion of fermentation was judged by observing the bubbles formed per min in the trap. The fermentation is over in 20 to 30 h (wild yeast) or 40 to 60 h (pure cultures). Sulphide formation occured mainly during the middle part of the fermentation and stopped when the fermentation ended.

2.3. Recovery of sulphide from toddy

The toddy sample was acidified with 2 ml of 98% H_2SO_4 (w.w)/100 ml toddy and gassed ($>$ 250 ml/min) with CO_2 for 45 min. Recovery experiments showed that the gas used was suitable for the purpose (section **3.1.**). In initial experiments a double trap containing zinc acetate (200 ml of 1.2% w/v) followed by Cadmium acetate (200 ml of 0.3% w/v) was employed. In subsequent experiments a special device was used to break up the gas bubbles (the gas was forced through small pores in an adaptor that was attached). This greatly facilitated sulphide absorption enabling quantitative recovery of $>$ 95% of H_2S using the Cadmium acetate trap.

Free sulphide was also determined by gassing without the addition of acid. This method of estimation was used only in the studies using metals and was not as reliable as acid conditions because increased period of bubbling gave rise to slight increases in free sulphide content. Generally the free sulphide content of toddy was 3 to 13 μmoles/l less than the total inorganic sulphide content.

2.4. Estimation of sulphide

Sulphide was estimated using NN diethyl p-phenylenediamine sulphate. The colour development technique was similar to that described in standard methods.[11] However, the strength of NN di-ethyl p.phenylene-diamine sulphate used was 0.81 g per 100 ml of 50% H_2SO_4 (w/v) and the intensity of colour formed was determined using a U.V. Spectrophotometer (UNICAM SP 500) at 660 nm. The estimation was done using a standard curve plotting S^{2-} content (Na_2S precipitated as ZnS and estimated by the I_2/ $Na_2S_2O_3$ titration) versus optical density. The test was most reliable over the range of 0.06 to 0.23 μmoles S^{2-} per sample. The standard curve had a gradient of 0.35 O.D./0.1 μmoles and a standard error of > 5% in this range. The test is specific for inorganic sulphide ; methionine and cysteine sent through the recovery procedure produced no colouration with the reagent.

2.5. Qualitative scoring system for volatile sulphide in toddy

This method was found to be useful for qualitative studies. Toddy (35 ml) was contained in a boiling tube (100 ml) plugged with cotton wool. A strip of filter paper of constant size, dipped in lead acetate, was affixed to the cotton wool plug. The extent of blackening of the paper was scored on a system of + signs from one to four.

Comparative tests with the quantitative method indicated that +, ++, +++ and ++++ as given in the text of this paper were in the ranges of 10, 10-30, 30-60 and > 60 μmoles/l respectively.

3. Results

3.1. Recovery of sulphide from toddy

Recovery of H_2S was tested by adding sulphide at concentrations in the range of 8 to 160 μmoles sulphide/l (as ZnS) into test solutions of (*a*) water (*b*) fermented H_2S free toddy. Solutions were gassed with CO_2 before addition of sulphide. Results showed that at least 90% of the sulphide could be recovered within 45 minutes using the traps, acid concentrations and rate of bubbling as described in section **2.3.** Sulphide-free toddy was obtained by fermenting toddy in the presence of 0.5% NH_4^+; aspiration in acid medium confirmed that no inorganic sulphide was present in this toddy.

3.2. Reproducibility

Reproducibility of the combined operations viz. the fermentation process, recovery of sulphide and quantitation of sulphide is reflected in the results in Table 1, which show that the method adopted was adequate to illustrate the effects described in this paper. However, it must be mentioned that extreme care must be taken in the fermentation stage with respect to : (1) volume of toddy used[3] (2) yeast cell density (3) the state of fermentation (Table 2) and (4) contamination. The first two factors must be kept constant within an experiment and the fourth scrupulously avoided.

TABLE 1. Reproducibility of assays

Sample	Inorganic sulphide formed (μmoles/l)		
	Experiment A	Experiment B	Experiment C
1	35.3	15.0	5.3
2	33.8	15.9	5.9
3	39.7		

In the three experiments above, parallel samples (triplicate in expt A and duplicate in expts B and C) were fermented. Reproducibility as above is a reflection of the combined effect of errors of the fermentation, recovery and detection stages of the process.

TABLE 2. A time course for sulphide formation in relation to sugar utilization.

Time (h)	Sulphide formed (μmoles/l)	Residual sugar (%)
0	0	16.4
10.5	1.0	12.5
21.5	128	9.0
27.5	172	7.9
32.5	225	7.2
50	228	5.0
70	228	3.3

Toddy was fermented in a series of bottles under identical conditions. Determinations were done on a separate ferment at the times given above. Total sugar was determined with Fehlings solution after inversion with HCI (sp. gr. 1.1029). Yeast culture No. 2 was used in this experiment.

3.3. Studies with wild yeast

Toddy samples showed a wide variation with respect to inorganic sulphide content ; of the 12 samples quantitatively studied, 7 produced $>$ 30 μmoles H_2S/l. The largest sulphide content observed was 150 μmoles/l.

The ammonium ion had a marked effect on sulphide formation.[3] In most cases, 0.01% ammonium was sufficient to eliminate H_2S formation (Table 3). However, there was a marked sample variation and in some instances 0.3% NH_4^+ was necessary for the purpose. However, in all cases 0.06% NH_4^+ was sufficient to reduce the H_2S level to $<$ 10 μmoles/l.

TABLE 3. Effect of NH_4^+ on sulphide formation during wild yeast fermentation.

Conc. of NH_4^+ added (%)	Sulphide formed (μmoles/l)		
	Expt A	Expt B	Expt C
0	116	150	16.0
0.005	91	19	4.1
0.01	—	<1.0	<1.0
0.02	N.D.	—	—
0.03	—	<1.0	N.D.
0.05	N.D.	—	—
0.1	N.D.	<1.0	—
0.2	N.D.	—	—

NH_4^+ added in the form of NH_4Cl to the same sweet toddy sample.

N.D. not detected.
— no experiment.

The source of NH_4^+ ion does not appear to be important but urea is not an effective inhibitor of sulphide formation (Table 4). Other experiments have shown that urea will inhibit at about 10 fold higher concentrations that NH_4^+. It was also found that addition of SO_4^{2-} (0.1%) did not affect the extent of H_2S formation.

TABLE 4. Effect of N sources on sulphide formation.

N source	Conc. added (as NH_4^+) (%)	Sulphide formed (μmoles/l)
NH_4Cl	0.02	<1.0
$(NH_4)_2SO_4$	0.02	<1.0
$(NH_4)_2HPO_4$	0.02	<1.0
$CO(NH_2)_2$	0.04	28.1
Control	0	29.7

The above sources of nitrogen were added to the same sweet toddy sample.

3.4. Studies with pure strains of yeast

Of the 34 strains of yeast tested 18 were able to produce > 30 μmoles H_2S/l. Generally top yeasts produced less H_2S than bottom yeasts. Of the 11 top yeasts used, only 1 produced > 30 μmoles H_2S/l. Results using some of these strains are shown in Table 5.

TABLE 5. Effect of yeast strain on sulphide formation.

Yeast culture No.	Source of yeast	Sulphide formed (μmoles/l)
2	Coconut toddy	59
15	Coconut toddy	128
17	Coconut toddy	<1
19	Coconut toddy	5.6
20	Coconut toddy	106
21	Coconut toddy	116
28	Palmyrah toddy	59
32	Mysore wine yeast	<1
40	Coconut toddy	N.D

N.D. not detected

The experiment was done with the same sweet toddy sample for all strains.

Different toddy samples with the same strain of yeast gave some difference in total sulphide content.

Two strains of yeast were chosen for further study. It was found that 0.05 %NH_4^+ was necessary to eliminate H_2S formation (Table 6).

TABLE 6. Effect of NH_4^+ on sulphide formation by high H_2S producing cultures.

Conc. of NH_4^+ added (%)	Sulphide formed (μmoles/l)		
	Culture 20	Culture 2	*Culture 20 and 2 mixed
0	112	125	185
0.005	78	112	—
0.01	59	69	69
0.03	—	—	5
0.05	—	—	<1
0.1	—	—	<1

Sweet toddy was fermented with the above strains of yeast.

*A different sample of toddy was used in this case.

— no experiment.

3.5. Studies using synthetic media

Addition of methionine and cysteine to a synthetic medium showed that : (1) of the two amino acids, cysteine produced H_2S more readily (2) the strain producing more H_2S from toddy produced more H_3S from the amino acids and (3) NH_4^+ inhibited H_2S formation (Tables 7, 8). This is consistent with H_2S being formed via the route of cysteine desulphydrase, the two latter points indicating a similar mechanism in toddy.

TABLE 7. Effect of methionine and cysteine on sulphide formation.

Yeast culture	Met. (μmoles/1)	Cys. (μmoles/1)	NH_4+ (%)	Sulphide formed (μmoles/1)
32	1677	0	0	<1.0
32	1677	0	0.7	N.D.
32	1677	0	0.2	N.D.
20	1677	0	0	11.8
20	1677	0	0.2	6.0
32	0	2067	0	122
32	0	2067	0.7	4.1
20	0	2067	0	209
20	0	2067	0.7	<1.0
20	0	0	0	N.D.

Methionine, Cysteine and NH_4+ were added in the above quantities to sweet toddy and the fermentation done with pure strains of yeast.

N.D. not detected.

TABLE 8. Effect of NH_4^+ on H_2S formation from cysteine and methionine.

NH_4 (%)	Methionine (μmoles)	Cysteine (μmoles)	Yeast culture no.	Volatile sulphide* formed	
				24 h	48 h
0	6.7	0	32	—	+
0.2	6.7	0	32	—	+
0.4	0	0	32	—	—
0.4	6.7	0	32	—	—
0.1	6.7	0	32	—	—
0	6.7	0	20	++	+++
0.2	6.7	0	20	—	+++
0.4	0	0	20	—	—
0.4	6.7	0	20	—	+
1.0	6.7	0	20	—	—
0.2	0	8.2	20	++++	++++
0.4	0	—	20	—	—
0.4	0	8.2	20	++++	++++
1.0	0	8.2	20	++++	++++
0	0	8.2	32	++++	++++
0.2	0	8.2	32	++++	++++
0.4	0	8.2	32	—	—
0.4	0	8.2	32	++	++++
1.0	0	8.2	32	++	+++

*Sulphide was detected by lead acetate paper (section 2.5). On a synthetic medium (section 2.1.3), other details as in Table 7.

3.6. The effect of metals on free H_2S content

Addition of metals to the ferment produced varying results (Table 9). Whereas Sn, Al and stainless steel produced no significant difference, copper (turnings) reduced free H_2S content, and mild steels increased free sulphide. The effect of copper appears to be due to the formation of CuS. The effect of the Fe is only partly inhibited by the NH_4^+ ion.

TABLE 9. Effect of metals on H_2S formation

Conditions	Sulphide liberated (μmoles/l)			
	Experiment 1	Experiment 2		Experiment 3
		Aspiratable	Acid liberatable	
+ Cu	4.4	—	—	<1.0
+ Al	13.1	—	—	—
+ Fe*	81	53	8.7	—
+ Sn	9.7	—	—	—
+ Mild steel	30.0	—	—	—
+ Bright steel	—	114	234	—
+ Stainless steel	—	17.5	24.7	—
+ Fe* + 0.1% NH_4^+	—	1.9	20.6	—
Control	9.4	21.9	25.6	54.4

Mass of metals, Experiment 1, 3g ; Experiment 2, 10g : and Experiment 3, 20g.

* B.D.H. electrolytically deposited iron

— no experiment.

3.7. The effect of NH_4^+ on the fermentation

Addition of NH_4^+ ion increases the rate of fermentation slightly but there is no significant effect on the efficiency of alcohol formation from sugar (Table 10). Flavour is altered and the distillate has no typical toddy odour, the odour being closer to that of a purer alcohol.

TABLE 10. Effect of NH_4^+ on the fermentation.

	NH_4^+ (0.05%) added		Control	
	21 h	48 h	21 h	48 h
Residual sugar (% w/v)	8.5	0.6	11.9	3.9
Alcohol (% w/v)	3.96	7.52	2.52	5.64
Ratio of alcohol formed to sugar utilized	0.50	0.47	0.52	0.45

Initial sugar, 16.6%. Alcohol was determined by specific gravity after distillation. Residual sugar was determined with Fehlings solution after inversion. Percentages are expressed as weight by volume. Yeast strain No. 20 was used in this experiment.

4. Discussion

The results clearly show that the inorganic sulphide produced in toddy varies markedly primarily due to differences in the characteristics of yeast in toddy. It is highly suggestive that the immediate source of H_2S is cysteine arising from the sweet toddy. The final enzyme in the pathway, cysteine desulphhydrase, has been isolated in bacteria.[6] The difference in extent of H_2S formed, using a single yeast strain with different samples of toddy could be due to variations in the sulphoamino acid content or concentrations of inhibitors in the different toddy samples.

Results show that even cultures that do not give H_2S with toddy (e.g. no. 32) may produce the enzyme in other media (as shown in the studies with the synthetic medium). The tendency to produce sulphide also varies from strain to strain, which is not surprising.

The inhibitory effect of ammonia could be due to either repression of synthesis of the enzyme cysteine desulphhydrase or to NH_4^+ acting as an inhibitor of the yeast enzyme (unlike the bacterial enzyme[6]).

The inhibition of inorganic sulphide formation as well as the reduction in intensity of flavour indicates a general 'sparing effect' of amino acids, well known in many alcoholic fermentations, which is probably connected with repression of synthesis of the enzymes concerned with amino acid degradation. A detailed study of the mechanism of inhibition by ammonia using cell-free extracts is in progress.

Another interesting point is that bottled sweet toddy (sterilized) produces small amounts of H_2S[8]. This may be due to incomplete deactivation of cysteine desulphhydrase or due to the participation of pyridoxal phosphate (a cofactor in the reaction) in a non-enzymic conversion of cysteine to H_2S, (remembering that the above cofactor can catalyse transaminase reactions in the presence of Fe^{3+}, Al^{3+} and Cu^{2+} at temperatures around 100°). Is this the mechanism for the enhancement of H_2S formation by Fe filings? Work is also proceeding on this aspect.

The study shows that H_2S and the consequent odour can be removed by : (1) use of selected yeast strains, (2) addition of small amounts of NH_4^+ and (3) addition of copper turnings or wire into the collection tank or even the still.

The fourth solution is only feasible for distilled products and may be applicable in the coconut arrack industry when methods of removing H_2S during the distillation procedure are not available.

Addition of NH_4^+ (0.01—0.03%) at collection points will prevent the formation of offensive odour of volatile sulphides. So will the use of selected strains of yeast on sweet toddy. The product will be a more pleasant sweet or fermented toddy, provided the increased salt content in the former case is acceptable. Distillation products of

NH_4^+ containing fermentation liquors would not have the characteristic flavour associated with coconut arrack and may be used as a spirit base for more selective purposes. Use of selected strains of yeast will be of use not only for the purpose of obtaining new flavours but also should result in increased yield of alcohol.

This study has not dealt with any aspect concerning organic sulphides in toddy ferments ; this line of study is worth investigating.

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