J. Natn. Sci. Coun. Sri Lanka 1976 4(2) : 121—138

Sulphhydryl Group determination by Amperometric methods and its application to studies with Coconut Toddy

M. K. G. S. KALYANANDA,* E. R. JANSZ AND E. E. JEYARAJ
Industrial Microbiology Section, Ceylon Institute of Scientific and Industrial Research (CISIR), P. O. Box, 787 Colombo 7, Sri Lanka

(*Paper accepted : 22nd September* 1976)

Abstract : A comparative study of different methods of amperometric titration of the sulphhydryl group is described. The factors varied included: (1) the microelectrode, (2) the titrant, (3) pH and (4) voltage. The method was successfully applied to coconut toddy to determine both RSH and RSSR content. Studies with coconut toddy gave further support to the theory that cysteine provides the sulphur for H_2S formation in fermenting toddy and also indicated why some yeast strains do not produce H_2S during the fermentation of (sweet) toddy.

1. Introduction

The sulphhydryl content of biological material can be measured by several chemical and polarographic methods.[3,4,5,9,11]. Amperometric titrations have often been employed in the determination of sulphhydryl (SH) groups in organic compounds and have been found to be superior to chemical methods of determination. The accuracy and precision of the results from amperometric titrations as well as the convenience of the method depend on several factors such as :

(i) microelectrode system
(ii) titrant
(iii) reaction conditions (pH, voltage).

Although considerable work has been done in this field, a comparative study of the different methods (varying the above factors) have not been reported. Furthermore, unlike in this study, most of the reported work has been done using semi-automatic or automatic polarographs.

Sulphhydryl groups can be titrated against a titrant containing any one of the following ionic species.[11]

(*a*) Titrants involving mercury.

(*b*) Titrants involving silver.

(*a*) *Titrants involving mercury.* The reaction of Hg(II) species with mercaptans can either be monovalent or divalent with respect to mercury.

*These studies form a part of the M.Sc. thesis University of Sri Lanka, Colombo Campus, of M. K. G. S. Kalyananda.

$$RSH + Hg^{+2} \longrightarrow (RS)_2Hg \qquad \text{Divalent} \ldots\ldots (1)$$

$$RSH + Hg^{+2} \longrightarrow (RS)_2Hg_2 \qquad \text{Monovalent} \ldots\ldots (2)$$

In these studies it was found that the relative stability of these two mercaptans and hence the valency of mercury in this reaction is governed by several factors such as titrant and the pH of the medium. Selection of these conditions fixes the valency of mercury in the above reaction.

(*b*) *Titrants involving silver species.* The silver ion can react with mercaptans according to the following equation.

$$RSH + Ag^{+} \longrightarrow RSAg.$$

However, titrations carried out in ammonia-ammonium nitrate or in "tris" buffer have given abnormally high values (about 130% of the theoretical values) on titration with known quantities of cysteine hydrochloride ($HS\text{-}CH_2\text{-}CH(NH_2)COOH.HCl$) This is because the silver ion reacts not only with the sulphhydryl group, but also with some other site in the cysteine molecule.[11] This error can be minimised by carrying out the titration in the presence of small concentrations (0.1M) of sodium sulphite. However, the presence of SO_3^{2-} complicates the interpretation of the results if the medium contains disulphides (RSSR) as the latter react with sulphite ions according to the following reaction resulting in the formation of reduced sulphhydryl (RSH) which in turn reacts with Ag^{+}, forming RSAg :.

$$RSSR + SO_3^{-2} \longrightarrow RSSO_3^{-} + RS^{-} \ldots\ldots (3)$$

Furthermore, a sharp end point is obtained when mercury(II) compounds are used as the titrants, because (i) mercury(II) mercaptides are much more stable and considerably less dissociated than the corresponding silver complexes, (ii) although mercaptides of both mercury and silver can form complexes with excess metal ion the difference in stabilities between normal Hg(II) mercaptides and the complex with excess metal ion is much greater than the corresponding differences of the silver mercaptan system.[11]

In the different amperometric titrations described below, compounds containing Hg(II) species were used as the titrant. This paper describes a comparative study of the different methods of amperometric determination of the SH group using a home made polarograph and also introduces the application of a rotating, hanging, mercury-drop electrode in amperometric titrations of the sulphhydryl groups. The paper also deals with the application of this method in studies of the mechanismn of hydrogen sulphide formation in fermenting toddy.

2. Experimental

2.1 RSH determination

2.1.1 *Polarograph*

A simple polarograph was constructed using two rheostats, microammeter, avometer, saturated calomel electrode and a 2V battery of 120 Amperage. The electrical circuit of the polarograph employed is shown in figure 1.

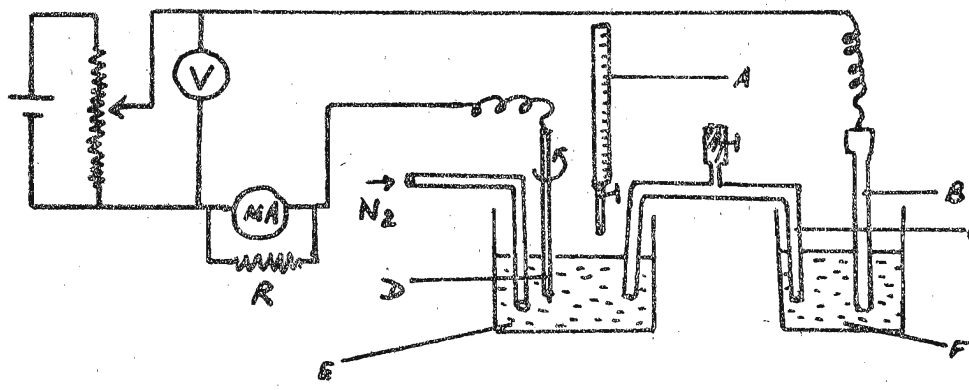

FIGURE 1—Diagrammatic representation of electrical circuit of the polarograph
A Microburette B Saturated calomel electrode
C Salt bridge (saturated solution of KCl with 3% Agar)
D Rotating hanging Hg drop electrode E Sample F Saturated KCl

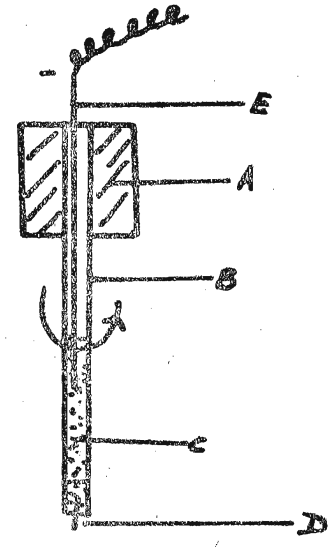

FIGURE 2—Rotating hanging Hg drop electrode
A Motor B Glass tube C Hg column D Gold plated Pt wire E Electrical connection

2.1.2 *Microelectrode system*

Two microelectrode systems were tried out.

(*a*) *Rotating Platinum Electrode.* A platinum wire (1 cm long, and 0.5 mm diameter) was sealed through the closed lower end of a glass tube (5 mm diameter) and electrical connections were made through a column of mercury.[11] It was fixed vertically to the axis of a motor. The effective length of the platinum electrode was 3 mm and the speed of rotation was 600 r.p.m.

(*b*) *Rotating hanging mercury drop electrode.* A platinum wire (1 cm long and 0.5 mm diameter) was sealed through the closed lower end of a glass tube so that 0.5 mm of the wire projected from the end of the rod. This projection was plated with a thin coating of gold and a drop of mercury, equivalent in size to one drop from a dropping mercury electrode, was attached to the gold plated wire. The electrical connection was made through a column of mercury.[3] (Figure 2). The electrode was fixed to a high speed mechanical motor rotating vertically (600 r.p.m.).

2.1.3 *Reagents*

Titrants : (*a*) 0.005M mercuric chloride solution (prepared using analytical grade $HgCl_2$) (*b*) 0.005M ethylmercury chloride (C_2H_5HgCl recrystallised) solution.

Samples : (*a*) 5×10^{-3}M cysteine solution.

(*b*) 5×10^{-3}M cysteine hydrochloride solution.

Buffers : (*a*) 0.02M acetate buffer pH 4.76

(*b*) 0.1M borax solution pH 9.5.

2.1.4 *The Amperometric Titration*

KCl (10 ml of 1M) was added to the sample (0—30 ml) in a clean beaker (150 ml). The solution was made upto 100 ml with buffer, and used as the titration cell in the circuit (fig. 1). This solution was de-aerated by a stream of nitrogen for 12 to 15 min. and the sulphhydryl group (SH) was titrated at a potential V with the titrant from the semimicroburette. The diffusion current corresponding to each titrant volume was recorded, and the endpoint determined from a plot of current and titrant volume (figures 3, 4 and 5). This titration was carried out under varying conditions, and after each addition of 1 ml of titrant a stream of nitrogen was passed through the sample for about 30 seconds.

2.1.5 *Experimental modification using toddy*

In the determination of RSH in toddy a known amount of toddy (40—80 ml) was taken into a 150 ml beaker and 1N NaOH was added to neutralise half of the total acid present in the toddy sample. The solution so obtained had a pH of about 4.75.

10 ml of 1M KCl was then added and the solution was made to 100 ml with acetate buffer (0.02M, pH 4.76). The rest of the procedure was the same as that described under section 2.1.4.

2.2. RSSR determination

The experimental procedure adopted in the determination of RSSR was the same as that described in section 2.1.4. except that the solution was made 0.1M with respect to sulphite prior to the titration, by adding the required quantity of solid Na_2SO_3 into the de-aerated medium. The final volume was adjusted to 100 ml with borax buffer. The addition of SO_3^{2-} converts all RSSR into RS^- and $RSSO_3^-$ according to equation (3).

2.3 RSH determination by ferricyanide method

Freshly prepared cysteine solution (0.20 mg/ml) was cooled in the water and phosphate buffer (0.2ml of 1M, pH, 6.7), sodium nitroprusside (0.1 ml of a 5% solution) and conc. NH_4OH (0.2 ml) were added. This resulted in a red colouration which was titrated rapidly with $K_3Fe(CN)_6$ (with continuous shaking). Standardisation of $K_3Fe(CN)_6$ was carried out with 3 different concentrations of cysteine solutions. Toddy (10 ml) was titrated with $K_3Fe(CN)_6$ using the same procedure.

2.4 H_2S determination

H_2S in fermented toddy was determined by the method described previously.[7]

2.5 Synthetic media

Czapex-Dox medium containing 15% glucose was used as the base to which was added the sulphur containing compound.

2.6 Materials and cultures

Sweet toddy suitable for these experiments was obtained by collecting the fresh sap of the coconut inflorescence in polythene bags. Yeast strains from the CISIR collection were used in this study and cultured as described previously.[6,7]

3. Results

3.1 RSH Determination

Results obtained under seven different methods (table 1) of amperometric titration are given in tables 2, 3, 4, 5, 6, 7 and 8 respectively. The relative advantages and disadvantages are described in section 4. The titration curves using these methods are given in figures 3, 4, and 5.

TABLE 1—Method used in RSH determination

Conditions	Micro-electrode system	Titrant	pH	Applied voltage	Other reagent added
Method 1	Rotating Pt electrode	$HgCl_2$	9.5	—0.2 V	—
Method 2	Rotating Pt electrode	$HgCl_2$	4.76	—0.45	—
Method 3	Rotating Pt electrode	EtHgCl	4.76	—0.45	—
Method 4	Rotating hanging Hg drop elec.	$HgCl_2$	4.76	—0.45	—
Method 5	Rotating hanging Hg drop elec.	EtHgCl	4.76	—0.45	—
Method 6	Rotating hanging Hg drop elec.	EtHgCl	9.5	—0.45	0.2M in Na_2SO_3
Method 7	Rotating hanging Hg drop elec.	EtHgCl	9.5	—0.70	0.2M in Na_2SO_3

See section 2 for details.

TABLE 2—Determination of cysteine by method 1.

Volume of 5×10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
2.50	3.10	2.50
5.00	6.10	5.00
7.50	9.40	7.50
10.00	12.00	10.00

The general pattern of the titration curve under this condition is shown in fig. 3.

See Table 1 and section 2.1 for details.

TABLE 3—Determination of cysteine by method 2.

Volume of 5 × 10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
10.00	5.00	5.00
20.00	10.20	10.00
30.00	15.00	15.00

The general pattern of the titration curve under these conditions is shown in fig. 3.

See Table 1 and section 2.1 for details.

TABLE 4—Determination of cysteine by method 3.

Volume of 5 × 10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
5.00	2.30	2.5
10.00	4.90	5.0
15.00	7.30	7.5
20.00	10.10	10.0

The general pattern of the titration curve is shown fig. 3.

See Table 1 and section 2.1 for details.

TABLE 5—Determination of cysteine by method 4.

Volume of 5 × 10^{-5}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
5.00	2.50	2.50
10.00	5.00	5.00
15.00	7.20	7.50
20.00	10.00	10.00

The general pattern of the titration curve is shown in fig 4.

See Table 1 and section 2.1 for details.

TABLE 6—Determination of cysteine method 5.

Volume of 5 × 10^{-3}M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
2.50	2.50	2.50
5.00	5.00	5.00
10.00	10.30	10.00
15.00	14.50	15.00

The general pattern of the titration curve is given in fig. 4.

See Table 1 and section 2.1 for details.

TABLE 7—Determination of cysteine by method 6

Volume of 5 × 10⁻³M cysteine (ml)	Observed end-point (ml)	Theoretical end-point (ml)
10.00	9.0	10.00
10.00	9.0	10.00
15.00	13.5	15.00
* 20.00	16.5	20.00
* 10.00	8.3	10.00

*Cysteine hydrochloride was used. The pattern of titration curve is given in fig. 5.

See Table 1 and section 2.1 for details.

TABLE 8—Determination of cysteine by method 7.

Volume of 5 × 10⁻³M cysteine hydrochloride (ml)	Observed end-point (ml)	Theoretical end-point (ml)
10.00	10.00	10.00
20.00	20.30	20.00

The general pattern of the curve under these conditions is shown in fig 5, curve 7.

See Table 1 and section 2.1 for details.

TABLE 9—Determination of RSSR by modified methods 6 and 7

Conditions	Cystine (mg)	Observed end-point (ml)	Theoretical end-point (ml)
Method 6	12.00	11.10	10.00
Method 6	12.00	9.20	10.00
Method 6	6.00	5.00	5.00
Method 7	12.00	9.30	10.00
Method 7	6.00	5.00	5.00

See Table 1 and section 2.2 for details.

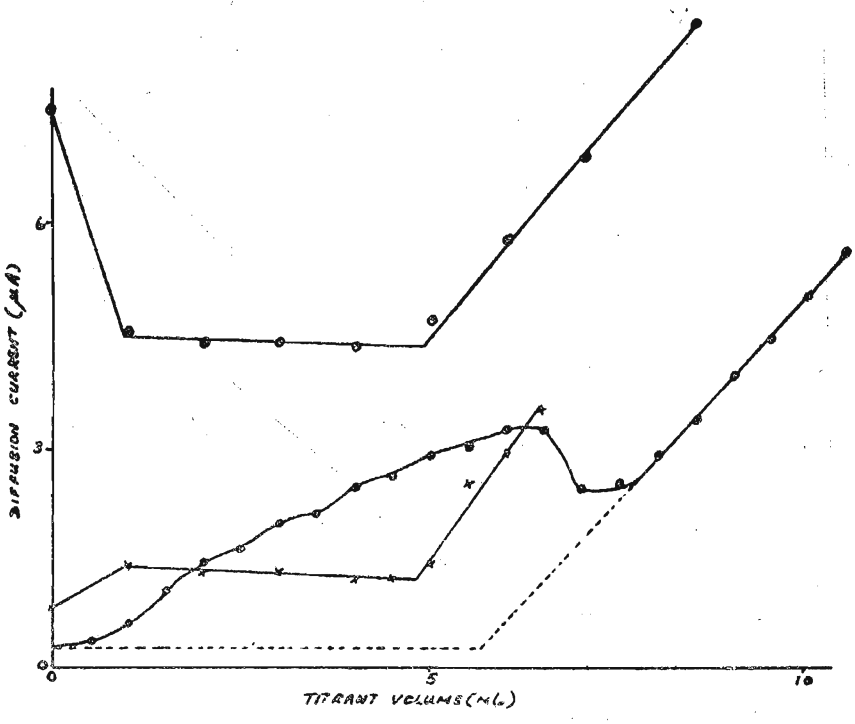

FIGURE 3—Titration curves for different methods of amperometric analysis

O — O, Method 1.
X — X, Method 2.
⊙ — ⊙, Method 3.

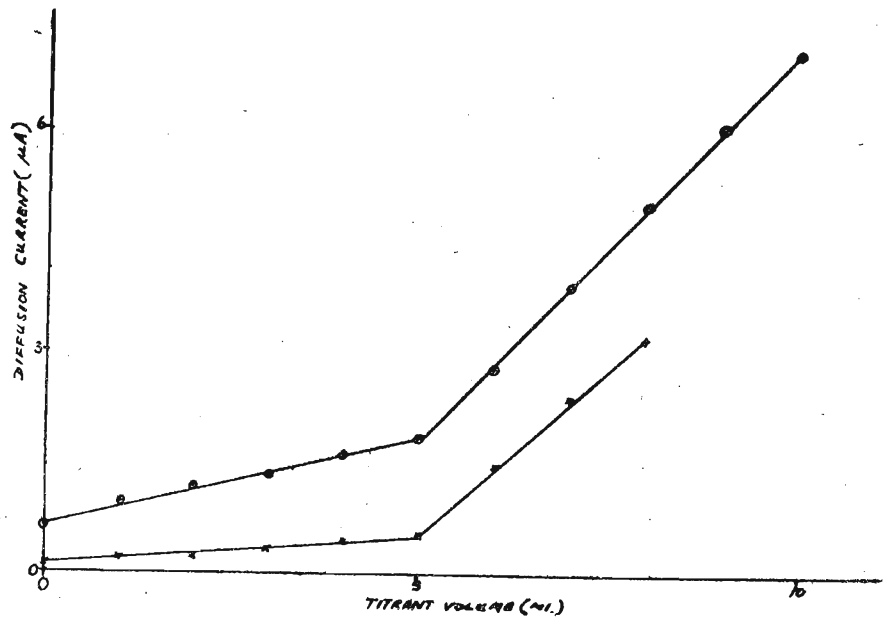

FIGURE 4—Titration curves for different methods of amperometric analysis

X — X, Method 4.
⊙ — ⊙, Method 5.

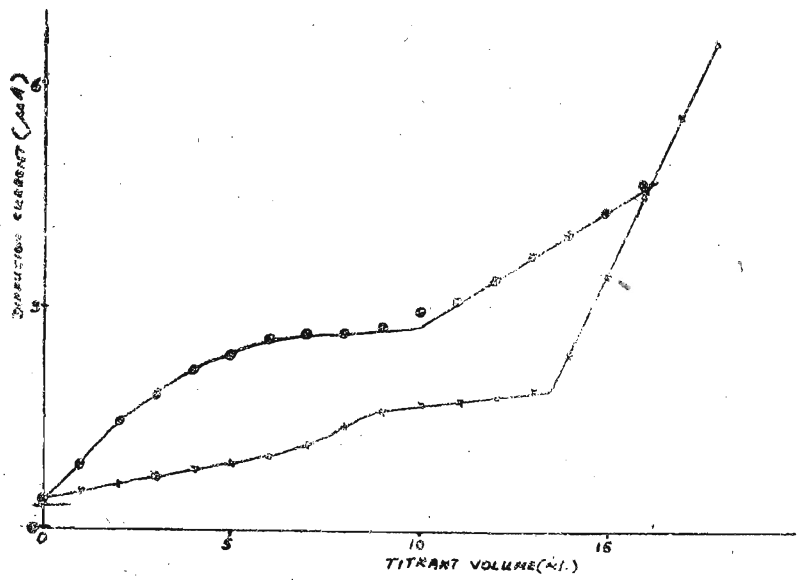

FIGURE 5—Titration curves for different methods of amperometric analysis

X—X, Method 6.
⊙–⊙, Method 7.

3.2 RSSR Determination

RSSR content was determined by the use of SO_3^{2-} as described in section 2.2 and discussed in section 4. Results are shown in table 9.

3.3 Application of method to toddy

3.3.1 *Preliminary Studies*

In using toddy the main problem was its low sulphhydryl content. In the determination of SH content in the above methods accurate results could be obtained only with a large quantity of toddy (40—80 ml). On the other hand when the volume of the sample (40—80 ml) was very large the procedure described under section 2.1.4. could not be used due to the difficulty of adjusting the pH of the medium (to 4.76) by the addition of acetate buffer. This problem was overcome by the method described under section 2.1.5 in which the pH of toddy was adjusted to 4.76 by neutralising half of the total acid (mainly acetic acid) present in toddy with 1N NaOH. The operation needs to be done with extreme care.

3.3.2 *Evidence for specificity*

As there can be no direct proof for the specificity of this method of determination of RSH content, several indirect methods were used. The evidence can be summarised as follows.

(*a*) Different amperometric methods used on toddy yielded the same result : e.g. methods 4 and 5 gave values of 1.21 and 1.21, 1.21 and 1.21, and 0.09 and 0.07 mM. SH respectively for three selected samples of toddy.

(*b*) Results using the amperometric method tallied with the $K_3Fe(CN)_6$ micro-titration[1,2] for SH group (using sodium nitroprusside as indicator).

(*c*) Analysis of the amperometric titration curve for toddy in the presence and absence of cysteine showed that the shift in end point corresponded to the amount of cysteine added. In addition there was no break in the titration curve until the new endpoint was reached.

(*d*) Analysis of the H_2S formed by fermentation of synthetic media showed that an initial cysteine content of about 1.6 mM produced about 0.13 mM H_2S. It was also found that the SH content of toddy (determined by the assay) that produced on fermentation the same quantity of H_2S was also of the order of 1.6 mM. In both sets of experiments the same strain of yeast was used.

3.3.3 *RSH and RSSR content of toddy*

The RSH content determined by method 5, showed that all samples of fresh sweet toddy analysed (over 25 samples), contained an SH content in the range of 1.4 to 1.9 mM, whereas the RSSR content of all these samples was negligible. However, on autoclaving sweet toddy, there was a dramatic decrease in RSH content and a corresponding increase in RSSR content. Further, on standing for a period of weeks the RSH content virtually declined to zero. Typical results showed that the —SH content of a sample of autoclaved toddy immediately after autoclaving and 7 days later was 0.6 mM and 0.45 mM respectively. At the latter stages RSSR content of autoclaved sweet toddy was sometimes as high as 0.9 mM (as SH).

When toddy was fermented with H_2S producing yeast strains (strains Nos. 2 and 20 of the CISIR yeast collection)[7,10] the final RSH content was generally zero although on one occasion a value of 0.09 mM was observed. RSSR content of fermented toddy on the other hand was high, generally in the range of 0.75 to 0.90 mM (as SH).

3.3.4 *Relationship between H_2S produced and RSH content*

Experiments carried out with autoclaved sweet toddy samples showed that variation of RSH content in these samples paralleled H_2S produced (table 10). Similar results were obtained using a synthetic medium under the same fermentation conditions (table 11).

TABLE 10—Effect of RSH content in autoclaved sweet toddy on H_2S produced

Sample	Initial RSH (mmoles/l)	H_2S produced (mmoles/l)
Tree No. 1	0.45	0.052
Tree No. 2	0.16	0.019
Tree No. 9	0.02	0.003
Tree No. 10	Nil	Nil

Aged autoclaved sweet toddy was analysed for SH content and fermented with a H_2S producing yeast strain (No. 20). H_2S was estimated after complete fermentation.

TABLE 11—H_2S formation in synthetic fermenting medium

Added cysteine (mmoles/l)	H_2S produced (mmoles/l)
0.3	0.015
0.6	0.043
0.9	0.110
1.2	0.097

To Czapex-Dox medium modified as in section 2.4 was added cysteine in the above quantities. The H_2S formed was determined after complete fermentation.

3.3.5 *Relationship between RSSR and H_2S formation*

Studies carried out with a synthetic medium (table 12) as well as those with toddy (table 13) led to the conclusion that cystine (RSSR) has no significant effect on H_2S formation during fermentation.

Samples with different initial RSH concentrations produced H_2S in quantities proportional to the initial RSH content. This was observed in the studies carried out on a synthetic medium (Czapek-Dox solution containing 15% sugar) using freshly grown yeast cultures 2 and 20.[7] The results showed that the RSH present initially was ultimately converted to RSSR and H_2S after fermentation. (Table 14) Further, it was found that the conversion of RSH to other forms (H_2S and RSSR) was quantitative, i.e. total sulphur in the form of H_2S, RSSR and RSH in the fermented product was often equal to the initial sulphur (present as RSH).

TABLE 12—Effect of RSSR on H_2S formation in a synthetic fermenting medium

Sample	Initial RSH (mmoles/1)	Initial RSSR (as SH) (mmoles/1)	H_2S produced (mmoles/1)
Synthetic medium (250ml)	Nil	Nil	Nil
Synthetic medium (250ml) + 11 mg cysteine	0.32	Nil	0.060
Synthetic medium (250ml) + 20 mg cystine	Nil	0.66	0.002

H_2S was determined after complete fermentation with H_2S producing strains (2 and 20).

TABLE 13—Effect of cystine (RSSR) on H_2S formation in fermenting toddy

Sample	Initial RSH (mmoles/l)	Initial RSSR (mmoles/l) (as SH)	H_2S produced (mmoles/l)
Autoclaved sweet toddy* (250ml)	Nil	0.76	Nil
Autoclaved sweet toddy (250ml) + 17mg cysteine	0.56	0.76	0.06
Autoclaved sweet toddy (250ml) + 30 mg cystine	Nil	1.78	Nil

Sweet toddy (aged and autoclaved) containing no —SH and 0.76 mmoles of RSSR was fermented after addition of cystine and cysteine and H_2S determined after complete fermentation.

*The sweet toddy sample used here was two weeks old.

3.3.6 *Results using attenuated yeast*

An interesting set of results was obtained when using attenuated yeast cultures. When yeast strains 2 and 20 (which are normally H_2S producing) were stored in a refrigerator at 5°C for about a week they no longer produced H_2S even though they could carry out fermentation efficiently. Study of a time-course of fermentation using these attenuated yeasts showed that even when fermentation was complete (at 36h) the SH content only fell from 1.65 mM to 1.50 mM and only traces of RSSR were formed ; H_2S was not detected. This showed that the yeast strains could not utilize RSH in toddy to any significant extent. It was also observed that the attenuated yeast could produce H_2S from added cysteine suggesting that the yeast differentiated between —SH in toddy and —SH of added cysteine. This failure to produce both H_2S and RSSR was of great interest because the same strain when not subjected to attenuation produced both H_2S and RSSR.

4. Discussion

(i) On comparing curves 1 to 7 it was found that all the titration curves were regular after the endpoint. However, the regularity of curves before the endpoint varied markedly, the rotating hanging mercury drop electrode (HMDE) being superior to the rotating platinum electrode for sulphhydryl determination. In using HMDE there were also other advantages such as (*a*) easy cleaning, (*b*) durability and (*c*) high sensitivity.

A sharp end point was not obtained only with method 1. Results using method 1 were precise to ± 1%[14] with a constant positive deviation of 27%. The end point of titrations obtained under conditions 2, 3 and 4 corresponded to the bivalent state of Hg whereas those obtained under methods 1, 5, 6 and 7 corresponded to the monovalent Hg.

The results of method 2 were precise to ± 1%. Although irregularities in the titration curve in method 2 were partly eliminated in method 3, a very high initial residual current was observed, which interfered with the estimation of low sulphhydryl concentrations (10^{-4} mg/l). However precision and accuracy remained unchanged at the ± 1% levels even under these conditions. The irregularities of method 3 were completely eliminated when the rotating platinum electrode was replaced by the rotating hanging mercury drop electrode and this is illustrated in the results obtained with methods 4 and 5 (fig 4) ; sensitivity was also increased under these conditions. This difference can be attributed to : (*a*) the high hydrogen overpotential and (*b*) the comparatively high cathodic range of mercury compared to platinum. Although results obtained were much more precise (± 1%) when method 4 was used instead of method 5, (which had a degree of precision of ± 2.5%), the latter method was more suitable for the estimation of small quantities of RSH. This was due to the fact that EtHgCl is monovalent when method 5 is used whereas $HgCl_2$ is divalent in method 4.

Of the two methods developed for the determination of RSSR, method 7 was more precise and accurate compared to method 6 ; a constant error of about 10% was observed in method 6. However, curve 6 of fig 5 reflects the superiority of method 6 over method 7 in the determination of small quantities of RSSR because of the low initial residual current of the former.

Method 5 was applied successfully in the determination of RSH in toddy. Sweet toddy was found to contain an SH content in the range of 1.4 to 1.9 mM. H_2S producing yeast strains (cultures No. 2 and 20) convert only a small fraction of this RSH into H_2S. However, the study demonstrates the involvement of the RSH group in the formation of H_2S confirming the conclusions of previous reports.[7,10] It is significant that this mechanism is very different from that of H_2S formation in beer which is brought about by the reduction of sulphite.[8,12,13]

Although evidence is by no means complete, we propose the following hypothesis: most of the cysteine (SH) in toddy is in the form of protein. In this state it will remain largely unoxidised unless the proteins are denatured (e.g. autoclaving as done in this study) or hydrolysed (as in the case of fermentation by yeast strain 2 and 20).

The lack of oxidation of RSH to RSSR in the experiment with attenuated yeast strains supports this. This is also supported by : (1) the absence of oxidation of SH in fresh toddy and (2) the differentiation between added cysteine and —SH in toddy by the attenuated yeast. That is, H_2S production in toddy can be visualized as a two-step process as given below :

	STAGE 1			STAGE 2	
Cysteine (bound in protein)	——→ Hydrolysis	Cysteine (free) Cystine (RSSR)	(R—SH)	——→ Cysteine de-sulphhydrase	H_2S

This two-step process involves the release of cysteine from protein in the first step which will explain the observation that H_2S and RSSR are always formed together or not at all (as in the case of attenuated yeast where both RSSR and H_2S were not formed).

This concept can account for the existence of non-H_2S producing strains of yeast (as shown in our earlier study).[7,10] This could be due to one of two reasons : (1) the lack of cysteine desulphhydrase as shown by yeast cultures No. 39 and 40 which cannot release H_2S from cysteine and (2) the lack of protein hydrolysing activity as suggested by attenuated cultures 2 and 20. More work is proceeding on our culture collection to screen strains of yeast for the absence of one or more of the necessary stages of H_2S production. A third possibility which is more remote is that RSH may be converted by some non-H_2S producers to a form which is not available for H_2S production.

Other results of ours have indicated that the effect of iron in increasing H_2S production may be due to an interference with the $2RSH \leftrightarrow RSSR$ equilibrium. Work on this aspect is continuing.

Our previous study showed that H_2S formation in toddy can be controlled by (*a*) choice of strain of yeast and (*b*) addition of NH_4^+. This study provides a clue to other methods of reducing H_2S formation which could take the form of (1) promoting the conversion of cysteine to cystine and (2) inhibiting protein hydrolysing activity.

The described method of estimation of RSH and RSSR appears highly promising and should be of immense value in furthering current studies on the mechanism of H_2S formation in coconut toddy and other biological systems.

TABLE 14—Fermentation in synthetic medium

Sample	Initial* RSH (mmoles/l)	Residual RSH in fermented sample (mmoles/l)	RSSR in ≠ fermented sample as SH (mmoles/l)	H_2S produced (mmoles/l)
1	0.375	0	0.27	0.10
2	0.75	0	0.54	0.21
3	1.125	0	0.91	0.23
4	1.50	0.24	N.D.	0.31
5	1.875	0.48	N.D.	0.42

*Initial RSH is from added cysteine hydrochloride.

≠ Method 6 was employed in RSSR determination.

H_2S, RSSR and RSH were determined after complete fermentation.

N.D., Not determined as instrument broke down.

See section 2 for experimental details.

Acknowledgements

The authors wish to thank the Director, CISIR, for laboratory facilities and the National Science Council for a grant to Mr. M. K. G. S. Kalyananda. The authors also thank Miss Kanthi Jayaratne for secretarial assistance.

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