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The production of H_2S by pure culture wine yeasts

by

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Die Bildung von H_2S durch Reinzuchthefen

Zusammenfassung. — Die Bildung von H_2S durch 12 Reinzuchthefen wurde mit verschiedenen Gärsubstratzusammensetzungen untersucht. Wenig- und viel-Sulfitbildende Hefen zeigten unterschiedliches H_2S-Bildungsvermögen: Waren Sulfat oder Sulfit die einzigen Schwefelquellen, dann konnten die Hefen nicht nur in Wenig- und Viel-Sulfit-Bildner, sondern auch in wenig- und viel-Sulfid-bildende Stämme eingeteilt werden. Letzteres zeigte sich auch, wenn die Hefen auf ABY-Agar ausgestrichen wurden. Auch ein Pantothenat-Mangelsubstrat bewirkte im wesentlichen die gleichen Ergebnisse. Wurde Cystein zugegeben, mit oder ohne Pantothenat, so reagierten nur einige Stämme mit verstärkter H_2S-Bildung. Alle Hefestämme produzierten gleich viel H_2S, wenn dem Gärsubstrat kolloidaler Schwefel zugegeben worden war. Die Vergärung von Traubenmost oder von synthetischem Substrat, dem Zentrifugentrub zugesetzt worden war, ermöglichte ebenfalls keine Gruppierung der Stämme. Es wird vermutet, daß die H_2S-Bildung bei der Vergärung von Traubensaft meistens durch kolloidalen Schwefel aus Spritzmittelrückständen verursacht wird. Es wird außerdem gezeigt, daß geeignetere Reinzuchthefen selektioniert werden können.

Introduction

The formation of H_2S during wine making is not yet fully understood (Eschenbruch 1974). Many variables interact making experimental results often difficult, if not impossible, to interpret. Variations in the composition of grape juices, the degree of clarification of juices, handling techniques during and after fermentations and spray residues, all influence the metabolism of the yeast cell and its capacity for H_2S production (Rankine 1963, Acree *et al.* 1969, Eschenbruch and Kleynhans 1974, Singleton *et al.* 1975, Eschenbruch 1976, Hysert and Morrison 1976).

Pure culture yeast strains are selected at random from natural populations associated with grapes. Significant differences exist between strains of the same species, particularly with regard to their physiological properties such as H_2S production (cf. Rankine 1968, Jansz *et al.* 1975). Winemakers often claim that pure culture yeasts sometimes produce faulty fermentations. Such fermentations may result from the specific metabolism of the strain reflecting environmental influences and changes.

In this paper the influence of different substrate compositions on the formation of H_2S by several pure culture wine yeasts is examined in a strictly defined medium. Also, differences between low- and high-sulphite forming strains are considered to assist the critical evaluation of uncertain aspects of sulphur metabolism.

Table 1

Identification and origin of yeast strains used
Identifizierung und Herkunft der verwendeten Hefestämme

Ruakura collection no.	Original number and place of isolation		Species
R 92[1])	WE 1	Oenol. Viticult. Res. Inst., Stellenbosch, S. Africa	*Sacch. cerevisiae*
R 93[1])	WE 14	do.	*Sacch. chevalieri*
R 100[1])	WE 353	do.	*Sacch. cerevisiae*
R 99[1])	Steinberg	FA f. Weinbau Gartenbau Getränketechnol. Landespflege, Geisenheim, Germany	*Sacch. cerevisiae*
R 102[1])	G 49	do.	*Sacch. cerevisiae*
R 104[2])	Mumm	do.	*Sacch. cerevisiae*
R 106[2])	Oberingelheim	do.	*Sacch. cerevisiae*
R 105[2]),[1])	729	Austral. Wine Res. Inst., Adelaide, Australia	*Sacch. cerevisiae*
R 107[3])	WE 372	Oenol. Viticult. Res. Inst., Stellenbosch, S. Africa	*Sacch. cerevisiae*
R 108[3])	WE 376	do.	*Sacch. cerevisiae*
R 109[3])	WE 377	do.	*Sacch. cerevisiae*
R 149[2])	St 36	LLVA f. Wein- Gartenbau Landwirtsch., Trier, Germany	*Sacch. pastorianus*

[1]) Commercially used strains, low-sulphite forming, except R 105.
[2]) High-sulphite forming yeasts.
[3]) New isolates, low-sulphite formers.

Materials and methods

Table 1 lists the different yeasts examined. A synthetic medium of pH 4.2 was prepared according to TOKUYAMA *et al.* (1973). Two vitamin treatments were used: complete medium and pantothenate deficient medium as described earlier (ESCHENBRUCH and BONISH 1976). Vitamins, L-cysteine, colloidal sulphur (80% sulphur, w/w, wettable powder) or slurry were added after the medium had been sterilized for 5 min at 1 atm. Sulphate (Na_2SO_4) was omitted when sulphite ($Na_2SO_3 \times 7H_2O$) was the only sulphur source. The slurry was that discharged during the clarification of pressed grape juice from a Westphalia Separator Model SAMR 3036. Grape juice slurry (unsterilized) and grape juice were kept frozen until used. Flasks were inoculated with 2 % of an exponentially growing culture propagated in either of the two synthetic media. From 4 l liquid cultures in 5 l flasks, kept at room temperature (20—22 °C), 25—300 ml samples were withdrawn daily for H_2S determinations during the course of the fermentation as described by ESCHENBRUCH and BONISH (1976).

The H_2S measured daily over a fermentation period of 10 d represented only that in solution, not the total H_2S produced.

The Acid Bismuth Yeast (ABY) solid medium recommended by NICKERSON (1953) was also employed to assess H_2S production. On this substrate colonies turn black or brown or remain white after 24 h, depending on the amount of H_2S produced. This method was originally used to distinguish strains and species of the genus *Candida*. The yeasts used for this investigation did not grow well on this ABY medium. When applied to this medium as a heavy streak of yeast cells which had been propagated for 48 h, five different colour shades developed after 24 h.

Results

The maximum levels of H_2S produced by 12 different yeasts during fermentation are listed in Table 2. The columns represent various substrate compositions and the results emphasize several points.

With sulphate as the only sulphur source (2nd column) two groups of strains can be distinguished with regard to their sulphide production. The high-sulphite forming strains (R 104, R 105, R 106, R 149) and also R 107 formed negligible H_2S whereas all the other yeasts produced significant amounts. H_2S formation was highest on day 5 or 6 of the fermentation. The formation of H_2S from sulphite (3rd column) was similar to that from sulphate. Again, maximum production occurred on day 5 to 6 of the fermentation process.

Addition of colloidal sulphur to the complete medium increased H_2S production dramatically with all strains. Maximum amounts were formed earlier in the fermentation, between day 2 and 4.

When the yeasts fermented a medium deficient in pantothenate H_2S production also occurred at an earlier stage. Under these conditions too, the high-sulphite forming strains plus R 107 are clearly separated from the other low-sulphite formers by their low H_2S production.

Addition of L-cysteine to the complete medium resulted in a different pattern. Several strains (R 92, R 99, R 102, R 107, R 108, R 109), all of which are low-sulphite formers, produced little H_2S, whereas the other strains formed high amounts. When L-cysteine was added to a pantothenate-deficient substrate the H_2S formation was amplified by all strains except the high-sulphite formers which produced similar amounts under either condition, i.e. L-cysteine addition to a medium with or without pantothenate. Maximum H_2S formation was in both cases on day 2 or 3 of the fermentation.

Columns 8 and 9 of Table 2 show the amount of H_2S formed when the complete medium was supplemented with slurry from Gewürztraminer and Müller-Thurgau varieties. In all strains H_2S production was substantial. This also occurred in the fermentation of pure grape juice: all yeast strains, whether low- or high-sulphite forming, produced significant though lesser amounts of H_2S. In these three cases maximum H_2S formation occurred at day 5 or 6 of the fermentation period.

The last column also classifies the 12 yeasts according to their H_2S producing capacity using the ABY agar method. Strains producing "dark brown" and "brown" streaks formed high amounts of H_2S. The colours "light brown", "ochre" and "cream" identified low-H_2S formers. In general, strain numbers R 92, R 93, R 99, R 102, R 109 and to a slightly lesser extent R 100 and R 108 produced H_2S more readily than the other yeasts.

Table 2

Maximum levels of H_2S formed by wine yeasts fermenting a synthetic substrate of varying composition or grape juice
Maximale Konzentrationen von H_2S gebildet bei der Vergärung von synthetischem Substrat wechselnder Zusammensetzung und von Traubenmost

	Yeast strain no.	SO_4^{--} only S-source	SO_3^{--} only S-source	SO_4^{--} + colloidal sulphur (100 mg wettable powder/l)	SO_4^{--} no pantothenate	SO_4^{--} + cysteine (300 mg/l)	SO_4^{--} + cysteine (300 mg/l) no pantothenate	Slurry[1] (500 ml/l)	Slurry[2] (500 ml/l)	Grape juice[3]	Streak on ABY agar
		Maximum H_2S content (μg/l) in fermentation liquid									
Low-sulphite forming	R 92	44	35	1353	60	25	155	53	100	45	dark brown
	R 93	56	46	1577	71	395	548	48	192	71	dark brown
	R 99	43	31	1609	74	39	90	55	174	88	dark brown
	R 100	16	20	1371	57	174	282	56	187	47	brown
	R 102	20	35	1686	61	61	80	68	160	47	dark brown
	R 107	1	1	1453	7	7	56	75	97	53	light brown
	R 108	16	33	1065	30	9	36	40	125	82	brown
	R 109	14	1	2864	21	13	53	70	147	66	dark brown
High-sulphite forming	R 104	2	1	1403	7	400	380	71	100	67	ochre
	R 105	1	1	960	2	327	254	84	145	80	ochre
	R 106	1	1	1001	9	275	267	69	300	63	light brown
	R 149	1	1	1545	1	125	94	35	135	16	cream
		Day of maximum H_2S production									
		5—6	5—6	2—4	2—4	2—3	2—3	5—6	5—6	5—6	

[1]) *Vitis vinifera* cv Gewürztraminer, vintage 1976, content of solids 22 % (10 min at 11630 × g).
[2]) *Vitis vinifera* cv Müller-Thurgau, vintage 1976, content of solids 10 % (10 min at 11630 × g).
[3]) *Vitis vinifera* cv Palomino, vintage 1975, sugar 17 %, total acidity 6.05 g/l, pH 3.4.

Discussion

The extent to which the formation of H_2S depends on the composition of the substrate is clearly shown by the results in Table 2. It also becomes quite obvious that different yeast strains respond differently to substrate variations.

If either sulphate or sulphite is the only sulphur source, high-sulphite forming yeasts produce little H_2S. The suggestion that such yeasts could not form significant amounts of H_2S, therefore, seems correct (Dittrich and Staudenmayer 1968). Increasing evidence supports this theory (Eschenbruch *et al.* 1973, Bonish and Eschenbruch 1976, Dott and Trüper 1976, Eschenbruch and Bonish 1976, Heinzel and Trüper 1976). However, the actual metabolic mechanisms and changes involved are not yet understood (Zambonelli *et al.* 1975, Bonish and Eschenbruch 1976, Dott and Trüper 1976, Heinzel *et al.* 1976) and may vary from strain to strain (Romano *et al.* 1976).

A different patterns occurs when the fermentation substrate contains colloidal sulphur. In this case all strains produce very large quantities of H_2S. Under practical conditions this type of sulphur would come mostly from fungicide residues from vineyard sprays (Rankine 1963, Acree *et al.* 1972). A similar picture is produced when the same yeasts ferment grape juice. No differences exist between low- and high-sulphite formers although the amounts produced are much smaller. This confirms earlier observations that high-sulphite forming yeasts fermenting grape juice produce as much H_2S as normal strains (Eschenbruch 1972, 1974, Eschenbruch *et al.* 1973). Again, when grape juice sediment is added to the complete synthetic medium, the H_2S production of all yeasts is equally high and pronounced; slurries from both Müller-Thurgau and Gewürztraminer juice are as effective.

From these results a more defined picture of H_2S formation during fermentation emerges. High-sulphite forming yeasts produce negligible H_2S from sulphate or sulphite alone but large amounts with other supplementary sulphur sources such as colloidal sulphur, cysteine, grape juice sediment or, to a lesser extent, grape juice itself. Therefore, H_2S production from grape juice and grape juice slurry by high-sulphite formers cannnot solely arise from sulphate via the normal sulphate reduction pathway. It seems likely that the H_2S produced by the low-sulphite forming strains fermenting grape juice or slurry arises in the same way as that produced by the high-sulphite forming strains, i.e. not from sulphate or sulphite. This is supported by some preliminary results which show that key enzymes of the sulphate reduction pathway (ATP-sulfurylase, sulphite reductase) are greatly depressed in yeasts grown in grape juice, compared to the levels of activity found in yeasts grown in synthetic medium (Bonish, unpublished).

The question arises as to which components of the grape juice or slurry cause increased H_2S formation. Since cysteine or methionine can be ruled out (Eschenbruch 1974), spray residues of elemental sulphur on the skins of grape berries could be a possible alternative (Rankine 1963, Acree *et al.* 1972). If this were so, then the degree of juice clarification would influence H_2S formation, as was shown by Hsia *et al.* (1975) and Singleton *et al.* (1975). Whether elemental sulphur is reduced by chemical reaction with thiol groups or enzymatically, involving a heat labile system, is not clear. Both reactions seem to occur simultaneously and indications are that it is a non-specific process (cf. Roy and Trudinger 1970).

Addition of Gewürztraminer slurry results in generally lower H_2S formation than does the addition of Müller-Thurgau slurry. The total sulphur content of the first was 6 mg/g dry weight compared with 22 mg/g dry matter for the second. How-

ever, possible correlations can only be substantiated through more experimental information including identification of the sulphur components involved.

Although pantothenate deficiency increases H_2S formation from sulphate with all strains, the amount produced by high-sulphite formers remains low. According to WAINWRIGHT (1970), this deficiency restricts the conversion of cysteine to methionine. Pantothenate deficiency in the presence of cysteine results in substantially higher H_2S formation by all strains except the high-sulphite formers, by which it is slightly lower, compared to the non-deficient medium containing cysteine. Since these strains grow slower — a linear growth curve as against a sigmoidal curve for low-sulphite formers — the conversion rate of cysteine to methionine may already be restricted. Consequently a pantothenate deficiency would be less critical.

It is interesting to find the low-sulphite forming R 107 showing a very similar H_2S producing pattern to the high-sulphite formers. This newly isolated pure culture yeast has been shown to have other advantageous characteristics. It is non-foaming (ESCHENBRUCH and RASSELL 1975) and produces more favourable wines when compared with other pure culture strains (VAN WYK and ESCHENBRUCH 1973, unpublished Research Report Oenology Dept., University of Stellenbosch, South Africa).

From these results it can be understood why many wineries in Australia and New Zealand favour the high-sulphite forming strain R 105 (cf. RANKINE 1968). Its H_2S formation from sulphate and sulphite is low and even from colloidal sulphur is less than in the other strains tested. As, however, the selection of R 107 shows clearly, low-sulphite producing strains can be found with an equally favourable H_2S forming pattern.

The results suggest several different stages of H_2S formation: Pantothenate deficiency, high levels of cysteine and also residues of colloidal sulphur can contribute to increased formation of H_2S early in the fermentation process, i.e. 2nd to 3rd day after initiation (Table 2). In grape juice, after the addition of slurry as well as from sulphate or sulphite H_2S is formed at a later period, 5 to 6 d after the onset of fermentation. A third stage of H_2S formation can occur after the completion of the fermentation when the yeast lees autolyses (cf. RANKINE 1963). Whether these differences can be attributed to different metabolic mechanisms or varying levels and activities of key enzymes of the sulphur metabolism remains to be confirmed. Screening yeast strains for H_2S production from various sulphur sources is, therefore, a useful tool to improve the quality of pure culture yeast material.

Summary

Production of H_2S by 12 pure culture wine yeasts on different substrates including grape juice has been studied. Under these conditions low- and high-sulphite forming yeasts showed different patterns of H_2S formation: When grown with sulphate or sulphite as the only sulphur source the strains could be divided not only into low- or high-sulphite (SO_2) formers but also into low- or high-sulphide (H_2S) forming yeasts. Growth on the ABY solid medium also separated high- from low-sulphide formers. A deficiency of pantothenate produced a similar pattern though with increased levels of H_2S. The addition of L-cysteine with or without pantothenate caused a substantial increase in H_2S production with only some strains. Addition of colloidal sulphur produced high concentrations of H_2S with all strains, such that they could not be differentiated. Neither did the fermentation of grape

juice nor the addition of grape juice slurry to the synthetic substrate allow any differentiation of the yeasts. It is concluded that H_2S formation in grape juice comes mostly from residual colloidal sulphur from fungal sprays. It is also shown that more suitable yeast strains can be selected.

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