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Relationship Between the Sterol Content of Yeast Cells and Their Fermentation Activity in Grape Must

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In grape must of high sugar concentration, yeast growth, the viability rate of "resting" yeast cells, and fermentation activity were stimulated under certain conditions of aeration and temperature. This stimulation might be interpreted as being a result of the yeast cell sterol content. The addition of certain sterols to the fermenting medium was able to increase this sterol content. According to aeration conditions of the medium, which determined the sterol content of yeasts, the sterols added in the medium acted as (i) growth factors, (ii) fermentation inhibitors, and (iii) survival factors for the yeast.

Ergosterol is the predominant sterol in yeast cells, but zymosterol (18), 5-dehydroergosterol (17), and 24,28-dehydroergosterol (4) are also present. In the cells, sterols account for 6% by weight of the protoplasmic membrane dry matter (11) and are bound to the mitochondrial particles (12). The sterol content of the cells also differs with the strain of yeast (7) and the conditions of culture (13). Demel and de Kruyff (6) show that sterols are regulatory; they can liquefy and condense the membrane.

It is known that molecular oxygen is necessary for yeasts to synthesize sterols (15). The work of Gordon and Stewart (8) established that there is a definite increase in the sterol content of yeast exposed to air for a long period after being cultured anaerobically. Kirsop (9) demonstrated the relationship between sterol biosynthesis and the oxygen requirements of yeasts. He found that these requirements are satisfied by supplementing the medium with ergosterol in Tween 80.

Works by Andreasen and Stier (1) and by Brechot (2, 3) have demonstrated that certain sterols (ergosterol, lanosterol, and cholesterol) as well as oleanolic acid (which is an essential constituent of grape cuticle wax [14]) are yeast anaerobic growth factors.

Recently, Lafon-Lafourcade et al. (10) showed that certain substances act as "survival factors" when added to grape must aerated and inoculated with aerobically cultivated yeast. Cellular multiplication is not affected; however, the resting cells maintained their viability and fermentative activity.

In this "survival state," these resting cells are responsible for the later stages of fermentation in grape must of high sugar concentration. These survival factors include: (i) certain sterols (ergosterol, lanosterol, cholesterol); (ii) oleanolic acid; and (iii) a peptidic hormone, oxytocin.

In this present work, an attempt had been made to establish a relationship between the sterol content of yeast cells and their fermentation activity and also to determine the mode of action on yeast of exogenously supplied sterols.

MATERIALS AND METHODS

Medium and yeast strains. The culture medium used was either pasteurized or fresh grape must. The pasteurized must was inoculated with commercial dried wine yeast, identified as *Saccharomyces cerevisiae*.

Aeration conditions. Anaerobic conditions were achieved with a special air lock filled with mercury and equipped with two taps, one attached to a vacuum and the other to a nitrogen gas supply. The fermentation vessel could be degasified then filled with nitrogen via the mercury air lock.

Semiaerobic conditions were achieved by leaving the fermenting grape must in a flask stoppered with a cotton plug.

For a short aeration, grape must fermenting anaerobically was decanted into a sterile container with a wide opening, the volume of which was about 10 times that of the must. The open container was then shaken manually for 5 min, putting the must in contact with a large quantity of air.

Chemical analysis. After fermentation, residual sugar was analyzed (16).

Addition of sterols in fermenting medium was made after their dissolution in a hot mixture of absolute ethanol, Tween 80 (2 ml/liter) and oleic acid (20 mg/liter); an equivalent volume of ethanol was added to the control medium.

The sterols were extracted from yeast cells by the Napias technique (C. Napias, Ph.D. thesis of Sciences University of Bordeaux, France, 1975).

Wet cells of a known weight were treated by solvent reflux. The solvents used were chloroform (1), followed by chloroform-methanol (2:1) (1.5 h) and methanol (1

h). The successive extracts were pooled, evaporated to dryness, and dissolved again in a solution of chloroform-methanol-water (47:3:50). The solution obtained was washed with distilled water until clear and then evaporated to dryness. The purified extract was saponified with N-KOH in methanol for 1.5 h.

The nonsaponified residue was extracted three times with diethylic ether. The resulting solution was washed with distilled water until the washing water was neutral.

The Liebermann-Burchard reaction was used to simultaneously analyze (i) a known volume of extract, and (ii) the same volume of extract with 1 mg of ergosterol. The sterol content was expressed as a percentage of yeast dry weight:

$$T(\%) = [De/(Ds - De)] \times (100/P)$$

where P dry weight of yeasts treated; De optical density of the sample of extract; Ds optical density of the sample of extract supplied with 1 mg of ergosterol.

Replicates obtained from other extractions by the same weight of yeast gave similar results (within ±0.1%).

RESULTS

Effect of anaerobic conditions of fermentation. In a must aerated before inoculation, the initial sterol content of the inoculum was 1.5% (milligrams of sterols per 100 mg [dry weight] of yeast; Table 1). The sterol concentration showed little change by day 2 of fermentation, but decreased rapidly between day 2 and 5 and progressively thereafter, until at day 19 of fermentation there was only about 0.3%.

When the same fermentation medium was supplemented with ergosterol, the maximal sterol content of the yeast was hardly affected for the first 2 days. However, this content became the double of that in the control cells from day 9 and remained at that level until the end of fermentation. By contrast when oleanolic acid was added to grape must, the sterols content of the cells remained nearly the same as that in the control. Nevertheless, in both cases, the number of viable yeasts in the decline phase was increased in the presence of ergosterol or oleanolic acid; consequently a greater quantity of sugar was consumed at the end of fermentation (F. Larue, 3rd cycle thesis, University of Bordeaux II, France, 1978).

Effect of semiaerobic conditions of fermentation. After 2 days the sterol content of the yeast was much greater (80%) than in the initial inoculum and was also greater (70%) than in the yeast fermenting anaerobically. The sterol content of the yeast fermenting under semiaerobic conditions remained about three times greater than in the yeast fermenting anaerobically. Consequently, after 19 days, the fermentation was more rapid and more sugar was fermented in the semiaerobic conditions than in the anaerobic conditions.

At the end of fermentation, in grape must supplemented in ergosterol, the sterol content of yeasts fermenting under semiaerobic conditions was lower (20%) than in the control, and the quantity of sugar consumed was lessened by 12 g/liter.

Effect of a short aeration during anaerobic fermentation. In grape must aerated before inoculation, the sterol content of the yeasts fermenting anaerobically was already decreasing significantly by day 3, as shown in Table 2.

On day 3 some samples of must were aerated. Other samples were aerated on day 10. We observed the following results.

(i) Six hours after the aeration, the sterol level in the yeast was greatly increased. It was approximately the same as in yeast grown in must supplemented with cholesterol and fermenting anaerobically.

(ii) The results on day 33 showed that aeration

TABLE 1. *Effect of anaerobic and semiaerobic conditions of fermentation on the sterol content of the yeast cells and on their fermentation activity*[a]

Condition	Sugar fermented (g/liter) after:			Viable yeast (cells × 10^6/ml) after:			Sterol content of yeast cells (% of dry wt) after:		
	2 days	9 days	19 days	2 days	9 days	19 days	2 days	9 days	19 days
Anaerobic conditions									
Control	37	164	170	22	5	0.5	1.60	0.40	0.30
Addition of ergosterol	36	169	199	20	7	0.5	1.40	1.00	0.60
Addition of oleanolic acid	23	154	185	17	5	0.1	1.70	0.30	0.20
Semiaerobic conditions									
Control	30	187	246	22	20	18	2.70	1.20	1.00
Addition of ergosterol	27	175	234	16	16	12	2.80	1.10	0.80
Addition of oleanolic adid	24	155	211	21	20	16	2.30	0.70	0.40

[a] Initial sugar, 246 g/liter; dry yeast: *S. cerevisiae* (initial sterol content, 1.5%); initial viable yeast, 2.2×10^6 cells per ml; ergosterol, 25 mg/liter; oleanolic acid, 50 mg/liter.

of the grape must on day 10 was ineffective.

(iii) Aeration carried out on day 3 had the same effect as the addition of cholesterol. Also, the two effects were additive.

(iv) At the end of the fermentation, the greater quantity of sugar was consumed by the yeast with the greatest sterol content. These results suggested that there was a relation between the sterol content of yeast and their fermentative capacity.

Effect of certain growth conditions during fermentation. On day 3 of grape must fermentation at 35°C, an important difference in the sterol content of the yeast was observed in comparison with yeast fermenting at 30 or 25°C (Table 3). The sterols content of the yeast fermenting at 35°C was much lower (50%); as a result, the yeasts were less active in fermenting and the quantity of sugar consumed at the end of the fermentation was lower (38%).

The addition of cholesterol to the must kept the sterol content of yeast cells at a high level, and the number of viable yeasts was also higher than in the control (F. Larue, 3rd cycle thesis, University Bordeaux II, France, 1978); but at 35°C such an addition did not restore the fermentation to a normal level. Changes in pH, the initial sugar concentration, and the addition of ammonium monophosphate had no effect on the sterol content of the yeast.

TABLE 2. *Effect of aeration on day 3 of the anaerobic fermentation of a grape must aerated before inoculation on the sterol content of the yeast cells and on their fermentation activity*[a]

Condition	Sugar fermented (g/l)		Sterol content of the yeast cells (% of dry weight)	
	6 h after aeration	Day 33 of fermentation	6 h after aeration	Day 33 of fermentation
Anaerobic condition				
Control grape must	79	178	0.45	0.20
Addition of cholesterol	80	215	0.85	0.40
Aerated on the 3rd day				
Control grape must	86	217	0.70	0.40
Addition of cholesterol	90	240	1.10	0.55

[a] Initial sugar, 298 g/liter; dry yeast, *S. cerevisiae* (initial sterol content, 1.50%), initial viable yeast, 3.6 × 10^6 cells per ml; cholesterol, 365 mg/liter.

TABLE 3. *Influence of the temperature on the sterol content of the yeast cells and on their fermentation activity*[a]

Condition	Temp (°C)	Sugar fermented (g/liter) after:		Sterol content of the yeast cells (% of dry wt) after:	
		3 days	33 days	3 days	33 days
Control	25	53	192	0.60	0.20
	30	87	169	0.50	0.15
	35	76	119	0.30	0.20
Addition of cholesterol	25	55	236	1.10	0.55
	30	90	192	1.10	0.50
	35	79	142	0.90	0.40

[a] Initial sugar, 285 g/liter; dry yeast, *S. cerevisiae* (initial sterol content: 1.5%); initial viable yeast, 3.2 × 10^6 cells per ml.

DISCUSSION

In grape must, the sterol content of the yeast fermenting under anaerobic conditions reached the maximum at the early stage of the fermentation; then this content decreased to about 0.3% at the end of fermentation. Consequently, the fermentation rate was relatively slow. Conditions which increased the sterol content in yeast promoted a more active fermentation and a greater quantity of sugar was consumed.

This suggested that the fermentative metabolism of the yeast was directly linked with their cellular sterol content. This cellular content depended mainly upon the following: (i) the inoculum reserves, which depended on preculture conditions; (ii) the thermal conditions (less than 35°C) which affected the cellular loss in sterols; and (iii) the possibility of sterol biosynthesis occurring, due to contact between the yeast and oxygen for a period as short as 5 min during the exponential phase of growth. Grape must is rich in dextrose and levulose, and under these conditions (2, 5) the oxidative catabolic pathway (respiration) is inhibited (Crabtree effect, or "contre-effet Pasteur"). Even when oxygen is present the sugar is catabolized by the fermentation pathway. Consequently, the end products are therefore the same as those under strict anaerobic conditions. The effect of oxygen was to allow the biosynthesis of certain essential metabolites, notably sterols (9).

An increase in the cellular sterol content could be obtained equally by the addition of sterols to anaerobically fermenting grape must. Such an addition to the must under semiaerobic conditions did not influence the cellular content in sterols, but it restricted the fermentation. From these results the following conclusions concern-

ing the different functions of sterols might be derived.

The inhibitor effect was observed under semiaerobic conditions (Table 1). It could be assumed that the sterol biosynthesis of the yeast cells was sufficient to ensure a satisfactory cellular structure. Addition of sterol to the culture medium would modify this balance by decreasing membrane permeability and suppressing the exchange of substances between the cell and the culture medium and so slowing down fermentation.

The growth factor effect is observed with an inoculum predeveloped in anaerobic conditions, fermenting in anaerobic conditions (1-3). All sterols synthesis is now impossible. The inoculum reserves were insufficient to enable the population to develop to the potential maximum permitted by the nutrient content of the growth medium. The sterol deficiency of the population became a growth-limiting factor. The addition of a sterol to the medium removed the inhibition, and thus the sterol acted as a growth factor.

The survival factor effect was observed with an inoculum predeveloped in aerobic conditions, fermenting under anaerobic conditions in grape must, initially aerated (Table 1). The yeast accumulated sterols before fermentation, and biosynthesis occurred during the first few hours of fermentation. It appeared, therefore, that sterol requirements were satisfied during the exponential and stationary phases of growth. However, the high sugar concentration meant that the fermentation period was long, and so the sterol reserves were eventually depleted. Consequently, the yeast lacked sterols towards the end of fermentation. An addition of sterol to the grape must alleviated this deficiency in the yeast cells during the decline phase and improved the exchange between the cells and the medium. Thus, sterols functioned as survival factors. The fermentation of grape must by indigenous yeasts was a characteristic case in which this occurred.

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