

# Relationship between Ergosterol Concentrations in Wine Yeast and Sugar Fermentation at Different Temperatures\*

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**The effect of cellular ergosterol on the fermentation ability of four different *Saccharomyces cerevisiae* wine yeast strains was studied. Fermentations were carried out at three temperatures and ergosterol concentrations determined at six fermentation stages. The lower ergosterol concentrations at the higher temperatures are ascribed to the higher growth rate of the yeast cells resulting in a more rapid dilution of the ergosterol. Growth of yeast during fermentation was linked with the ergosterol content of the cells. The ergosterol concentration of the yeast cells was not directly correlated to specific fermentation rates.**

The plasma membrane of *Saccharomyces cerevisiae* is a lipoprotein bilayer structure (Andreasen & Stier, 1953). The membrane contains a mixture of phospholipids, including sphingolipids, and cardiolipin. In addition, yeast synthesizes and incorporates sterols into its membranes. The principal sterol is ergosterol (Starr & Parks, 1962; Rattray, Schibeci & Kidby, 1975; Parks, 1978). Dulaney, Staply & Simpf (1954) measured ergosterol production in yeast cultures covering 60 species in 20 genera. Most of the cultures produced about 0,1% of their dry weight as ergosterol, but some strains of *Saccharomyces* produced 7-10% ergosterol dry weight; the average in this genus is 2-5%.

The lipid composition affects membrane functions to a great extent. However, the lipid composition of the yeast membrane is affected by culture conditions. Furthermore, mutants with defects in lipid metabolism contain membranes with altered lipid compositions. Growth of *Sacch. cerevisiae* under anaerobic conditions results in requirements for both a sterol and an unsaturated fatty acid. However, growth rates similar to those found under aerobic conditions can be maintained under anaerobic conditions if the medium is supplemented with ergosterol and oleic or linoleic acid (Haukeli & Lie, 1976; Watson & Rose, 1980). The endogenous levels of unsaturated fatty acids and sterols in *Sacch. cerevisiae* are lowered by dilution as yeast cells multiply under anaerobic conditions in the absence of lipids; growth ceases when the concentration decreases to approximately 25% of the values obtained under aerobic conditions (Guidici & Guerzoni, 1982). The limiting value of ergosterol is 1 mgg<sup>-1</sup> yeast (dry weight) (Aries & Kirsop, 1977). However, Strydom, Kirschbaum & Tromp (1982) stated that ergosterol is probably not directly responsible for the formation of new cells, but may be concerned only with the physiological state of the cell, permitting it to maintain a higher level of activity and enabling it to complete fermentation rapidly. Oxygen deficiency during beer fermentation results in restricted yeast growth, reduced

yeast viability, slow and incomplete fermentation and changes in beer flavour (Jakobsen & Thorne, 1980).

Molecular oxygen is required for cyclization of squalene to squalene epoxide and subsequent demethylation of lanosterol to zymosterol (Rattray *et al.*, 1975). A proportion of each sterol exists in a combined form, presumably as fatty acid esters. Zymosterol is present almost entirely in this form (Aries & Krisop, 1978). According to Bailey & Parks (1975) the physiological state of the cells reflects the rate of esterification of yeast sterols with long chain fatty acids. A culture growing exponentially on a good carbon source in a rich medium contains low levels of sterol esters. Once sterol intermediates are esterified, they are effectively prevented from being further metabolized to ergosterol (Bailey & Parks, 1975). Under anaerobic conditions, sterol esters are metabolized to free sterols (Aries & Kirsop, 1978; Ahvenainen, 1982).

Yeasts containing sterols with an unsaturated side chain at C<sub>17</sub> (ergosterol and stigmasterol) are more alcohol tolerant than those containing a saturated side chain (campesterol and cholesterol) (Thomas, Hossack & Rose, 1978). Hossack & Rose (1976) suggested that the double bond in the sterol side chain increased stabilization in the distal part of the fatty-acyl chain in contact with the sterol side chain. This increased stabilization could lead to the formation of a more effective barrier against ethanol molecules entering yeast cells which were suspended in ethanol solutions (Thomas *et al.*, 1978). This barrier effect could be even greater if an asymmetrical distribution of sterol molecules in the yeast membrane exists. Thus, if the sterol content is low in the plasma membrane, ethanol tolerance will be lower, and ethanol will have a greater effect on the cell metabolism.

The major significance of sterols in yeast is their influence on the dynamic state of membranes (Proudlock *et al.*, 1968). They influence phospholipid-protein interactions, membrane permeability and activity of membrane-bound enzymes

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(Rattary *et al.*, 1975; Ahvenainen, 1982). Parks & Starr (1963) reported a relationship between sterol biosynthesis and the respiratory capacity of yeast cells.

This study was undertaken to determine how fermentation at different temperatures affects the ergosterol concentration of four wine yeast strains, and to establish whether there is a relationship between the ergosterol concentration and fermentation rate.

#### MATERIALS AND METHODS

**Yeast strains:** *Saccharomyces cerevisiae* strains WE 452, WE 500, WE 14 and WE 372 from the VORI collection were used.

**Medium:** The medium of Usseglio-Tomasset, Ciolfi & Pagliara (1982) was used with 200 g l<sup>-1</sup> sucrose as carbon source.

**Culture conditions:** The medium was filter-sterilized and transferred aseptically into glass flasks which had been pre-sterilized with 80% ethanol and subsequently rinsed twice with sterile distilled H<sub>2</sub>O. The inoculum was prepared by inoculating stock cultures of each yeast strain in 1/ flasks containing 400 ml medium. Yeast cells were cultured stationary at 25°C for 3 days and then transferred into a 10/ flask and again incubated at 25°C for 3 days. These cultures were standardised spectrophotometrically at an optical density of 1,0 at 660 nm and this was then used to inoculate the medium (4% v/v inoculum). Fermentations were carried out in triplicate at 10,15°C and 20°C in 10/ flasks which contained 8/ medium and were sealed with a bung fitted with a fermentation trap.

**Sampling technique:** The medium and suspended yeast cells were thoroughly shaken by rotating the fermentation flask prior to sampling. Sampling was done through the fermentation trap by means of CO<sub>2</sub> (oxygen-free) pressure (Osumi *et al.*, 1978). Samples (360 ml) were taken at intervals during which the sugar concentration decreased by about 12,5 g l<sup>-1</sup>. The yeast cells were washed twice in distilled H<sub>2</sub>O by centrifugation (13 000 g x 10 min). The pellet was finally suspended in 4 ml distilled H<sub>2</sub>O and stored at -20°C. The supernatant was stored at 0°C until analysis.

**Yeast dry mass:** Washed yeast cells were dried at 105°C to constant mass.

**Sugar analysis:** The total reducing sugars as (g l<sup>-1</sup> invert sugar) were determined by employing the official methods of the AOAC (Anon, 1975).

**Ergosterol analysis:** One ml yeast suspension was added to 1 ml distilled H<sub>2</sub>O, and the ergosterol concentration determined by the method of Breivik & Owades (1957) using a Beckman Model 25 spectrophotometer.

**Fermentation rate:** Fermentation rates (g l<sup>-1</sup> sugar fermented day<sup>-1</sup>) were determined for the intervals 0-24, 13-37, 38-62, 63-87, 88-112 g l<sup>-1</sup> and 113-137 g l<sup>-1</sup> sugar fermented which corresponded to six fermentation stages. These values were considered to indicate the activity of the yeasts at each fermentation stage and were also used to determine specific fermentation rates (SFR) as g l<sup>-1</sup> sugar fermented day<sup>-1</sup> 100 mg<sup>-1</sup> yeast cells.

**Standardization of data:** Yeast dry mass and ergosterol concentrations were plotted against sugar concentration. Standardised values corresponding to 12, 25, 50, 75, 100 g l<sup>-1</sup> and 125 g l<sup>-1</sup> sugar fermented (fermentation stages 1-6) were then obtained from the graphs.

**Statistical analysis:** A completely randomised experimental design with three replicates was employed. Three measurements were taken at each of the six fermentation stages. Consequently an analysis, taking account of the potential correlations between measurements at the various stages, was performed. The analysis was performed using the REPEATED command of the SAS procedure GLM and the POLYNOMIAL transformation keyword for fermentation stages. The PRINTE option was used to perform a sphericity test, as described in the SAS User's Guide (Anon, 1985). Separate analyses were performed for ergosterol concentration and fermentation rate. A standard VORI factorial statistical software package was used to test significant differences among treatment means (identical fermentation stages). The same program was used to determine correlation coefficients.

#### RESULTS AND DISCUSSION

For both variables (ergosterol concentration and fermentation rate) strain x temperature x stage interactions were indicated by the multivariate test (the sphericity test was rejected in both cases) and separate analyses were, therefore, performed for each yeast strain. In the ergosterol concentration analyses by strain, the approximate significance levels (SL) were 0,0018 (WE 14), 0,1598 (WE 372), 0,2423 (WE 452) and 0,3788 (WE 500). The sphericity test was therefore only rejected in the case of WE 14. In the case of fermentation rate only the WE 500 sphericity test was not rejected (SL = 0, 1546). The multivariate test performed when the sphericity test was rejected are illustrated in Table 1 for ergosterol concentration in WE 14. The univariate analysis of variance, valid when the conditions for failing to reject the sphericity test are met, is illustrated in Table 2 for WE 372 on the same variable.

TABLE 1

Multivariate analysis of variance over six fermentation stages for WE 14 at three temperatures.

Source of variation	df		Significance Level (Wilks, Lambda, or Pillai's trace, or Hotelling-Lawley trace, or Roy's greatest root)
	Numerator	Denominator	
Stage	5	2	0,0403
Stage x Temp. (Linear)	5	2	0,0360
Stage x Temp. (Quad.)	5	2	0,0365

TABLE 2

Univariate analysis of variance for WE 372 at three temperatures and over six fermentation stages.

Source of variation	df	Error Term	Significance Level
Stage = S	5	$S^2_2$	0.0001
Linear = $S_1$	1	$S^2_{2,1}$	0.0001
Quadratic = $S_2$	1	$S^2_{2,2}$	0.0301
Cubic = $S_3$	1	$S^2_{2,3}$	0.1219
Quartic = $S_4$	1	$S^2_{2,4}$	0.6350
Quintic = $S_5$	1	$S^2_{2,5}$	0.1591
Temperature	2	$S^2_1$	
Linear = $T_1$	1	$S^2_1$	0.8568
Quadratic = $T_2$	1	$S^2_1$	0.6787
Stage x Temperature 10 $S \times T_1$	5	$S^2_2$	0.4316
$S_1 \times T_1$	1	$S^2_{2,1}$	0.8701
$S_2 \times T_1$	1	$S^2_{2,2}$	0.0615
$S_3 \times T_1$	1	$S^2_{2,3}$	0.4898
$S_4 \times T_1$	1	$S^2_{2,4}$	0.6080
$S_5 \times T_1$	1	$S^2_{2,5}$	0.4388
$S \times T_2$	5	$S^2_2$	0.1307
$S_1 \times T_2$	1	$S^2_{2,1}$	0.0764
$S_2 \times T_2$	1	$S^2_{2,2}$	0.6585
$S_3 \times T_2$	1	$S^2_{2,3}$	0.7296
$S_4 \times T_2$	1	$S^2_{2,4}$	0.3185
$S_5 \times T_2$	1	$S^2_{2,5}$	0.1547
Flasks within Temperatures	6	$=S^2_1$	
Stage x Flasks within Temp <sup>†</sup> .	30	$=S^2_2$	
$S_1 \times$ Flasks within Temp <sup>†</sup> .	6	$=S^2_{2,1}$	
$S_2 \times$ Flasks within Temp <sup>†</sup> .	6	$=S^2_{2,2}$	
$S_3 \times$ Flasks within Temp <sup>†</sup> .	6	$=S^2_{2,3}$	
$S_4 \times$ Flasks within Temp <sup>†</sup> .	6	$=S^2_{2,4}$	
$S_5 \times$ Flasks within Temp <sup>†</sup> .	6	$=S^2_{2,5}$	
Total (corrected for mean)	53		

The mean times needed by each of the different yeasts to reach the various fermentation stages are presented in Table 3. At 10°C yeast strain WE 500 fermented 125 g l<sup>-1</sup> sugar in the shortest time, followed by WE 452, WE 14 and WE 372. At 15°C and 20°C, the fermentation time needed to reach the sixth fermentation stage was similar for strains WE 452 and WE 500, somewhat longer for strain WE 14 and almost twice as long for strain WE 372. The mean times needed by yeast strain WE 372 to ferment 125 g l<sup>-1</sup> sugar were 2,28, 1,81 and 1,44 times longer at 10, 15°C and 20°C respectively than those needed by strain WE 500, therefore, strain WE 372 seems to be sensitive to lower fermentation temperatures.

The ergosterol content of the yeast cells decreased during fermentation for all strains (Fig. 1). The rate of decline of ergosterol per 100 mg cells from the first to the sixth fermentation stage differed significantly between the three temperatures for all strains. Yeast ergosterol appears to be an individ-

ual characteristic of *Sacch. cerevisiae* strains as indicated by the temperature x strain x stage interaction. Guidici & Gueroni (1982) also concluded that cell ergosterol content is a strain characteristic and proposed that cell ergosterol content of yeast can be used for yeast selection for vinification.

The ergosterol results in Fig. 1 were obtained with no oxygen in the synthetic medium other than that present at the start of fermentation. Andreasen & Stier (1953, 1954) showed that when *Sacch. cerevisiae* was grown anaerobically in Yeast Nitrogen Base (YNB) medium, a nutritional requirement for an unsaturated fatty acid and a sterol was induced. The addition of Tween 80, oleic, linoleic and linolenic acid to these anaerobic cultures allowed growth. These findings have been corroborated by other investigators (Alterthum & Rose, 1973; David & Kirsop, 1973; Hossack & Rose, 1976). However, Macy & Miller (1983) reported that growth does occur in YNB medium in the absence of ergosterol and oleic acid. Our

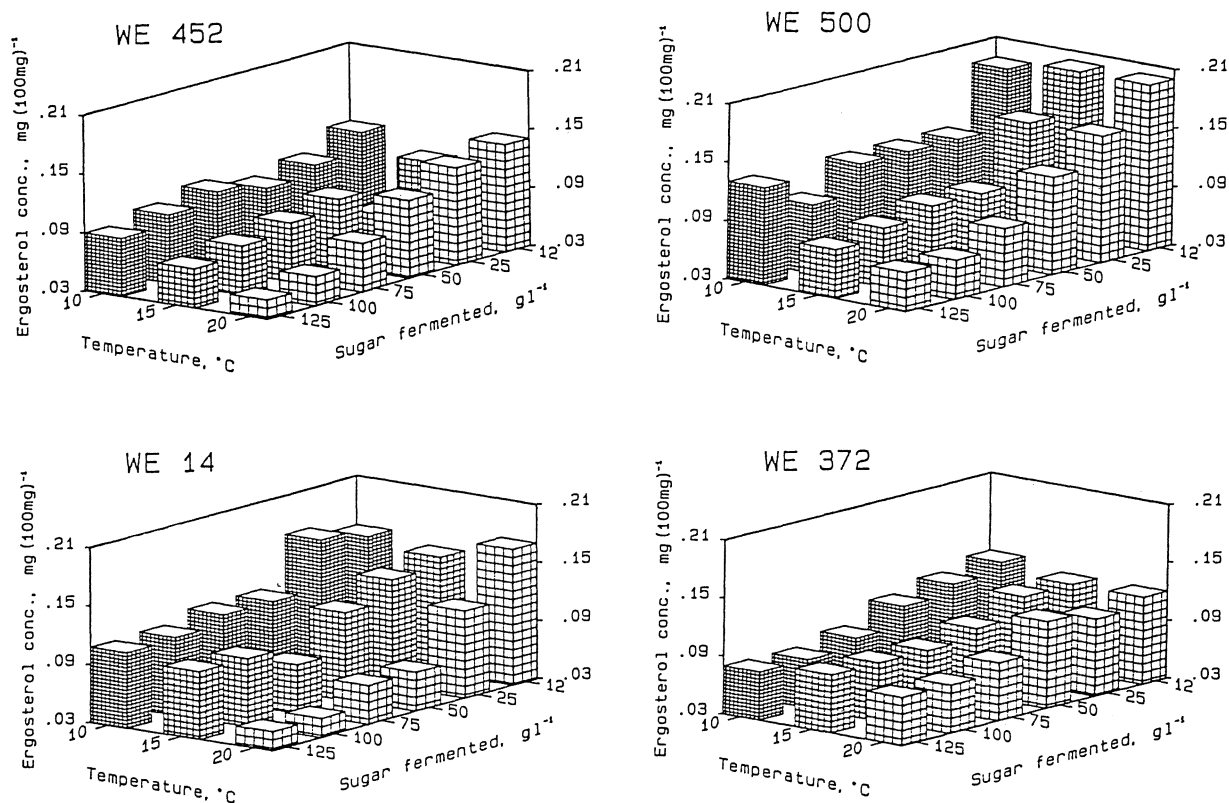


FIGURE 1

Ergosterol concentration in cells of *Saccharomyces cerevisiae* strains WE 452, WE 500, WE 14 and WE 372 during fermentation at three temperatures.

TABLE 3

The mean time needed for four yeast strains to reach specific fermentation stages at three temperatures.

Yeast strain	Temperature (°C)	Time(days)					
		S1 12 <sup>a</sup>	S2 25	S3 50	S4 75	S5 100	S6 125
WE 452	10	6,43	9,73	14,73	20,10	24,13	33,03
WE 500		3,30	6,27	11,80	16,03	21,77	27,90
WE 14		5,17	9,17	15,13	23,77	35,53	51,40
WE 372		9,03	15,77	26,67	41,70	58,10	63,75
WE 452	15	1,73	3,20	5,17	6,80	9,10	11,93
WE 500		1,93	3,30	5,17	6,80	9,10	11,67
WE 14		2,07	3,17	5,30	8,07	11,43	15,53
WE 372		3,07	5,27	7,50	10,40	14,87	21,07
WE 452	20	0,80	1,57	2,87	4,00	5,37	7,37
WE 500		1,00	1,83	3,10	4,27	5,60	7,17
WE 14		0,98	1,80	3,03	4,47	6,17	8,68
WE 372		1,33	2,37	3,87	5,30	7,50	10,33

S = fermentation stage

a = sugar fermented (g l<sup>-1</sup>)

results confirmed that *Sacch. cerevisiae* cells grow under non-aerated conditions in the absence of exogenous sterols and unsaturated fatty acids during the initial stages of fermentation. The yeast dry mass increased considerably over the first three stages, but little or no yeast growth was observed over the final three stages (Table 4).

However, the endogenous levels of these lipids were depleted under these conditions. The ergosterol content of the cells at the fourth stage was between 0,09% and 0,13% at 10°C, between 0,09% and 0,12% at 15°C and between 0,07% and 0,09% at 20°C for all four yeast strains (Fig. 1). Further growth of the yeasts in this study could well have been inhibited by a growth-limiting concentration of approximately 0,1% ergosterol (David & Kirsop, 1973; Haukeli & Lie, 1976; Aries & Kirsop, 1977) as this compound is not produced under anaerobic conditions, as well as by the ethanol in the medium. The lower ergosterol concentrations at the higher temperatures are ascribed to the higher growth rate of the yeast cells resulting in a more rapid dilution of the ergosterol. The decrease in ergosterol content of yeast strain WE 452 in the absence of growth during stages 5 and 6 is surprising and can probably be ascribed to cellular wastage of this sterol.

interaction could be simplified to stage x temperature (linear). In the case of WE 14 the former interaction had SL = 0,5588 while the latter had SL = 0,0014, while for WE 452 the corresponding values were 0,0542 and 0,0033 respectively. The data and the polynomial equation (that passing through the "treatment" means) were plotted three-dimensionally in Fig. 2. The different patterns indicated the strain x temperature x fermentation stage interactions. The fermentation rates of all four yeast strains at similar stages of fermentation decreased with decreasing temperature. The maximum fermentation rates were reached between the second and the fourth fermentation stages with yeast strains WE 452, WE 500 and WE 372 at all three temperatures, but from the first to the third stage with WE 14 (Fig. 2). Yeast strain WE14 was thus the fastest starter among the four strains.

Figure 3 shows that the specific fermentation rates (SFR) decreased as the fermentation proceeded. Higher fermentation temperatures resulted in higher SFR. The SFR were the highest for WE 14 at 15 and 20°C during the first two fermentation stages, followed by the SFR of WE 452, WE 500 and WE 372. The SFR of WE 14 decreased drastically as the fermentation proceeded. Strain WE 14 is possibly more sensi-

TABLE 4

Mean dry mass of yeast cells produced by four yeast strains at six fermentation stages at three temperatures.

Sugar fermented (gl <sup>-1</sup> )	Yeast dry mass (mg ml <sup>-1</sup> )											
	WE 452			WE 500			WE 14			WE 372		
	10°C	15°C	20°C	10°C	15°C	20°C	10°C	15°C	20°C	10°C	15°C	20°C
12	14	17	21	18	25	32	14	16	16	22	25	25
25	26	32	40	37	44	49	22	26	26	34	38	41
50	41	47	53	40	49	56	28	42	50	38	48	54
75	45	52	55	40	50	58	33	46	57	40	51	60
100	47	53	55	38	52	63	39	48	55	41	48	59
125	44	53	55	36	52	65	40	47	55	41	46	54

The analysis of ergosterol by yeast strain showed stage x temperature interaction in all cases except WE 372. Table 2 shows no significant differences between temperatures and a quadratic curve is sufficient to describe the reduction in ergosterol concentration during fermentation. The pattern of change for the other three strains is one of stage x temperature (quadratic) interaction, i.e. curved responses over temperature which differ depending on the stage involved (Fig. 1).

The analysis of fermentation rate by yeast strain showed similar stage x temperature (quadratic) interaction, although there was a general tendency suggesting that the nature of the

tive to ethanol than the other three strains. The ergosterol concentration of WE 500 at the first fermentation stage (Fig. 1) probably influenced the physiological state of the cell and permitted the cells to maintain higher SFR (although not significant compared to WE 452) at the sixth fermentation stage than the other three strains at all three temperatures (Table 5). These data for strain WE 500 supported the statement of Strydom *et al.* (1982), viz. that a high ergosterol concentration enables the cells to complete fermentation more rapidly. However, in contrast, no significant difference was found between the ergosterol concentrations of WE 372 and

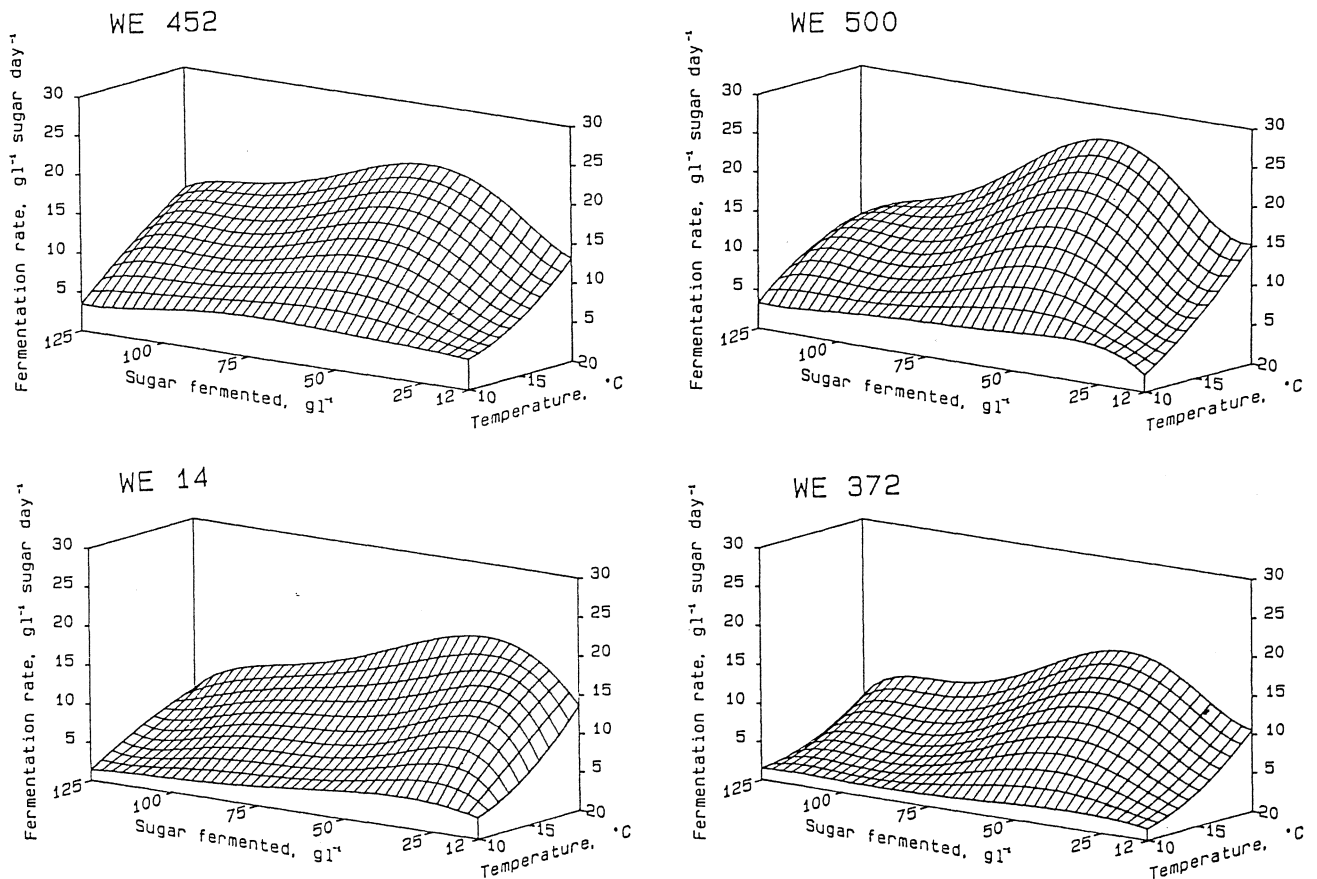


FIGURE 2

Rates of sugar fermentation by *Saccharomyces cerevisiae* strains WE 452, WE 500, WE 14 and WE 372 at different stages of fermentation at temperatures from 10 to 20°C.

WE 500 at 15°C and 20°C, but WE 500 had significantly higher SFR values than WE 372 at the sixth fermentation stage (Tables 5, 6 & Fig. 3). At 10°C, strain WE 500 contained the highest cell ergosterol concentration (Table 6) and maintained the highest SFR at the sixth fermentation stage (Table

5). Thus, these conflicting results showed that a high ergosterol concentration alone does not always ensure a high fermentation rate, resulting in a negative correlation ( $r = -0,57\%$ ) between the SFR and the ergosterol concentration during the sixth fermentation stage.

TABLE 5

Mean specific fermentation rate of four yeast strains at three temperatures during the sixth fermentation stage.

Temp. °C	Specific fermentation rate (gl <sup>-1</sup> sugar day <sup>-1</sup> (100 mg cells) <sup>-1</sup> )			
	WE 452	WE 500	WE 14	WE 372
10	7,49ab	9,52 bc	3,49a	3,65a
15	15,97def	18,34fg	11,51bcd	7,45ab
20	19,40fg	22,50g	14,96def	13,83cde

The letters a-g designate groups which are significantly different ( $P \leq 0,05$ )

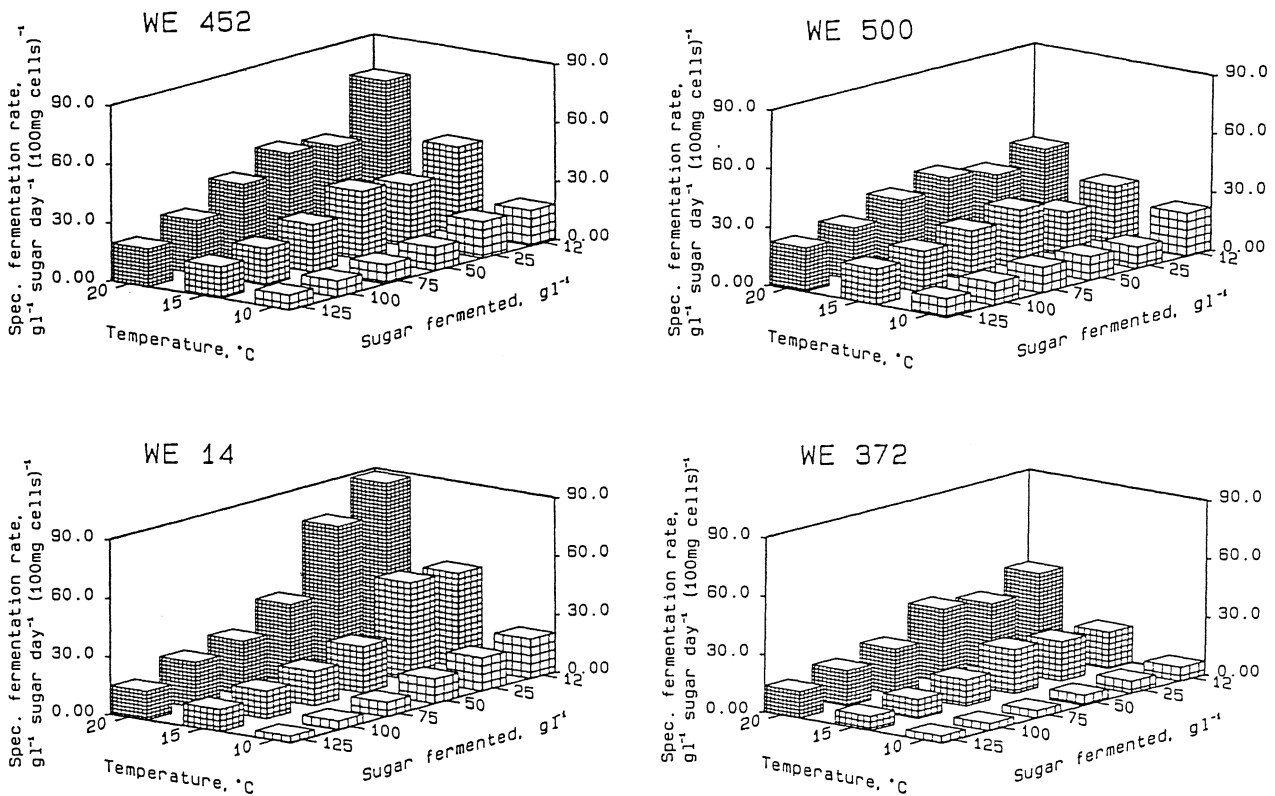


FIGURE 3

Specific fermentation rates of *Saccharomyces cerevisiae* strains WE 452, WE 500, WE 14 and WE 372 at three temperatures.

TABLE 6

Mean ergosterol concentration of four yeast strains at three temperatures during the sixth fermentation stage.

Temp. (10°C)	Ergosterol concentration (mg (100mg) <sup>-1</sup> )			
	WE 452	WE 500	WE 14	WE 372
10	0,09bc	0,13d	0,10c	0,08bc
15	0,07ab	0,08bc	0,10c	0,09bc
20	0,05a	0,07ab	0,05c	0,08bc

The letters a-d designate groups which are significantly different ( $P \leq 0,05$ )

### CONCLUSIONS

During anaerobic growth (fermentation) yeast cellular growth depended mainly on the yeast strain and the thermal conditions during fermentation. Lower ergosterol concentrations at the higher temperatures are ascribed to the higher growth rate of the yeast cells resulting in a more rapid dilution of the ergosterol. The fermentation rates, therefore, cannot be

directly related to ergosterol concentration, because of the temperature x strain x stage interaction. High ergosterol contents did not always result in high fermentation rates during the later stages of fermentation. It must be borne in mind that this experiment was done in a synthetic medium and the results must still be confirmed in a complex medium such as must.

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