Vitis 21, 5—14 (1982)

Istituto di Industrie Agrarie, Università di Bologna, Italia

Sterol content as a character for selecting yeast strains in enology

by

P. GIUDICI and M. ELISABETTA GUERZONI

La teneur en stérols comme un caractère de sélection de souches de levures en oenologie

Résumé. —Nous avons établi que l'accumulation de stérols dans les cellules est un caractère individuel et reproduisible des *Saccharomyces cerevisiae* dans des conditions égales. Cette accumulation est influencée par la concentration du glucose dans le milieu. Toutes les 18 souches ne subissent pas la répression catabolique de la synthèse de stérols due au glucose.

Il n'y a aucune corrélation entre la teneur en stérols en aération continuelle et dans des conditions de semianaérobiose (culture statique). Dans des conditions statiques nous avons trouvé une corrélation significative positive entre la teneur en stérols et la production d'éthanol. Par contre, dans des conditions d'érobiose, les teneurs en stérols présentent aux différentes concentrations de glucose des corrélations significatives négatives avec la production d'éthanol des souches.

Dans des conditions de développement statiques la teneur en stérols peut être utilisée comme un nouveau critère de sélection pour obtenir des souches de levures productrices de degrés élevés d'alcool.

Introduction

Saccharomyces cerevisiae is regarded as a facultative anaerobe; oxygen may be required for the growth of the organism. This requirement is suppressed if the growth medium is supplemented with ergosterol and is absent if aerobically grown cells are used as the inoculum (DAVID and KIRSOP 1973). The study of sterol biosynthesis showed that molecular oxygen is required for the oxidative cyclization of squalene to lanosterol (POPJÀK and CORNFORTH 1960). The only known exception to this behaviour is *Schizosaccharomyces japonicus* (BULDER 1971). *S. cerevisiae,* grown anaerobically in the absence of unsaturated fatty acids and sterols, diminished its endogenous levels of these lipids by dilution as yeast numbers and mass increased. When the levels of unsaturated fatty acids and sterols reached approximately one quarter of those found in aerobically grown cells, the organisms stopped dividing and there was a decline in the protein synthesizing activity of the cells and the mitochondrial oxidative phosphorylation became progressively uncoupled (ASTIN and HASLAM 1977). In the mitochondria this was due to a loss of high molecular weight RNA. In the cytoplasm the effect was at the level of the ribosomes, but it was not caused by a loss of cell integrity. When oxygen was supplied, protein synthesis in mitochondria and cytoplasm was rapidly reactivated, even in the absence of cell growth. This reactivation was accompanied by a rapid resynthesis of unsaturated fatty acids and ergosterol (GORDON and STEWART 1972).

The literature of the 25 years, following the first observations of ANDREASEN and STIER (1953, 1954) on sterol requirement under anaerobical conditions in *S. cerevisiae,* never mentioned that the requirement of sterols and unsaturated fatty acids would be specific (PROUDLOCK *et al.* 1968). WILSON and MCLEOD (1976) connected the loss of viability in anaerobically grown cells during starvation to an impaired membrane function

caused by a deficiency in sterols and unsaturated fatty acids. They also observed that, throughout the entire period of starvation, while all the other cell constituents diminished, sterol proportion increased during 72 h of starvation from 0.11 to 0.61 (on a dry weight base) for anaerobically grown cells. This clearly confirms their important role in maintaining cell viability. According to RADLER (1968), RIBÉREAU-GAYON *et al.* (1975), LAFON-LAFOURCADE *et al.* (1977), LARUE *et al.* (1979), and RIBÉREAU-GAYON (1979) the sterols can be considered as a survival factor for yeasts. In particular, RIBÉREAU-GAYON (1979) showed that the addition of exogen ergosterol, by increasing the rate of viability of anaerobical yeast cultures, permitted the extension of fermentative activity and consequently the increase of final alcohol yield in wine. Considering the positive results obtained from the addition of ergosterol, we have taken into account the possibility of selecting *S. cerevisiae* strains which are capable to accumulate increased amounts of endogenous sterols.

The frequent failure in the use of yeast strains, selected on the basis of such a complex phenotypical characteristic as alcohol resistance, motivated us to look for a physiological charactersitic, genetically determined and much less dependent on environmental variations. The alcohol tolerance is in fact the result of various unknown genotypical characteristics and its manifestation is subject to a too large number of variables.

Preliminary to this selection we had to clarify:

1. whether the sterol content is an individual characteristic in *S. cerevisiae;*
2. whether there is a correlation between cellular sterol content and alcohol production.

The variability of the sterol content of different yeast species has been taken into account, above all to clarify the relationship between lipid composition and taxonomy (KANEKO *et al.* 1976) but there is no information available on the variability between the different strains of a yeast species.

Material and methods

18 strains of *S. cerevisiae* from the collection of the Istituto di Industrie Agrarie and from the Istituto di Microbiologia e Industrie Agrarie (Università di Bologna) presenting different alcohol production have been studied.

The following synthetical standard medium was used: glucose 20 %; $(NH_4)_2SO_4$ 0.5 %; KH_2PO_4 0.2 %; $MgSO_4 \cdot 7\ H_2O$ 0.25 %; $CaCl_2$ 0.25 %; H_3BO_3 1 mg/l; $ZnSO_4$ 1 mg/l; $MnCl_2$ 1 mg/l; Tl_2SO_4 1 mg/l; $FeCl_3$ 0.5 mg/l; $CuSO_4$ 0.1 mg/l; KJ 0.1 mg/l. Vitamin content was: biotin 25 µg/l; thiamine 300 µg/l; Ca-pantothenate 300 µg/l; nicotinic acid 300 g/l; pyridoxine 300 µg/l; inositol 25 mg/l. When different amounts of glucose were added, the concentrations are reported in the results.

Yeast inoculation usually was done at a 2.5 % level and the cultures (500 ml) were incubated at 25 °C until the early steady state, determined on the basis of optical density at 420 nm.

Total sterol content in the freeze dried cells was determined by the reaction of Liebermann-Buchard with the following modifications:

1. Acetic anhydride-sulfuric acid reagent: 4 parts of acetic anhydride and 1 part of concentrated sulfuric acid were mixed slowly and constantly stirred in an ice-cooled flask just prior to use.
2. Ergosterol standard: 100 mg of ergosterol were dissolved in cloroform and adjusted to 100 ml at refrigerator temperature.

This stock solution was diluted 1:10 with chloroform at the same temperature as the standard. Both solutions were stored in the refrigerator.

Procedure: 100 mg of freeze-dried cells were saponified with 5 ml of alcoholic KOH at 40 % in a water-bath at 80 °C for 30 min; after cooling the unsaponifiable fraction was extracted with 4 ml of n-heptane: 3 ml of the solution were transferred into a tube and completely dried. Then 3 ml of chloroform and 2 ml of cold acetic anydride-sulfuric acid reagent were added to each sample and stirred. The colour reaction was left for 15 min, measured at 620 nm and compared with the standard over a range of 0.1 —0.5 mg of ergosterol.

Results and discussion

1. Sterol content as a strain character

It is known that the sterol composition of a yeast culture is not constant but may depend on environmental conditions (BAILEY and PARKS 1975, WILSON and MCLEOD 1976).

One might therefore question the validity of the conclusions drawn from this set of data. It should be noted, in connection with this, that in all cases the same medium composition was used and sampling took place at the same physiological stage whenever possible, i.e. in the early steady phase.

The experiment was repeated 10 times with 5 strains grown on standard medium and in a static culture.

The results given statistically in Table 1 demonstrate that sterol cell content is an individual and reproducible characteristic in S. cerevisiae strains.

2. Influence of sugar concentration

Considering the variability of the sugar concentrations in the musts, the influence of this parameter on the sterol content of the 18 strains grown under static and shaken conditions has been evaluated (Fig. 1a and b). The data are averages of three experiments.

Three types of behaviour can be recognized: 1. strains with a high sterol content in shaken and in static cultures (635, P 3); 2. strains with a relatively low sterol content in

Table 1

Variability of sterol content in 5 strains of *Saccharomyces cerevisiae* under standard conditions

Variabilité de la teneur en stérols en 5 souches de *Saccharomyces cerevisiae* cultivées en conditions standard

Strains	Means (mg sterol/100 mg dry cells)
692	0.188[a1])
9109	0.161[a]
635	0.127[b]
P 0	0.105[bc]
W 2	0.083[c]

[1]) The data were analysed by Duncan's statistical method; values not followed by the same letter differ significantly at P = 0.05.

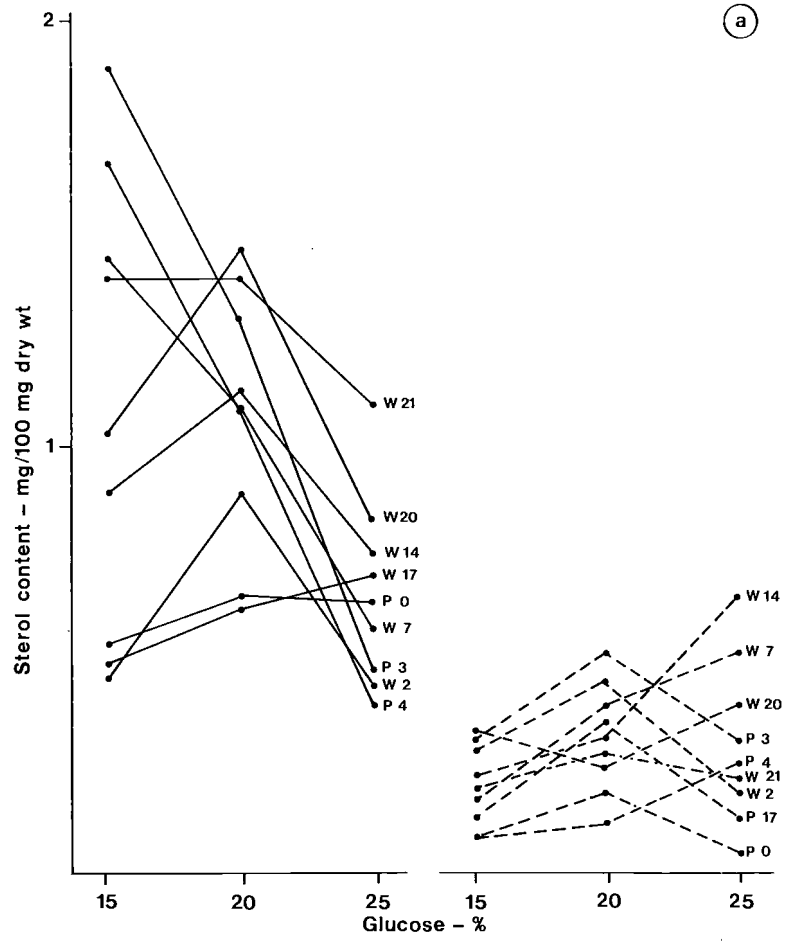
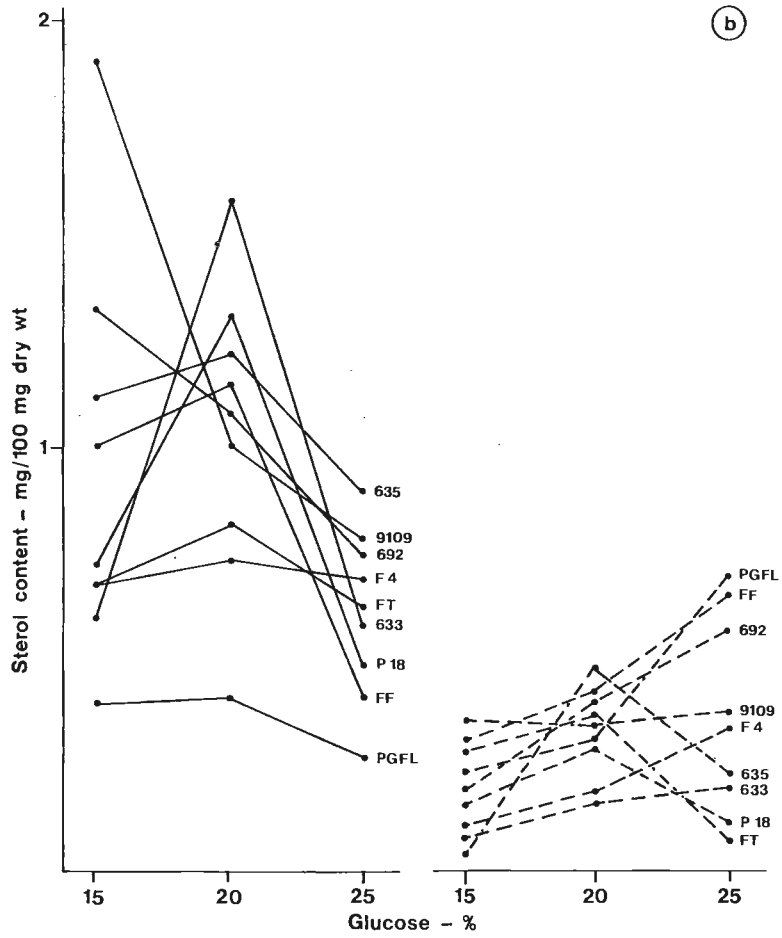


Fig. 1a and b: Influence of the glucose concentration on cell sterol content in various strains. Dashed lines = cells grown statically; solid lines = cells grown under shaken conditions.

Effet de la concentration en glucose sur les stérols des différentes souches. Lignes discontinues = cellules cultivées sans aération; lignes continues = cellules cultivées en aérobiose.

aerobiosis and a relatively high content in static cultures (W 2, PGFL, 692, FT); 3. strains with a relatively high sterol content in aerobiosis and relatively low content in static cultures (633, W 21, W 20, P 18).

As indicated in Fig. 1, there is a prevalent tendency in both the static and shaken cultures: Most of the strains show a maximum in total sterols when grown at 20 % of glucose. Some strains, as 692, 9109, P 3, P 4, W 7, reveal a maximum sterol production when grown at 15 % glucose content, while, in static culture, the total sterols of the strains W 14, W 7, W 20, P 4, FF, PGFL, 692, F 4 increase with the increase of sugar concentration. Data obtained with a glucose content higher than 25 % have not been taken



into consideration in order to avoid interference with the inhibitory effects, deriving from the concentrations themselves, on the biosynthetic activity.

Tests for a linear correlation between the sterol contents of the strains and the different glucose concentrations showed that, under static conditions, at all sugar concentrations, the coefficients were low and the correlations non-significant. Under shaken conditions, the total sterol content at the three different concentrations provided a greater significance, though there was only one case with a confidence level at 95 % between sterols at 20 % and sterols at 15 % ($r=0.441$, confidence level < 99, > 95).

On the other hand, there was no significant linear correlation between the sterol contents of the 18 strains under static and shaken conditions ($r=0.04$ for cells grown at 20 % glucose and $r=0.240$ at 25 % glucose). This means that a strain can be a relatively high sterol producer in aerobiosis but relatively low in static cultures and viceversa.

QUINE and HASLAM (1979) established that sterol synthesis was also subject to catabolite repression even by 5 % glucose concentration and was derepressed as this was transformed into alcohol. On the basis of our data (Fig. 1a and 1b), the catabolite

repression was only operative in shaken cultures and only in some strains: 692, 9109, P 3, P 4, W 7. Most of the strains reveal a maximum sterol total with a high concentration of glucose at 20 %, both in static and shaken cultures, and are not subject to catabolite repression.

As stated above, the sterols provide survival factors, prolonging life time and the maintenance of cellular metabolic activity after reaching the stationary phase. The sensibility to the catabolite repression of the sterol synthesis, for the yeasts which it undergo, should diminish survival in the late steady phase and the fermentative efficiency in the very sugary musts. Owing to the interesting technological implications of this regulatory system, it should be clarified whether the yeasts which do not undergo catabolite repression even with 25 % glucose content in static cultures are those which best present high alcoholic percentage at a high sugar concentration. However, based on the data shown in Fig. 1 and Table 2 it does not seem that there is a relation between susceptibility to catabolite repression and alcohol production at high sugar concentration in these 18 strains.

3. Survival and alcohol yield

With the hypothesis that the extension of the viability and metabolic activity of the cells is a precondition for obtaining a high alcohol yield, two factors have been taken

Table 2

Influence of medium glucose concentration on mass and ethanol production

Effet de la concentration en glucose sur la production cellulaire et sur le rendement alcoolique

Strains	Glucose in growth medium (%)				
	15	20	25	20	25
	Cells statically produced (mg dry wt/100 ml medium)			Ethanol produced (% v/v)	
W 7	72	90	100	11.1	14.6
W 2	100	100	80	10.5	14.3
W 14	80	100	100	8.3	12.9
W 20	80	90	90	8.3	11.5
W 21	70	80	90	8.6	12.0
P 0	70	70	60	9.3	13.5
P 3	55	70	80	9.7	13.3
P 4	70	70	60	9.4	12.7
P 17	100	100	100	11.0	13.3
P 18	74	60	60	9.1	10.8
692	55	50	70	11.3	14.5
9109	60	50	80	9.6	12.8
633	70	60	60	10.0	9.8
635	77	80	70	8.0	11.9
FF	73	100	60	10.5	12.8
FT	75	70	80	11.3	13.2
F 4	50	60	60	11.0	10.9
PGFL	40	30	50	11.4	13.5

into account which could play an important role in survival in the later steady phase of must fermentations: 1. the capacity of the strain to produce cellular mass in static semiaerobic cultures: 2. its capacity to produce sterols in shaken cultures. These two variables were compared to the ethanol yield produced under the same conditions.

The data about the cellular mass production of single strains in relation to sugar concentration are shown in Table 2; two patterns of hebaviour can be seen: strains in which the mass production increases with the increases of sugar concentration (W 7, W 14, W 21, P 3, 692, 9109, PFGL), and strains in which the cellular yield diminishes with the increase of sugar concentration (from 20 to 25 %).

It could be observed, without yet putting forward any hypothetical explanation, that the first group coincides with the yeasts which are not susceptible to glucose catabolic repression, as far as sterol synthesis is concerned. A significant positive linear correlation has been found between cellular mass produced in static condition at 20 % in glucose and ethanol produced under the same conditions ($r = 0.483$); confidence level < 99, > 95.

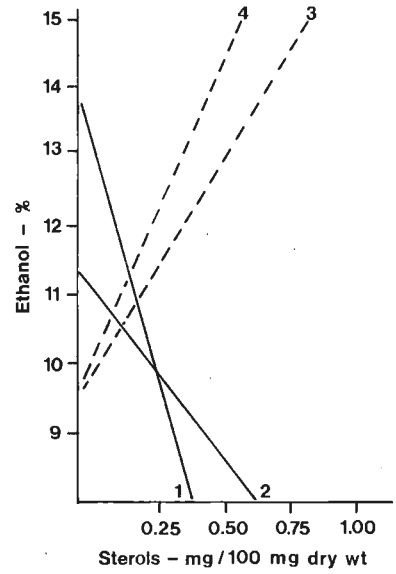


Fig. 2: Regression lines between the sterol content in the cells of 18 strains and the relative final alcoholic degree under different culture conditions. Dashed lines = cells grown statically; solid lines = cells grown under shaken conditions. 1: 25 % sugar, $r = 0.598$, confidence level > 95 %, < 99 %; 2: 20 % sugar, $r = 0.223$, confidence level < 95 %; 3: 20 % sugar, $r = 0.100$, confidence level < 95 %; 4: 25 % sugar, $r = 0.493$, confidence level > 95 %, < 99 %.

Lignes de régression entre la teneur en stérols des cellules de 18 souches et le degré alcoolique final dans des diverses conditions de culture. Lignes discontinues = cellules cultivées en conditions statiques; lignes continues = cellules cultivées en culture aérée. 1: 25 % en glucose, $r = 0{,}625$, niveau de confiance > 95, < 99 %; 2: 20 % en glucose, $r = 0{,}223$, niveau de confiance < 95 %; 3: 20 % en glucose, $r = 0{,}100$, niveau de confiance < 95 %; 4: 25 % en glucose, $r = 0{,}493$, niveau de confiance > 95 %, < 99 %.

Even if it is plausible that a greater alcohol yield can be obtained whenever the strain is able to produce a consistent cellular mass at the beginning of the process, nevertheless there are strains, like PGFL, 692, FT, in which the high yield cannot be a consequence of the increased cellular production.

No significant linear correlation has been found between the capacity of the 18 strains to produce cellular mass and sterol content either in shaken or static cultures.

In Fig. 2, the regression lines between the sterol content in the cells of the 18 strains and the final alcoholic degree under different culture conditions are shown. The average alcoholic degree obtained in three fermentations of the same must with different amounts of glucose added (20 and 25 %) is considered. In static cultures, a positive linear correlation between the sterol content as the independent and ethanol as the dependent variable was observed, with a good confidence level at higher sugar concentration. A negative linear correlation, with a good confidence level at higher sugar concentration, was obtained in shaken cultures.

From these data, which are in line with the lack of correlation between sterol content in static cultures and shaken cultures, it can be seen that the alcoholic yield of a strain is linked to the sterols that can be accumulated in static culture independent of the amount accumulated in shaken cultures.

In fact, the strains which present large amounts of these lipidic constituents in shaken cultures are those with the least alcoholic yield. This negative correlation agrees with the results of LARUE *et al.* (1979) who have shown that ergosterol added to aerobically grown cells exerts an inhibitory effect.

The only hypothesis reasonably explaining the present results is that there is an accumulation, in static but not in shaken cultures, in the high alcohol producing strains of one or more specific sterols. These, even in small quantities, are capable to initiate a resistance to high alcohol concentration. The explanation coincides with the hypothesis of THOMAS and ROSE (1979) who established that the extraordinary tolerance to alcohol conferred by the presence of doubly unsaturated fatty acid residues was greater when the plasma membrane was enriched with a sterol containing an unsaturated side chain at C_{17} (ergosterol, stigmasterol) than with a saturated side chain (campesterol, cholesterol).

In order to prove this hypothesis, further studies are in progress to identify the individual free and esterified sterols present in the cytoplasmatic membrane of various strains and mutants affected in the sterol biosynthesis. Furthermore, a comparison of qualitative sterol composition of *S. cerevisiae* with those of a high fermenting yeast as *S. bayanus* and low fermenting yeasts can supply useful suggestions.

Conclusions

The presented data allowed to draw some conclusions:

1. The cellular content in sterol is an individual character in *S. cerevisiae* strains under the same culture conditions and at the same physiological age. Only some strains undergo catabolite repression by glucose in sterol biosynthesis. This different behaviour can determine the loss of correlation found between the sterol contents of the 18 strains studied when grown at different sugar concentrations.
2. There is no significant linear correlation between the sterol content in cells grown in static and in shaken cultures.
3. The alcohol yields in the strains at all sugar concentrations have shown a significant positive correlation only with the sterol contents of the cells grown statically;

on the contrary, in shaken cells at all sugar concentrations highly significant negative correlations were found. Thus, the sterol accumulation in static cultures may be considered a promising selection criterion to obtain strains producing high alcoholic degrees.

Summary

It could be established that the total sterol content is an individual and reproducible characteristic in *Saccharomyces cerevisiae* under the same cultural and physiological conditions. Its accumulation in the cells is affected by glucose concentration to different extents in the 18 strains studied. Only some strains undergo glucose catabolite repression in sterol biosynthesis.

There was no linear correlation between sterol content in the cells of the 18 strains grown under static or shaken conditions. Under statical conditions significant positive correlations were found between sterol contents and ethanol average production in the 18 strains. On the contrary, in aerobically grown cells significant but negative correlations were found between sterol contents and alcohol production.

A new yeast selection characteristic for vinification is proposed.

References

ANDREASEN, A. A. and STIER, T. J. B., 1953: Anaerobic nutrition of *Saccharomyces cerevisiae:* ergosterol requirement for growth in a defined medium. J. Cell. Comp. Physiol. **41,** 23-36.

ASTIN, A. M. and HASLAM, J. M., 1977: The effect of alterated membrane sterol composition on oxidative phosphorylation in a haem mutant of *Saccharomyces cerevisiae.* Biochem. J. **166,** 278-298.

BAILEY, R. B. and PARKS, L. W., 1975: Yeast sterol esters and their relationship to the growth of yeast. J. Bacteriol. **124,** 606-612.

BULDER, C. J. E. A., 1971: Anaerobic growth, ergosterol content and sensitivity to a polyene antibiotic of the yeast *Schizosaccharomyces japonicus.* Antoine van Leeuwenhoek **37,** 353-358

DAVID, M. H. and KIRSOP, B. H., 1973: Yeast growth in relation to the dissolved oxygen and sterol content of worth. J. Inst. Brew. **79,** 20-25.

GORDON, P. O. and STEWART, P. R., 1972: Effect of lipid status on cytoplasmic and mitochondrial protein synthesis in anaerobic cultures. J. Gen. Microbiol. **72,** 231-242.

KANEKO, H., HOSOHARA, N., TANAKA, M. and ITOH, H., 1976: Lipid composition of 30 species of yeast. Lipids **11,** 837-844.

LAFON-LAFOURCADE, S., LARUE, F., BRECHOT, P. et RIBÉREAU-GAYON, P., 1977: Les stéroides facteurs de survie des levures au cours de la fermentation alcoolique du moût de raisin. C. R. Acad. Sci. (Paris), Sér. D, **284,** 1939-1942.

LARUE, F., LAFON-LAFOURCADE, S. et RIBÉREAU-GAYON, P., 1979: Les différents rôles fonctionelles des stéroides sur le levures dans le moût de raisin en fermentation: notion de facteur de survie. Ann. Microbiol. (Inst. Pasteur), Sér. A, **130,** 231-243.

POPJAK, G. and CORNFORTH, J. W., 1960: Biosynthesis of cholesterols, Adv. Enzymol. **22,** 281-335.

PROUDLOCK, J. W., WHEELDON, L. W., JOLLOW, D. J. and LINNANE, A. W., 1968: Role of sterols in *Saccharomyces cerevisiae.* Biochim. Biophys. Acta **152,** 434-437.

QUINE, D. E. and HASLAM, J. M., 1979: The effects of catabolite depression on the accumulation of steryl esters and the activity of β-hydroxymethylglutaryl-CoA reductase in *Saccharomyces cerevisiae.* J. Gen. Microbiol. **111,** 343-351.

RADLER, F., 1978: Les activateurs du développement anaérobie de la levure. Ann. Technol. Agric. **27,** 203-213.

RIBÉREAU-GAYON, F. J., PEYNAUD, E., RIBÉREAU-GAYON, P. et SUDRAUD, E., 1975: Sciences et techniques du vin, Vol. 2. Dunod, Paris.

RIBÉREAU-GAYON, P., 1979: Les facteurs de survie de la levure au vinification. Proc. Cong. Intern. Microbiol. et Ind. Aliment., Octobre 1979, Paris.

THOMAS, D. S. and ROSE, A. M., 1979: Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. Arch. Microbiol. **122**, 49-55.

WILSON, K. and MCLEOD, B. J., 1976: The influence of conditions of growth on the endogenous metabolism of *Saccharomyces cerevisiae:* effect on viability. Antoine van Leeuwenhoek **42**, 397-410.

Eingegangen am 14. 9. 1981

Prof. M. ELISABETTA GUERZONI
Istituto Industrie Agrarie
Università di Bologna
V. S. Giacomo 7
40126 Bologna
Italia