13
Inhibition of Alcoholic Fermentation

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Introduction

Alcoholic fermentation is a characteristic of yeast cells, especially of *Saccharomyces*. However, some bacteria, particularly *Zymomonas mobilis*, can also produce ethanol from glucose (Rogers *et al.*, 1982).

The different enzymatic steps of glycolysis are well known. The regulation of respiratory and of fermentative metabolism (Crabtree and Pasteur effects) of yeasts have been extensively studied, especially for *Saccharomyces* (Nord and Weiss, 1958; Sols, Gancedo and de la Fuentes, 1971; Lagunas, 1981), *Brettanomyces* (Scheffers and Nanninga, 1977); *Kluyveromyces* (Chassang-Douillet, 1973; Moulin and Galzy, 1978; Royt and MacQuillan, 1979), and other species (Pons, Guiraud and Galzy, 1975; Moulin *et al.*, 1976). However, notwithstanding its industrial importance, the inhibition of alcoholic fermentation has been much less studied.

Proof of inhibition

It is generally recognized that ethanol has three inhibitory effects: inhibition of cell multiplication (*Figure 1*); inhibition of fermentation (*Figure 2*), and a lethal effect on cells.

Holzberg, Finn and Steinkraus (1967) observed that a *Saccharomyces cerevisiae* Hansen strain stopped growing on a grape-juice medium when the ethanol content reached 68·5 g/ℓ, whereas the final ethanol concentration rose to 108 g/ℓ. Similarly, Rose (1980) showed that for five different *Saccharomyces cerevisiae* strains, cell growth was stopped when ethanol content of the medium reached 55 g/ℓ to 103 g/ℓ depending on the strain. Benitez *et al.* (1983) found that of 632 strains isolated from wine, 35 were able to grow at up to 118 g/ℓ ethanol. Several authors have shown that the growth rate of yeast cells decreases with higher ethanol concentrations (*Table 1*). Cell death rate is more difficult to assess, because it is low under experimental conditions where the yeast cells remain in a good physiological state.

Biotechnology and Genetic Engineering Reviews — Vol. 2, October 1984
0264-8725/84/02/365-18$10.00 + $0.00

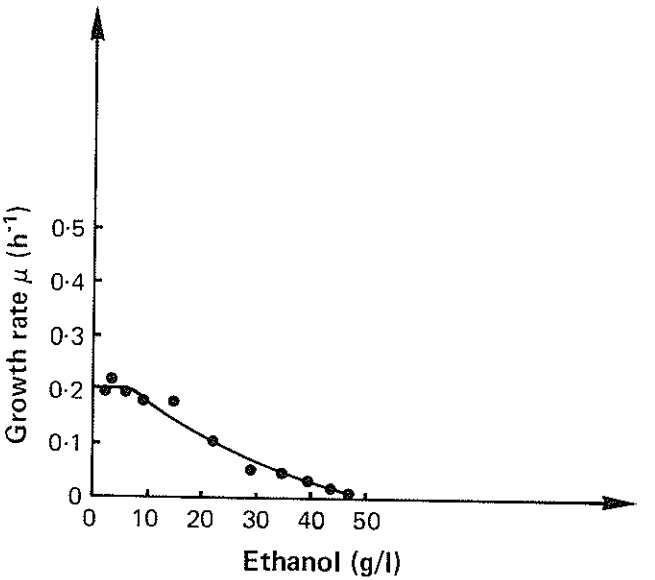

Figure 1. Growth rate μ (h^{-1}) of *Candida pseudotropicalis* under strict anaerobic conditions versus ethanol. Carbon substrate: lactose 150 g/ℓ.

In the case of a rapid fermentation in the presence of a high cell population (8×10^8 cells per ml), the cell viability is closely linked with fermentation conditions. Cell mortality is higher with a faster fermentation (Nagodawithana, Castellano and Steinkraus, 1974; Nagodawithana and Steinkraus, 1976).

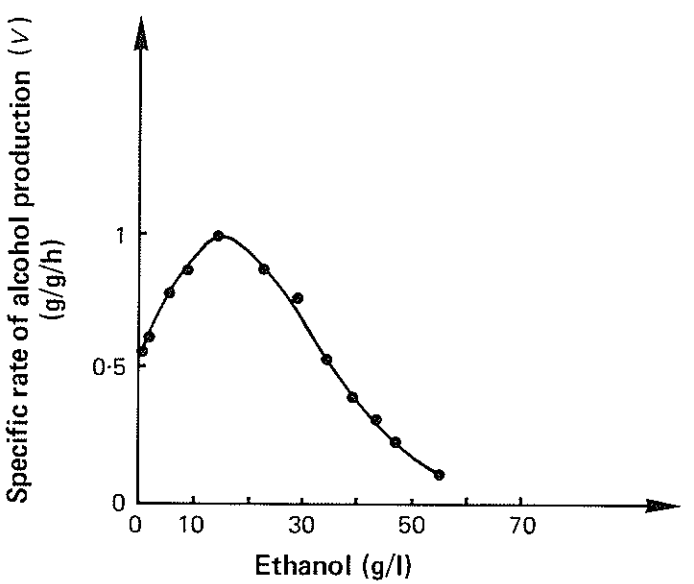

Figure 2. Specific rate of alcohol production V (g/g/h). *Candida pseudotropicalis* under strict anaerobic conditions, versus ethanol. Carbon substrate: lactose 150 g/ℓ.

Table 1. Effect of ethanol concentration (P) on specific growth rate (μ) of some yeasts in batch culture

(A) *Saccharomyces cerevisiae* NRRL–Y–132		(B) *Saccharomyces cerevisiae* ATCC 4126		(C) *Saccharomyces cerevisiae* NCYC 479	
P_0(g/ℓ)	μ(h^{-1})	P_0(g/ℓ)	μ(h^{-1})	P_0(g/ℓ)	μ(h^{-1})
0	0·4	0	0·44	0	0·280
24	0·264	50	0·36	20	0·251
50·4	0·17	60	0·36	40	0·200
66·0	0·091	80	0·28	60	0·139
80·2	0·043	100	no growth	80	0·018
90	no growth			100	0·024

(A) from Ghose and Tyagi, 1979a; (B) from Bazua and Wilke (1977);
(C) from Brown *et al.*, 1981

Lafon-Lafourcade *et al.* (1977) found that in the case of grape musts, cell viability decreased during the whole fermentation process. In addition, according to Geneix, Lafon-Lafourcade and Ribereau-Gayon (1983), ethanol is not the only inhibitor of alcoholic fermentation: fatty acids (C_6, C_8 and C_{10}) produced by yeasts in grape musts are chiefly responsible for the mortality of the yeast populations.

Methodology

The oldest methodology used for the study of alcoholic fermentation was based on batch cultures. During the fermentation process, it was possible to monitor the evolution of biomass, of ethanol concentration and of substrate content. Under these experimental conditions, it was possible to determine the maximum concentration of ethanol produced by a yeast strain from a well-defined medium. It was also possible to determine the ethanol concentration which would stop cell growth.

The inhibition of fermentation without involving cell growth could also be studied by adding different concentrations of ethanol. This method usually depended on measurement, with the Warburg apparatus, of the amounts of CO_2 evolved under a nitrogen atmosphere per hour and per mg dry matter ($Q^N_{CO_2}$). This technique has been used by different authors (Suto, Tagaki and Uemura, 1951; Nagatani, Shoda and Aiba, 1968), and was used to study the effects of concentration and nature of the carbon substrate (Moulin, Boze and Galzy, 1980). Brown *et al.* (1981) monitored the production of CO_2 with a Gilson respirometer.

The continuous fermentation technique was used for the study of cell growth, and fermentation in the presence of different concentrations of added ethanol under steady-state conditions.

Aiba, Shoda and Nagatani (1968) proposed equations (1) and (2) to describe inhibition of cell growth and fermentation rate by ethanol. In 1977, Bazua and Wilke proposed equations (3) and (4). Equations (5) and (6) were put forward by Ghose and Tyagi (1979a):

$$\mu = \mu_0 e^{-K_1 P} \qquad \text{Eq. (1)}$$

$$V = V_0 e^{-K_2 P} \qquad \text{Eq. (2)}$$

$$\mu_{max} - \mu_0 \left(1 + \frac{\bar{P}}{P_{max}}\right) \tfrac{1}{2} \qquad \text{Eq. (3)}$$

$$V_{max} = V_0 \left(1 + \frac{\bar{P}}{P_{max}}\right) \tfrac{1}{2} \qquad \text{Eq. (4)}$$

$$\mu = \mu_{max} \left(1 - \frac{P}{P_m}\right) \qquad \text{Eq. (5)}$$

$$V = V_{max} \left(1 - \frac{P}{P'_m}\right) \qquad \text{Eq. (6)}$$

In these equations

μ = growth rate (h^{-1}) = $dX/dt \times 1/x$, where X is the concentration of cells;
V = specific rate of ethanol formation (g/g/h) = $dP/dt \times 1/X$, where dP is the variation of production of ethanol, and X is the concentration of cells;
μ_0 = maximum specific growth rate at $P = 0$;
V_0 = maximum specific ethanol production rate at $P = 0$;
P = ethanol concentration (g/ℓ)
P_{max} = maximum value of P at which cells still viable;
$\bar{P}$ = average value of P in the continuous runs;
P_m = ethanol concentration at which there is no cell multiplication;
P'_m = ethanol concentration above which there is no fermentation.

In all three models the authors assume that the inhibition of cell growth and of fermentation by ethanol is of a non-competitive type.

The inhibitory action of ethanol added to the medium could be studied with these models. *Table 2* shows the ethanol concentrations at which the rate of growth and of the specific rate of ethanol production equal half of their maximum values. These results clearly show that the growth rate was inhibited by lower ethanol concentrations than the specific rate of ethanol production. Similarly, the ethanol concentration at which there was no more cell growth was always lower than those where fermentation was completely stopped. The values given in *Table 2* are either experimental results or are estimated according to the proposed equations. The model proposed by Aiba, Shoda and Nagatani (1968) cannot be used for the calculation of the alcohol concentration where fermentation was stopped, because it assumed that fermentation would go on to infinity.

The above models were studied by adding ethanol to the medium. However, during the alcoholic fermentation process, ethanol concentrations within the cell and in the medium are not identical (Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978; Thomas and Rose, 1979; Beaven, Charpentier and

Table 2. Ethanol concentrations for which different parameters were modified

Reference	Ethanol concentration A	B	C	D
Aiba *et al.*, 1968	24	46	76	—
Basua *et al.*, 1977	40	41	93	94
Ghose *et al.*, 1979a	43	57	87	214

(A) Ethanol concentration (g/ℓ) where $\mu = \mu_{max}/2$
(B) Ethanol concentration (g/ℓ) where $V = V_{max}/2$
(C) Ethanol concentration (g/ℓ) where cell growth was completely stopped
(D) Ethanol concentration (g/ℓ) where fermentation was completely stopped

Rose, 1982; Dasari *et al.*, 1983). Taking this into account, different authors studied the inhibitory action of ethanol produced in the fermenter during continuous-culture conditions by the yeast cell itself. Under these conditions, the ethanol concentration at which growth rate was reduced by half was respectively found by Cysewski and Wilke (1976), and Hoppe and Hansford (1982) to be 5 g/ℓ and 5·2 g/ℓ. These values are significantly lower than those obtained when ethanol was added to the medium (*Table 2*(B)). Novack *et al.* (1981) also showed that for the same yeast strain, this value was 3·8 g/ℓ for produced ethanol and 105·2g/ℓ for added ethanol. These results show the limits of the methodology used where ethanol was added to the medium.

Effect of different factors on the sensitivity of yeast to ethanol

EFFECT OF THE SUBSTRATE

The carbon substrate could have a inhibitory action on the alcoholic fermentation. Strehaiano, Moreno and Goma (1978) observed, during anaerobic culture of *Saccharomyces cerevisiae* UG 5, a strong inhibitory effect of glucose on cell growth. Ghose and Tyagi (1979b) attributed the inhibitory effect of sugars from hydrolysed bagasse to the presence of unfermented xylose, cellobiose, cellotriose and cellotetraose. During a study with the Warburg apparatus, Moulin, Boze and Galzy (1980) also observed inhibition of the fermentation process by the substrates. Fermentation of glucose by the *Candida pseudotropicalis* strain was inhibited by sugar concentrations above 100 g/ℓ. Under the same conditions, lactose was less inhibitory than glucose. In both cases, there was a synergistic action between ethanol and the carbon substrate. However, the inhibitory action of the substrates appeared only at high concentrations and remained weak compared with the action of ethanol.

EFFECT OF TEMPERATURE

It is a well-known fact that the traditional fermentation process can be brought to a complete halt by an excessive increase in temperature. The influence of temperature on yeast physiology has been reviewed (Stokes, 1971). The optimal temperature for the specific rate of fermentation is 5 – 10°C higher than

that for cell growth (Gray, 1941; Stokes, 1971; Brown and Oliver, 1982a; Lee, Williamson and Rogers, 1980). The optimal fermentation temperature also depends on the ethanol content of the medium. Gray (1941) showed that for *Saccharomyces cerevisiae* the optimum temperature fell from 38°C to 32°C when the ethanol content increased from 0 to 46 g/ℓ. Similarly, Aiba, Shoda and Nagatani (1968) defined a law for the action of temperature on the rate of fermentation. Navarro and Durand (1978) observed that *Saccharomyces uvarum* stopped growing at lower ethanol concentrations as the temperature of the medium increased.

During their work on 'rapid fermentation', Nagodawithana, Castellano and Steinkraus (1974) also studied the effect of temperature. These authors observed under very special conditions that, to reach 95 g/ℓ alcohol, the fermentation periods were 3, 4, 5 and 6 hours when the medium was at a temperature of, respectively, 30°C, 25°C, 20°C and 15°C; the cell viability increased, in parallel, from 13% at 30°C to 48%, 80% and 94% at the lower temperatures. Thus, in this case, an increase of the rate of fermentation was observed with an increase in temperature; a parallel decrease in cell viability was also observed. The greater sensitivity of yeast cells to ethanol when the temperature increased could be linked with the rapid accumulation of intracellular ethanol. These experiments were performed with 83×10^8 cells/ml which constitute an exceptionally high population.

Van Uden, Abranches and Cabeca-Silva (1968), Van Uden and Madeira-Lopez (1970), Van Uden and da Cruz-Duarte (1981) studied the effect of temperature on cell growth and cell mortality. These authors defined an optimum growth temperature (T_{op}), a final maximum temperature for growth (T_{maxf}), and an initial maximum temperature for growth (T_{maxi}). According to Van Uden and da Cruz-Duarte (1981), as long as the ethanol concentration in the medium remained very low, the cell population retained its normal fundamental temperatures (T_{op}, T_{maxf} and T_{maxi}). As more ethanol was produced these values decreased and were found to be 37°C and 25°C for T_{op}; 40°C and 33°C for T_{maxf}; 44°C and 36°C for T_{maxi} respectively in the absence of ethanol and in the presence of 60 g/ℓ ethanol added to the medium, for a respiratory-deficient strain of *Saccharomyces cerevisiae*. Identical results were obtained with *Saccharomyces cerevisiae* IGC 4072 (Loureiro and Van Uden, 1982) and with *Kluyveromyces fragilis* IGC 2671 (Sa-Correira and Van Uden, 1982). Ethanol content reached a critical value when T_{maxf} equalled the 'process temperature', as cell growth becomes nil. It appeared that the optimum fermentation temperature was always higher than the optimum growth temperature; the optimum 'process temperature' must take into account these two values. However, these values decreased with higher ethanol concentrations. The optimization of a fermentation process with regard to temperature would require the reduction of process temperature as more ethanol was produced.

ROLE OF LIPIDS IN THE MEDIUM

The addition of ergosterol and of oleic acid to the culture medium was required for the growth of *Saccharomyces cerevisiae* under anaerobic conditions

(Andreasen and Stier, 1953, 1954). These results showed that molecular oxygen was necessary for one or several steps in the biosynthesis of sterol and of unsaturated fatty acids. Studies of the sterol biosynthesis pathway of yeasts indicated that oxygen was also required for the appearance of the secondary alcohol function of sterol (Popjack and Cornforth, 1960; Kirsop, 1974; Rattray, Schibeci and Kidby, 1975; Henry, 1982). Under anaerobic conditions, these two phenomena did not occur. The sterol and unsaturated fatty acid contents of cells grown under anaerobic conditions were also relatively low compared with those of cells grown under aerobic conditions (Gordon and Stewart, 1972; Alterthum and Rose, 1973; David and Kirsop, 1973; Rogers and Stewart, 1973; Hossack and Rose, 1976; Kirsop, 1977; Watson and Rose, 1980). The role of oxygen was thus especially important for alcoholic fermentation. These results explain the importance of sterols and unsaturated fatty acids of the fermentation medium regarding resistance to ethanol.

Many authors have shown that lipids have an important role in ethanol resistance of yeast cells. Gray (1948) and Troyer (1953) observed that the strains most resistant to ethanol were also those richest in lipids. Kawaharada, Hayashida and Hongo (1970) showed that the *Saccharomyces sake* Kyokai 7 strain produced 158 – 182 g/ℓ ethanol at 20°C in sake mash containing koji mould mycelia (*Aspergillus oryzae*). A proteolipid extract of this *Aspergillus oryzae* provided similar results on a synthetic medium (Hayashida, Feng and Hongo, 1974). This complex acted in anaerobic culture by increasing the growth rate, the fermentative activity and the 'alcohol endurability' of yeasts (Hayashida *et al.*, 1976). The spheroplasts of cells grown under anaerobic conditions in the presence of this proteolipid complex remained stable in a solution containing 158 g/ℓ ethanol, whereas spheroplasts of cells grown in the absence of this complex burst in this alcohol solution (Hayashida and Ohta, 1978); thus the membrane of the former spheroplasts had become 'alcohol endurable', i.e. resistant to alcohol. Electron-microscopic study of the fine structure of the cells revealed lipid deposits with characteristic lamellar structures on the peripheral part of the cytoplasm of cells grown under anaerobic conditions in the presence of the complex. This complex contains 26·7% proteins and 58·5% phospholipids (78·3% phosphatidylcholine, 18·1% sphingolipids and 3·2% lysophosphatidylcholine). The fatty acids comprised 77·5% of unsaturated fatty acids, with linoleic acid being the main component. The addition of phosphatidylcholine to a synthetic medium increased cell growth and the fermentative activity of *Saccharomyces sake* Kyokai 7, while the addition of ergosteryl-oleate increased resistance to ethanol. This was determined by measurement of the fermentation process with yeast cells previously treated for 48 hours with 158 g/ℓ ethanol at 15°C. The addition of these two compounds increased the three parameters cited above. These two compounds have a complementary effect on the physiological activity of yeast cells grown under anaerobic conditions (Hayashida and Ohta, 1980).

Some sterols which are described as growth factors for yeasts under strict anaerobic conditions (Andreasen and Stier, 1953) may act under various conditions according to different mechanisms (Lafon-Lafourcade *et al.*, 1977; Lafon-Lafourcade, Larue and Ribereau-Gayon, 1979). Larue, Lafon-Lafourcade and Ribereau-Gayon (1978, 1980) proposed three functional roles

for sterols in yeasts depending on the fermentation conditions: (1) an inhibitory role was demonstrated under permanent moderate aerobic conditions—the supplementary addition of sterol modified the membrane structure and decreased the cell permeability; (2) a growth-activator role under anaerobic culture conditions; (3) a survival-factor role, in the case of fermentation of grape musts with high sugar contents.

Thomas and Rose (1979) showed that under anaerobic conditions the growth rate of *Saccharomyces cerevisiae* NCYC 366 was less reduced in the presence of ethanol when the growth medium contained linoleic acid instead of oleic acid. In addition the cells which grew in the presence of linoleic acid contained less ethanol than those which grew in the presence of oleic acid. This could be attributable to a slight increase in ethanol excretion when the cells were grown in the presence of linoleic acid. In both cases, the intracellular ethanol content remained higher than that in the culture medium. Thomas, Hossack and Rose (1978) also found that the membrane incorporated into its lipids the fatty acid provided in the medium. Similarly Watson (1982), showed that unsaturated fatty acid residues in membrane phospholipids (but not ergosterol) are essential for resistance of *Saccharomyces* to high concentrations of ethanol (103 – 122 g/ℓ).

Yeast physiology

SACCHAROMYCES SPP.

In *Saccharomyces* spp., the biosynthesis of respiratory enzymes is induced by oxygen. In an aerated medium, glucose has a strongly repressive effect on this biosynthetic process (Slonimski, 1953, 1958). This inhibitory mechanism of glucose is known as the reverse-Pasteur effect or the Crabtree effect (Ephrussi *et al.*, 1956; Sols, Gancedo and de la Fuentes, 1971). This means that in the presence of glucose, under aerobic conditions, more than 80% of the substrate can be converted into ethanol (Lemoigne, Aubert and Millet, 1954).

Fermentation in an aerated medium is never at the maximum rate, because of the Pasteur effect. Measurements of fermentation with the Warburg apparatus in the presence of air ($Q^{air}_{CO_2}$) are always lower than those under a nitrogen atmosphere ($Q^{N}_{CO_2}$) (Sols, Gancedo and de la Fuentes, 1971). However, moderate aeration increases the rate of cell multiplication and improves the physiological state of the cells and their resistance to ethanol, thus favouring the fermentation process. The above features, especially the Crabtree effect, have led to the widespread use of *Saccharomyces* spp. in traditional fermentation industries.

BRETTANOMYCES SPP.

Besides the *Saccharomyces* spp., the Crabtree effect also affects the genus *Brettanomyces* and its ascosporogenous counterpart *Dekkera*. Their lipid composition is akin to that of *Saccharomyces* (Johnson and Brown, 1972; Moulin *et al.*, 1975).

This group, however, differs from *Saccharomyces* by demonstrating a strong Custer effect (Wiken, Scheffers and Verhaar, 1961; Scheffers, 1966). Oxygen from air activates fermentation in these species, i.e. their $Q^{air}_{CO_2}$ is higher than their $Q^{N}_{CO_2}$. This is a reversal of the situation observed with the Pasteur effect for *Saccharomyces* species. The Custer effect has been explained as a net reduction of NAD^+ to NADH in yeast cells by the formation of acetic acid (Scheffers, 1966; Scheffers and Nanninga, 1977). Under strict anaerobic conditions, the production of acetic acid (even if very slight) is sufficient to prevent the reoxidation of NADH, which would significantly slow down the fermentation process. In an aerated medium NADH can easily be reoxidized to NAD^+ through the respiratory pathway. The fermentation process is thus activated by the presence of oxygen. The inhibition of cell growth and fermentation by ethanol has been little studied in *Brettanomyces* species: in fact, for these species, the accumulation of acetic acid has been as much a serious problem as the production of ethanol (Blondin, 1983). *Brettanomyces* species are used in practice only for the production of a special beer—the 'Lambric' of Brussels.

YEASTS LACKING THE CRABTREE EFFECT

In the past, alcohol production has always been carried out using yeast strains with a strong Crabtree effect. Thanks to this regulatory mechanism, effective alcoholic fermentation can be achieved despite the aeration required for the synthesis of sterols and unsaturated fatty acids. In the absence of the Crabtree effect, excessive aeration would permit intensive production of cell material at the expense of ethanol production. *Saccharomyces* species can ferment only a limited number of substrates, some of which must undergo preliminary hydrolysis. Yeast species lacking the Crabtree effect can, on the other hand, ferment unconventional substrates such as lactose (Moulin and Galzy, 1984), inulin (Guiraud, Deville-Duc and Galzy, 1981; Vandamme and Derycke, 1983); starch (Calleja *et al.*, 1982; Frelot, Moulin and Galzy, 1982; Wilson, Khachatourians and Ingledew, 1982; Dhawale and Ingledew, 1983) or cellobiose (Gonde *et al.*, 1982).

These yeasts may produce maximum amounts of ethanol in the same production range as do the *Saccharomyces* species. Again, in this case, ethanol affects growth rate, fermentation rate and cell death (Moulin, Boze and Galzy, 1980, 1982; Janssen *et al.*, 1983). However, these yeasts possess particular properties which modify the inhibition mechanisms of the alcoholic fermentation process. In contrast to strains showing the Crabtree effect, these yeasts are rich in linolenic acid (Johnson and Brown, 1972; Moulin *et al.*, 1975). In some instances, important alternative pathways have been described (Henry, Ramaide-Deplus and Nyns, 1974; Heritage, Tribe and Whittaker, 1981; Blondin, 1983).

The Pasteur effect in these strains is very effective when fermentation occurs under micro-aerobic conditions, even though the Pasteur–Meyerhoff coefficient is weaker than that of the *Saccharomyces* species. Respiratory-deficient mutants can be obtained from these strains by using ethidium bromide. These

mutants may ferment under aerobic conditions without excessive cell growth and without the Pasteur effect. It is thus possible to obtain a very active fermentation process by using these non-traditional species in distilleries (Moulin, Boze and Galzy, 1982).

The nature of ethanol inhibition

Effect on glycolytic enzymes

Nagodawithana, Whitt and Cutaia (1977) have studied the action of ethanol on the main enzymes of the glycolysis pathway. The function of several of these enzymes was found to be inhibited. Hexokinase was the most sensitive to a non-competitive type of inhibition. It is probable that ethanol also acts at different levels, because cell growth was inhibited at concentration levels lower than those required for inhibiting glycolysis.

Working with whole cells, Navarro (1980) confirmed the sensitivity of hexokinase towards ethanol. This author reported an irreversible inhibition: however this seemed to be linked with a very high accumulation of intracellular ethanol. Millar and Scopes (1981) did not find any inhibition of hexokinase within the cell for ethanol concentrations below 10% (w/v). The above results clearly show that the action of ethanol could be abnormally important under particular experimental conditions, such as poor minimum medium or extreme temperature. Under such conditions, ethanol excretion is probably insufficient.

Effect on plasma membrane

The lipid content (40%) and especially the sterol content (6%) of the plasma membrane warranted the study of their effects on membrane permeability and resistance to ethanol. Some antibiotics, such as nystatin, form a complex with sterols and thus modify cell permeability (Marini, Arnow and Lampen, 1961). An increase in sterol content of the membrane on the other hand would facilitate change from the gel state to the liquid state (Cabon and Haslam, 1973). Demel and De Kruyff (1976) attributed to sterols a regulatory role on the physical state of the membrane. Other authors also proposed that the toxic action of ethanol occurs at the plasma membrane level, especially by modifying the membrane fluidity, the transfer mechanisms and the activities of enzymes associated with membranes (Ingram, 1976; Thomas and Rose, 1979; Berger, Carty and Ingram, 1980; Leao and Van Uden, 1982).

The plasma membrane has a major role in regulating sugar and nutrient transport into the cell, as well as the excretion of metabolic products such as ethanol. Thomas and Rose (1979) showed that the incorporation of ethanol (0·5M) in buffer (pH4·5), into cell suspensions containing glucose, L-lysine, or L-arginine reduced the rate of accumulation of these compounds in the cells. The reductions were smaller when the cells had been grown in a medium containing linoleic acid (18:2), rather than oleic acid (18:1). According to these authors, a possible explanation of the protective effect of linoleyl residues on

the inhibition of solute uptake is that, whereas the decrease in fluidity caused by the presence of ethanol inhibits the action of one or more of the proteins involved in the transport of compounds into cells, this inhibition is partly compensated for, in cells with plasma membranes enriched in linoleyl residues, by the greater mobility of these residues.

Leao and Van Uden (1982) showed that alcohols inhibited the V_{max} of the glucose transport system of *S. cerevisiae*. Similarly, the transport of maltose was inhibited non-competitively by ethanol and other alcohols (Loureiro-Dias and Peinado, 1982). In both cases, the authors observed a positive correlation between the solubility of lipids in the alcohols studied and the inhibitory effects of the alcohols. These authors attributed the inhibitory action to a modification of lipid contents in the environment of the sugar transport system.

Many reports have confirmed in different ways the relationship between the 'action of ethanol' and the 'action of membrane lipids' on the functions of membranes. Thomas, Hossack and Rose (1978) showed that the viability of *Saccharomyces cerevisiae* cells suspended in phosphate buffer pH 4·5 containing ethanol, depended on the lipid composition of their plasma membranes. The cells with membranes enriched with linoleic acid (18:2) were less sensitive to ethanol than those enriched with oleic acid (18:1). In addition, besides the effect of unsaturated fatty acids, the protective effect was greater when the membranes were enriched with sterols with unsaturated side chains, such as ergosterol or stigmasterol, compared with sterols with saturated side chains, such as campesterol or cholesterol. These authors suggested that the presence of unsaturated fatty acids in the membrane increased fluidity and compensated for the adverse effect of ethanol. Hossack and Rose (1976) also showed that yeast spheroplasts enriched with sterols with unsaturated side chains C_{17} (ergosterol and stigmasterol) could better resist tension than when enriched with other sterols with saturated side chains. Hayashida and Ohta (1978) obtained similar results.

These various reports clearly indicate that the membranes have a fundamental role in the inhibition of alcoholic fermentation by ethanol. The 'fluidity' of the plasma membrane must be maintained at its optimum level in order to perform all the cellular exchange functions.

During the anaerobic growth of *Saccharomyces cerevisiae* NCYC 431, Beaven, Charpentier and Rose (1982) noted a decrease in fatty-acyl unsaturation and an increase in saturation. In addition, when 0·5 M, 1·0 M and 1·5 M ethanol were added to the cultures there was an increase in the proportion of mono-unsaturated fatty-acyl residues in phospholipids, especially 18:1 residues, and a decrease in saturated residues. In this case, ethanol induced modifications that increased resistance of the cells to ethanol. It was shown that the addition of linoleic acid to the medium provided the cells with a better resistance to ethanol. Thomas and Rose (1979) observed a difference in intracellular ethanol concentrations when the cultures were grown in the presence either of oleic acid or of linoleic acid. This difference was attributed by the authors to a faster ethanol excretion rate when the cells were enriched with linoleyl residues. Linoleic acid seemed to protect the cells against the entry of extracellular ethanol, while favouring the excretion of ethanol formed within

the cell. Thomas and Rose believed that an assymmetric distribution of unsaturated fatty acyl residues in plasma membrane phospholipids was responsible for these phenomena.

Conclusion

The inhibitory action of ethanol on all the yeast species with fermentative metabolism affects their growth and fermentation rates, the former being more sensitive to ethanol than the latter. Ethanol has a lethal effect on cells.

The factors influencing ethanol sensitivity (temperature, aeration, medium composition) act directly or indirectly by modifying the properties of the plasma membrane. The importance of membrane lipids has been clearly demonstrated by numerous reports. However, ethanol does not seem to have a single effect, but acts at several levels: modifications of membrane lipid properties, of solute transport systems, and action on some enzymes. The authors all agree that the intracellular level of ethanol is always greater than that in the medium.

Ethanol inhibition greatly depends on the balance between the rate of ethanol production and its rate of excretion. It is possible to improve industrial fermentation processes by maintaining the cells in a good physiological state and favouring ethanol excretion.

The inhibitory action of ethanol on growth rate and alcohol production quickly increases at higher ethanol concentration. This explains the difficulties encountered in the use of continuous fermentation processes. The experiments attempted with this technique are restricted to low ethanol concentrations, as in the vacuum fermenter of Cysewski and Wilke (1976, 1978).

Ethanol production is traditionally tied to the manufacture of different alcoholic beverages. In these processes, towards the end of the fermentation period, the yeast cells autolyse and release higher alcohols and fatty acids into the medium. These compounds are esterified and provide the beverage with its particular aroma. Any modification of the fermentation condition can thus drastically modify the organoleptic qualities of the product. Special technologies for the production of ethanol for industrial use (which must be as pure as possible), must also be borne in mind.

Genetic improvement of cells for ethanol resistance presents difficulties because the inhibitory action occurs at several levels. Ismail and Ali (1971 a,b) have shown that the ability to withstand ethanol was under polygenic control in *S. cerevisiae*. Interspecies crosses attempted by Day, Anderson and Martin (1975) did not significantly improve resistance. Brown and Oliver (1982b) observed a progressive increase in fermentation rate in a continuous culture, but the genetic mechanism of this phenomenon was not analysed. It may be possible to obtain a significant increase of resistance to ethanol by modifying the excretion systems or by modifying the physical and chemical structure of the membranes. In bacteria, the cloning of genes coding for the biosynthesis of some lipids has been achieved (Ohta *et al.*, 1981) and it may be possible eventually to modify some yeast-membrane properties by genetic engineering.

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