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Ethanol Induced Yeast Film Formation with Cell Surface Hydrophobicity as a Major Determinant

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Summary

In this study we have investigated the influence of ethanol on yeast film formation and cell surface hydrophobicity (CSH). A yeast strain (P3) previously isolated from film yeast was grown in a medium containing increasing ethanol concentration ranging from 0 to 14% (volume fraction). The growth of the film increased with higher concentration of ethanol up to 10%. We demonstrate for the first time using two different methods that ethanol induces an increase in cell surface hydrophobicity. Taking into account the increase in CSH with increasing ethanol concentration which leads to greater film development, it seems likely that CSH alteration constitutes an adaptation mechanism which allows the cell to rise to the surface where growth conditions are favoured, i.e. oxidative metabolism. The effect of CSH on yeast film formation has been confirmed using a wine strain (3079) able to form a film on the liquid surface. Thus we have shown that this yeast possesses a lower CSH (50%) compared with P3 strain (80%). However, CSH is not the only determinant of film formation since a respiratory deficient mutant (P3 rho$^-$) with high cell surface hydrophobicity could not form a film. Treatment of cells with lyticase which dramatically reduced CSH, suggests the protein or glycoprotein nature of the component responsible for CSH.

Keywords: cell surface hydrophobicity, yeast film formation, film yeast, *Saccharomyces cerevisiae (beticus, cheriensis, montuliensis)*

Introduction

In some types of wine at the end of the alcoholic fermentation process, some cells rise to the surface of the liquid which allows them to continue growing and form a film that can stay several months (sherry wine) and even several years (French »vin jaune«). These strains called film yeasts belong to different strains of *Saccharomyces cerevisiae* (*beticus, cheriensis, montuliensis*) *(1)*. The formation of yeast film after fermentation results in important changes in the characteristics of the wine due to the oxidative metabolism of the flor yeasts. The characteristics of these wines are the high ethanal (up to 500 mg/L) and ethanol (up to 15% volume fraction) content *(2)*.

The film formation allows the yeast to have an oxidative metabolism since at the liquid surface oxygen is not restricted. According to Martinez *et al. (3)* under such conditions yeast cells withstand high ethanol concentration. Oxidative metabolism seems to be essential, since petite mutants of the flor strain lose their ability to form velum *(4)*. Very little is known about the molecular and physiological characteristics of these yeasts and even less about the nature of the flor formation. Together with the oxidative metabolism, cell surface hydrophobicity (CSH) seems to be an important determinant in film formation *(5)*. These authors examined the difference in cell surface hydrophobicity between film and non-film yeast. They reported that the change from non-film to film stage was due to a change in cells from hydrophillic to hydrophobic. To gain more insight into the role of hydrophobicity on film formation versus ethanol, we have determined the cell surface hydrophobicity of film yeast and its mutant cultivated with increasing ethanol concentration.

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Materials and Methods

Strain

Saccharomyces cerevisiae P3 isolated from the film of »French Savagnin wine«, was used as a film strain. *Saccharomyces cerevisiae* 3079 is a commercial wine strain (Lallemand). Respiratory deficient mutant P3 (*rho*$^-$) was isolated from strain P3 cultivated in YPDE (15% volume fraction) as described by Jimenez and Benitez *(4)*. Exponential phase culture was spread onto YPDG; after incubation at 25 °C for 3 days, a small colony was selected.

Media

Yeasts were grown in YP medium supplemented with 2% glucose (YPD medium), 3% (volume fraction) glycerol and 0.1% glucose (YPDG medium) and different concentrations of ethanol (YPDE medium). Media were solidified by the addition of 2% agar.

For film formation we used the medium described by Fornachon *(6)*: 0.1% yeast extract, 0.05% $(NH4)_2SO_4$, 0.1% KH_2PO_4, 0.1% $MgSO_4$, 0.1% $CaCl_2$, 0.5% glycerol and 0.003% iron(III)citrate hydrate.

Culture conditions

Organisms preincubated in YPD medium at 25 °C for 24 h were inoculated at a final concentration of 10^6 cells/mL in 50 mL YPD or YPDE medium and incubated with rotatory shaking at 25 °C until the stationary phase was reached. To determine velum formation by flor forming yeast, 250 mL of Fornachon medium were inoculated to a final concentration of 10^6 cells/mL.

Determination of cell surface hydrophobicity (CSH)

1. Phase distribution method according to Rosenberg *et al.* (7).

Cells from YPD, YPDE or Fornachon medium were harvested in stationary phase of growth, centrifuged (10 min, 1000 g), rinsed with distilled water and resuspended in PUM buffer (pH = 7.1; 22.2 g $KH_2PO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and distilled water to 1000 mL) to an A_{620} of 1. Cells from the film were separated from the bottom cells (sediment) before being rinsed and resuspended in PUM buffer to an A_{620} of 1. Two milliters of hexadecane were added to the cell suspension and the phases were vortexed uniformly for 2 min. After 15 min, during which time the phases were allowed to separate, the optical density of the lower aqueous phase was measured at 620 nm. Hydrophobicity was determined as follows :

$$\text{Hydrophobicity}/\% = \frac{A_i - A_f}{A_i} \times 100$$

A_i = initial absorbance A_f= final absorbance

2. Magnobeads assay according to Straver and Kijne *(8)*

Adhesion of yeast cells to paramagnetic polystyren-coated latex beads (0.9 μm ; Sigma) was measured as follows: yeast cells were harvested at the stationary phase of growth, washed and resuspended in 50 mM sodium acetate buffer, pH = 4.5 to an A_{620} of 0.4 ($A_{initial}$). 15 μL of the beads were added to 1 mL cell suspension, this mixture was incubated for 20 min at room temperature under continuous shaking. After incubation, the glass tube was placed against a samarium cobalt magnet for 3 min and finally the A_{620} value of the remaining cell suspension was determined (A_{final}). The percentage of the cells that adhered to the beads was calculated as described in the first procedure.

Chemical and enzymatic treatment of the cells

The influence of dithiotreitol (DTT) and lyticase (Sigma) on cell wall hydrophobicity was determined by measuring CSH before and after the treatment.

Cells were harvested, washed and resuspended in 0.05 M phosphate buffer pH = 7.4 containing 0.6 M KCl (as an osmotic stabiliser). Dithiotreitol (1mg/mL) was added to the cell suspension, the treatment lasted 30 min at 37 °C with gentle shaking. The cells were then washed several times and an aliquot was resuspended in PUM buffer containing 0.6 M KCl at a final A_{620} of 1. Hydrophobicity was then determined using the phase distribution method. The rest of the cell suspension was resuspended in the phosphate buffer containing lyticase (0.1 mg/mL). Enzymatic treatment was carried out with gentle shaking at 30 °C during 30 min. After washing with PUM buffer containing 0.6 M KCl, CSH was determined.

Results and Discussion

Effect of ethanol on film formation and CSH

The occurrence of film yeast on liquid surface has been shown to be in part dependent on hydrophobicity *(5)*. These authors also reported that the cells grown in ethanol medium (5%, volume fraction) possessed more hydrophobic surfaces than those grown in the glucose medium. In order to better understand the role of ethanol in yeast film formation, we have studied the influence of ethanol on the CSH of cells from strains that were or were not able to form a film on the liquid surface.

As shown in Fig. 1, whatever the test used, P3 strain possessed a high CSH that was similar for both the cells present in the film and in the sediment. Ethanol induced a significant increase in CSH. The higher the ethanol concentration, the higher was the CSH. The number of cells present in the film *versus* those present in the sediment increases with ethanol concentration (Fig. 2). These results indicated that ethanol favours the film formation *via* high CSH. In this study we have shown for the first time that increase in CSH is linked to ethanol concentration.

To compare the CSH of strain P3 with strain 3079 and strain P3 (*rho*$^-$) which do not form any film, all the strains were cultivated in YPD medium under shaking at 0% and 10% ethanol for strains P3 and 3079, and 5% ethanol for the respiratory deficient mutant P3 (*rho*$^-$). The mutant strain does not grow at higher ethanol concentration; the lower ethanol resistance of *rho*$^-$ has already been reported *(9)*. Strain P3 cultivated in YPD medium under shaking possesses a CSH similar to that

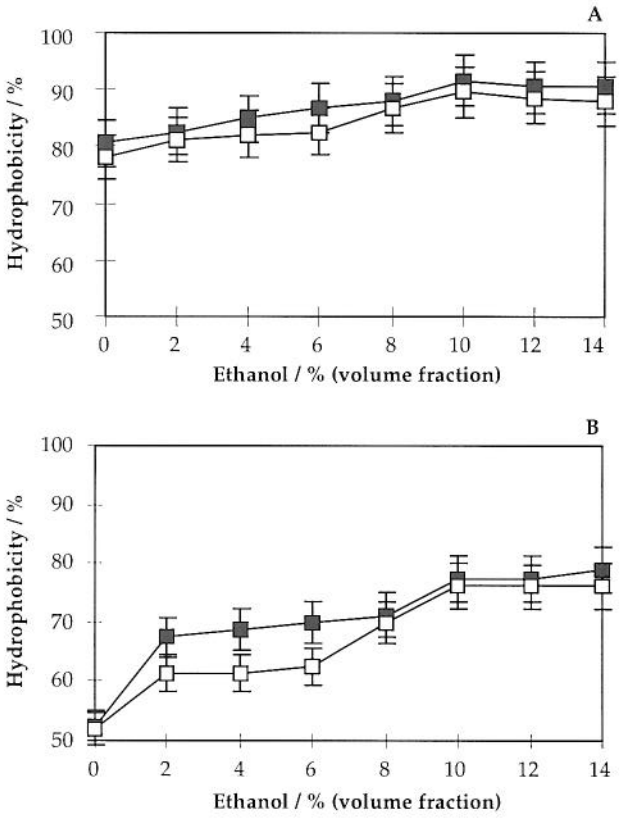

Fig. 1. CSH evolution of cells present in the film (■) or in the sediment (□). *Saccharomyces cerevisiae* strain P3 was grown in Fornachon medium containing increasing ethanol concentrations for 15 days at 25 °C. CSH was determined either by the phase distribution method (A) or the magnobead assay (B).Vertical bars indicate standard deviation of three independent experiments.

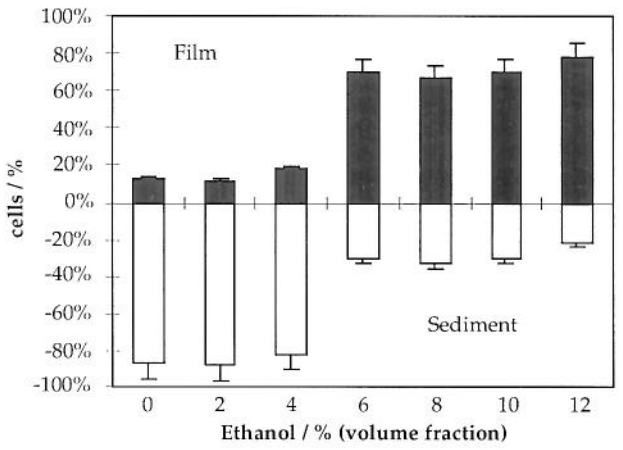

Fig. 2. Cell distribution between the film (■) and the sediment (□) for different ethanol concentration. Vertical bars indicate standard deviation of three independent experiments.

obtained with Fornachon medium, which allows the comparision of the strain P3 CSH with the other strains. The measured CSH of strain 3079 is significantly lower than that of P3 (Fig. 3). On the other hand, the respiratory deficient mutant although unable to form a film possesses a CSH similar to its parent (Fig. 3). Our data point out that under culture conditions where no film

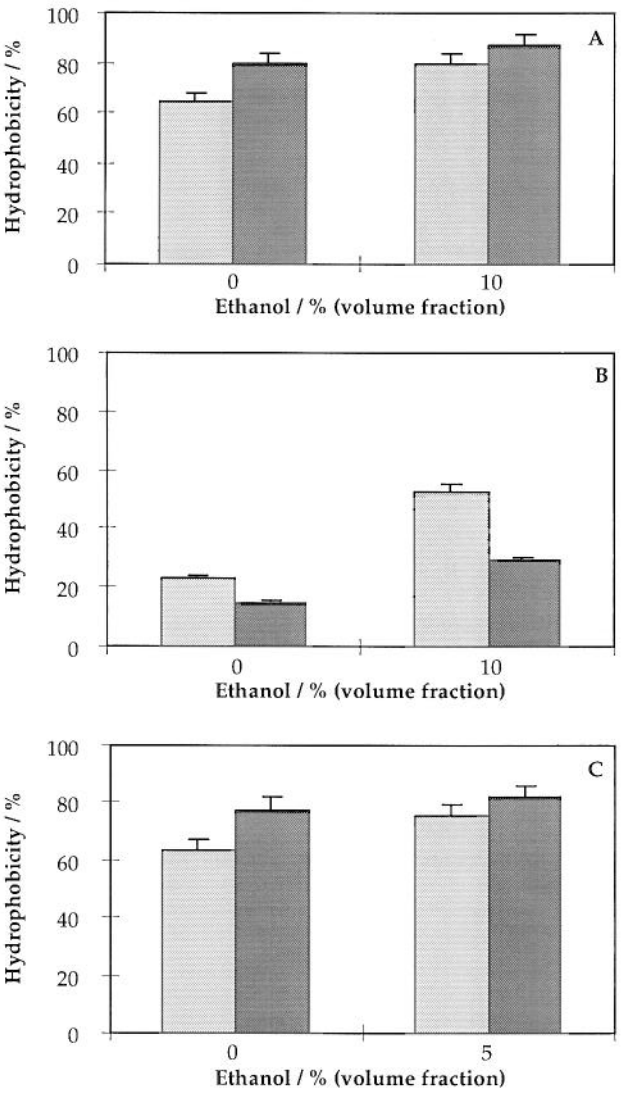

Fig. 3. CSH of strain P3 (A), strain 3079 (B) and strain P3 *rho*⁻ (C) cultivated in YPD medium with or without ethanol. CSH was determined either by the phase distribution method (▒) or the magnobead assay (▓).Vertical bars indicate standard deviation of three independent experiments.

occurs, *i.e.* shaking culture, ethanol induced an increase in CSH (Fig. 3). These results are consistent with an adaptation mechanism of the cells *versus* ethanol. High hydrophobicity seems to be necessary for film formation since strain 3079 with low CSH is unable to form a film. However, CSH is not the only determinant of film formation. The mutant P3 (*rho*⁻) possesses a high CSH but does not float. This result indicates that oxidative metabolism is essential to maintain cells on the liquid surface, which confirms previous findings *(5)*. However, when cells are cultivated in Fornachon Medium without ethanol, the sole carbon source is glycerol and in this condition the cells form a scanty film which sustains the important role of ethanol in film formation.

Effect of different treatments on CSH

Treatment of the three strains with dithiotreitol did not alter the measured CSH (Fig. 4), which indicates that

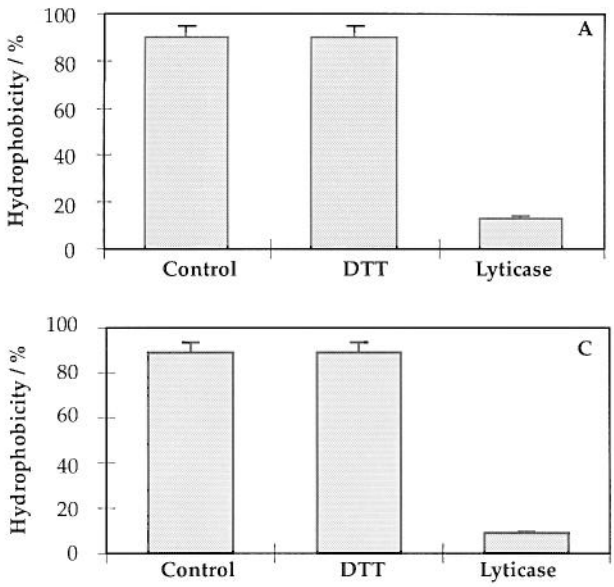

Fig. 4. Effect of dithiotreitol (DTT) and lyticase treatment on the CSH of strain P3 (A), strain 3079 (B) and strain P3 *rho*$^-$ (C). CSH was determined by the phase distribution method. Vertical bars indicate standard deviation of three independent experiments.

the non-covalently linked components are not responsible for CSH.

On the other hand, treatment with lyticase (β-glucanase) dramatically reduced the CSH of yeast strains P3 and P3 (*rho*$^-$) and to a lesser extent of the strain 3079. These results clearly supported the contention that cell wall of P3 contains hydrophobic components that are removed as a consequence of the partial degradation of the outermost surface of the wall by the action of lyticase.

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Etanolom inducirano stvaranje tankog filma kvasca uvjetovano hidrofobnošću stanične površine

Sažetak

U radu je ispitan utjecaj etanola na stvaranje filma i na hidrofobnost stanične površine. Soj kvasca (P3), prethodno izoliran iz kvasaca koji stvaraju film, bio je uzgajan u medijima koji su sadržavali rastuće koncentracije etanola od 0 do 14% (volumnog udjela). S povećanjem koncentracije etanola (do 10%), povećavao se i film. Prvi je put pokazano, koristeći dva različita postupka, da etanol inducira povećanje hidrofobnosti stanične površine. Uzimajući u obzir porast hidrofobnosti stanične površine kao posljedicu povećane koncentracije etanola, što dovodi do većeg stvaranja filma, izgleda da je ta promjena mehanizam adaptacije koji omogućava da se stanice podižu do površine podloge gdje su povoljniji uvjeti rasta, tj. gdje dolazi do oksidativnog metabolizma. Uloga hidrofobnosti stanične površine na stvaranje filma potvrđena je primjenom vinskog soja (3079) sposobnog da stvara film na površini podloge jer je pokazano da taj kvasac ima manju hidrofobnost površine stanica (50%) od soja P3 (80%). Međutim, hidrofobnost stanične površine nije jedini pokazatelj stvaranja filma jer respiratorno deficijentan mutant (P3 rho$^-$) s velikom hidrofobnosti stanične površine nije mogao stvarati film. Obradba stanica s litikazom, čime se bitno smanjuje hidrofobnost stanične površine, pokazuje da je komponenta odgovorna za hidrofobnost stanične površine po svojoj prirodi protein ili glikoprotein.