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Whole cell immobilization as a means of enhancing ethanol tolerance

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Cell immobilization by covalent linkage to an epoxide derivative of hydroxyalkyl methacrylate gel via glutaraldehyde-diamine spacers improves the tolerance of *Saccharomyces cerevisiae* cells to ethanol. This was attributed to membrane compositional changes accompanying this mode of cell attachment. The stability of the membrane alterations was tested under salt stress, and the character of stimuli inducing the phenotype changes of attached cells is discussed.

Keywords: cell immobilization; ethanol tolerance; lipid composition

Introduction

Microbial cell–solid surface contact has been recognized as providing phenotype changes in anchored cells that may be technologically important [2,11]. In this context, the aim of the present study was to assess the possible protective effect of yeast cell attachment against ethanol toxicity. Because ethanol damages yeasts at the level of the cell membrane and membrane composition determines the ethanol tolerance [24,25], this study focused on the effect of a model immobilization on the composition of yeast plasma membrane and the sensitivity of attached yeasts to ethanol. The covalent immobilization of yeast cells was accomplished via dialdehyde-diamine spacers to a physiologically inert hydroxyalkyl methacrylate gel [26].

Materials and methods

Organism and growth conditions

Saccharomyces cerevisiae A-17 used in this study was a wild type supplied by the culture collection of the Prague Institute of Chemical Technology. It was grown aerobically on a rotary shaker (90 rpm) at 28°C in Olson-Johnson [16] synthetic medium at pH 4.7. Cells in the logarithmic phase were separated by centrifugation (5500 × *g*, 10 min). The cell suspension for immobilization was prepared by washing harvested cells twice in 50 mM citrate-phosphate buffer (pH 4.7).

Cell immobilization

The support for covalent immobilization was prepared by incorporating epichlorhydrin into the hydroxyalkyl methacrylate gel Separon H-1000 according to Jirku and Turkova [7]. The epoxide derivative obtained (10.0 g) was suspended in 100 ml of 1 M tetra-methylenediamine solution. After 48 h stirring at room temperature, the suspension was transferred onto the column and the gel was washed with ten volumes each of water, 96.0% ethanol, 99.5% butanol, and ethanol again. The content of attached diamine was determined from the amount of nitrogen, quantified by Kjeldahl's method [10]. Unreacted epoxide groups were eliminated using overnight hydrolysis with 0.1 M $HClO_4$. The NH_2-Separon obtained was suspended in 25 ml of 10% v/v glutaraldehyde. After stirring the gel for 40 h it was washed with water until reaction with 2,4-dinitrophenylhydrazine, indicating the presence of free glutaraldehyde, was negative. Afterwards the gel was washed with 500 ml of 50 mM citrate-phosphate buffer (pH 4.7). The suction-dried support was placed in the yeast cell suspension (70 ml, 10^9 cells ml^{-1}). After 5 h stirring at 20°C, the support was harvested and washed with 1000 ml of 50 mM citrate-phosphate buffer (pH 4.7, 28°C). The column reactor and the support material were sterilized in an autoclave at 121°C.

Ethanol tolerance

Resistance to ethanol-induced leakage of UV-absorbing substances has been used as a means of rapid screening of yeast's ethanol tolerance [20]. The free cells as well as the support particles were collected by centrifugation, washed with sterile citrate-phosphate buffer (50 mM, pH 4.7, 28°C) and suspended in the same buffer containing ethanol (5, 10, and 15% v/v; 5% v/v is close to the minimal lethal concentration at 28°C for the yeast strain used). These suspensions were shaken slowly (40 rpm) at 28°C. Samples (10 ml) were centrifuged at 5000 × *g* for 5 min and the supernatants were immediately examined for UV-absorbing compounds (260 nm, Shimadzu UV-1201 spectrophotometer, Kyoto, Japan). The support material showed no sorption capacity for UV-absorbing material if contacted with the above supernatants. The pellets of free and immobilized cells were used for control lipid analyses.

Viable cell count

Samples of cultivation medium devoid of support were diluted and 0.2-ml aliquots were spread in duplicate on agar plates containing all components of Olson-Johnson [16]

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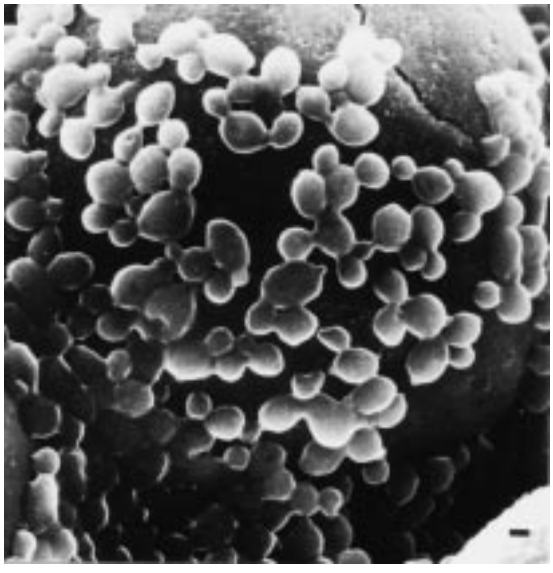

Figure 1 Scanning electron micrograph of growing *S. cerevisiae* cells immobilized via glutaraldehyde-diamine spacer. Bar equals 1 μm.

Table 1 Time course of the change in the amount of bound cell dry weight and free cell counts

Cultivation time (h)	Bound cell dry weight (mg g^{-1} dry support)[a]	Free cells (CFU ml^{-1})[b]
0	2.1	–
4	3.8	37
6	4.6	85
8	5.7	127
10	6.4	245

[a] Determined on the basis of the amount of nitrogen [10].
[b] See Material and methods.
[a,b] Values are the means of duplicate determinations; standard deviation is 7% or less; (*S. cerevisiae* A-17 dry weight-viable count correlation: 1 mg = 8×10^6 CFU).

medium. Plates were cultivated (48 h; 28°C) for a viable cell count (CFU).

Salt stress

For studies on the effect of variation in salinity, Olson-Johnson medium [16] was prepared without NaCl and specified amounts of NaCl were added prior to autoclaving the medium.

Analytical determinations

Washed cells were extracted following the procedure of Bligh and Dyer [1]. Fatty acid profiles were analysed by gas chromatography [23], using a Carlo Erba Fractovap 2450 (Milan, Italy) chromatograph. Individual phospholipids were separated by two-dimensional thin layer chromatography [4] and determined by phosphorus estimation [14]. Ergosterol and squalene were quantified by HPLC [18] performed on a TSP 3500 apparatus (Thermo Separation Products, Riviera Beach, FL, USA). The total content of phospholipids was calculated from the concentration of inorganic phosphate in the extracted lipids [12]. The total sterol content was determined according to Longley *et al* [13].

Scanning electron microscopy

Immobilized cells were fixed with 5.0% glutaraldehyde in 0.1 M sodium cacodylate buffer 1:1 (vol/vol) for 20 h at 5°C. Samples were dehydrated through an acetone series to 100% and dried by injection of CO_2 in a critical point drying apparatus (CPD-030 Balzers, Balzers, Liechtenstein, Germany). Samples were then coated with a gold layer (Balzers Union SCD-040, Germany) and observed on a Jeol JXA-840A Electron Probe Microanalyser (Akishima, Japan).

Results and discussion

Multipoint, covalent attachment of yeast cells (Figure 1) is accomplished by interaction of free, reactive aldehyde

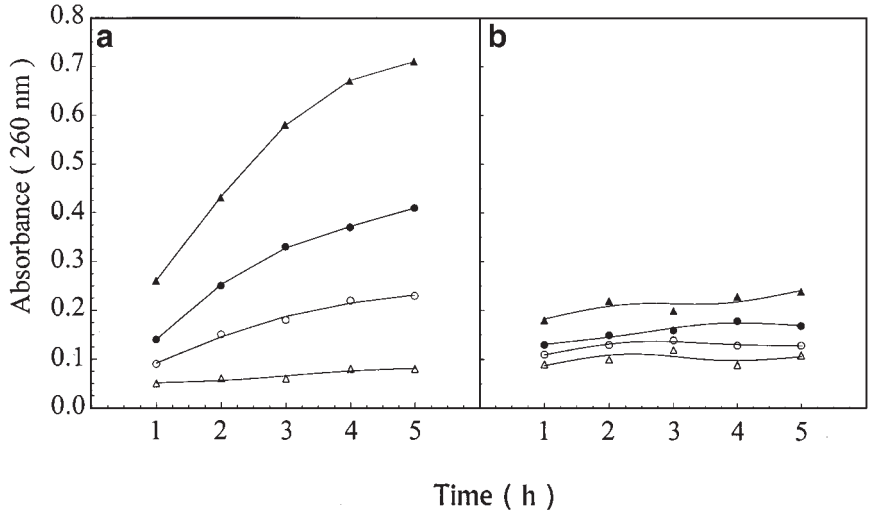

Figure 2 UV-absorbing compounds leaked from free (a) and covalently immobilized (b) *S. cerevisiae* cells incubated in buffer (pH 4.7) at 28°C in the presence of 5% (○), 10% (●) and 15% (v/v) (▲) ethanol. Cells not exposed to ethanol (△).

Table 2 The content of lipid components in *S. cerevisiae* cells exposed and not exposed to the effects of immobilization and salt stress*

Cultivation time (h)	Fatty acids (%)[a]												Phospholipids (μmol lipid phosphorus g^{-1} dcw)												Sterols (% dcw, w/w)					
	16:0			16:1			18:0			18:1			Phosphatidyl choline			Phosphatidyl ethanolamine			Phosphatidyl inositol			Phosphatidyl serine			Ergosterol			Squalene		
A																														
	F	I	S	F	I	S	F	I	S	F	I	S	F	I	S	F	I	S	F	I	S	F	I	S	F	I	S	F	I	S[bc]
3	9	**22**	*8*	23	*12*	**26**	15	**25**	*10*	35	*23*	**36**	18.7	**24.3**	**25.3**	33.5	**44.2**	*30.1*	20.7	19.6	*13.2*	10.6	**20.3**	*8.7*	0.08	**0.25**	0.15	0.03	0.02	0.02
5	8	**21**	*7*	24	*15*	**28**	17	**24**	*10*	26	*15*	**35**	19.1	**25.2**	**25.0**	34.7	**43.5**	*26.5*	21.3	19.8	*11.5*	10.1	**19.5**	*8.3*	0.11	**0.30**	0.15	0.03	0.03	0.04
7	12	**20**	*8*	21	*12*	**26**	13	**24**	*9*	21	*12*	**35**	19.4	**26.1**	**24.7**	35.1	**42.8**	*26.7*	19.8	20.2	*12.6*	10.3	**18.8**	*9.0*	0.13	**0.31**	0.11	0.04	0.02	0.02
10	9	**24**	*9*	25	*10*	**29**	15	**26**	*10*	23	*12*	**33**	18.5	**25.7**	**25.1**	33.8	**44.3**	*25.3*	20.5	19.4	*12.3*	10.7	**21.3**	*7.9*	0.09	**0.28**	0.15	0.02	0.03	0.04
B																														
		S			S			S			S			S			S			S			S			S[c]		
3		9			25			10			36			26.2			31.0			12.3			9.2			0.14			0.04	
5		8			28			11			34			25.3			27.0			11.8			8.8			0.11			0.03	
6					( )																									
	S		SI	S		SI	S		SI	S		SI	S		SI	S		SI	S		SI	S		SI	S		SI	S		SI[cd]
7	8		**20**	24		*11*	11		**26**	35		*20*	25.2		24.8	26.2		**43.8**	11.2		**20.1**	8.3		**19.8**	0.11		**0.30**	0.03		0.04
10	9		**21**	25		*12*	11		**25**	33		*17*	24.8		25.2	27.0		**44.2**	10.8		**19.6**	8.2		**20.3**	0.10		**0.35**	0.03		0.02
11	9		**23**	27		*12*	9		**25**	33		*15*	25.0		25.4	25.8		**43.7**	12.0		**19.3**	7.8		**19.5**	0.14		**0.33**	0.02		0.02

[a] % of total; [b]F, free cells; I, immobilized cells; [c]S, free cells exposed to salt stress (0.4 M NaCl); [d]SI, cells immobilized and cultivated under salt stress (0.4 M NaCl).
* Values are the means of duplicate determinations; standard errors did not exceed 4.7%; (bold/italics illustrates an **increase**/*decrease*).

groups which bind with amino acid groups in accessible cell surface proteins [8]. Counts of free cells in the medium and the time course of changes in dry weight of bound cells (Table 1) show that yeast cells immobilized in this manner grow and divide without a significant leakage of progeny into the medium. The immediate immobilization of daughter cells during their growth is assured by the content of homogeneously coupled diamine arms (737 μmol g^{-1} dry support), extended with monomeric glutaraldehyde. The extent and homogeneity of this modification is determined by the basic composition [26] and previous epoxidation [7] of the support.

Figure 2 shows the pattern of leakage of UV-absorbing compounds in free and attached cells exposed to ethanol, indicating that covalently immobilized cells can tolerate higher ethanol concentrations. To prove that the acquired resistance to ethanol is accompanied by an alteration in the plasma membrane composition, the contents of selected fatty acids, phospholipids and sterols in free and immobilized cells were compared (Table 2, Part A:F,I). Immobilized (more ethanol-resistant) cell populations showed an increase in the proportion of saturated and a decrease in the proportion of unsaturated fatty acids as well as an increase in the content of three phospholipids and ergosterol. A comparison of the sterol/phospholipid ratio, between free and attached cells (not shown), exhibited no notable difference; however, cell attachment stimulates an increase in the total amounts of both of these lipids. Changes in the membrane composition in attached cells are reproducibly detectable after 1 h cultivation in the immobilized state (not shown). In spite of the fact that the above results suffer from the possible shortcoming that the analyzed lipids were extracted from whole cells and some compositional changes may be masked, the long-term stability of the alterations seen (Table 2, Part A) suggests that their development is not stimulated by transiently acting stimuli in the microenvironment of covalently attached cells. In order to prove these stimuli potent and permanently acting, the development of this attachment-induced response was tested under the effect of Na^+ stress, known to alter lipid composition of bacterial and yeast species [17,22], however, stimulating the development of an almost opposite pattern of changes in the concentration of analyzed lipids (Table 2, Part A:S). Free cells exposed to salt stress were immobilized (Table 2, Part B, 6 h) and further cultivated in the presence of Na^+. Lipid analysis revealed (Table 2, Part B) a full suppression of the manifestation of salt stress effect by immobilization, suggesting a higher potency of the second effect to induce membrane compositional changes. Comparing the character and stability of the observed compositional alterations, induced by immobilization of cells not exposed or exposed to increased external osmolarity (Table 2, Part A:I; Part B:S,I), no difference was found.

Salinity has two possible toxic effects on cells: a nonspecific osmotic effect and a specific toxicity of the ions on defined cellular systems [21]. The observed dominance of the cell attachment effect over the effect of salt stress indicates that covalently attached cells respond to more potent signal(s), permanently acting in the variable microenvironment of immobilized cells. The paucity of information about the existence of a transductive system, participating in a cell response to cell-support contact, and a range of diverse effects of the attached cell microenvironment [15,19], make a clear-cut explanation of these phenomena impossible. Moreover, altered compositional or behavioral patterns of immobilized cells also may result from the 'switching on' of some genes at the cell surface as described by Dagostino *et al* [5,6]. Clearly, the results obtained support the hypothesis that a multipoint, cell–solid surface contact results in significant modulations of cellular structures and functions [9,11], in this case possibly a modulation of membrane integrity/fluidity enhancing ethanol tolerance [3,27]. In summary, the results of this study support the idea that whole cell immobilization by a multipoint attachment to an insoluble support could allow us to manipulate the sensitivity of yeast cell growth or fermentation capacity to ethanol.

Acknowledgements

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