J. Microbiol. Biotechnol. (2001), **11**(1), 17–20

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Reduced Susceptibility of a Model *Saccharomyces cerevisiae* Biofilm to Osmotic Upshifts

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Received: June 2, 2000
Accepted: September 4, 2000

Abstract Whole-cell attachment by covalent linkage, thereby simulating natural and specific attachments, improves the osmotolerance of *Saccharomyces cerevisiae* cells. The enhanced osmoresistance is correlated with a decrease in the intracellular concentration of trehalose and accompanied by membrane compositional changes. The results obtained indicate that yeast cell-support (physical) contact is sensed and responded to.

Key words: Yeast biofilm, covalent linkage, osmotolerance

Industrial microorganisms must be able to tolerate the stressing osmotic fluctuations of nutrients and metabolic products [28]. Therefore, efforts are focused on optimizing technological applications of such microorganisms by modulating their osmosensing. Three aspects of the yeast (osmotic) stress response, which have received extensive attention, are trehalose level [5], plasma membrane composition [24], and high-osmolarity glycerol response [19]. Moreover, microbial populations forming multicellular consortia, which require cells to (physically) contact not only the extracellular matrix but also the neighbor and support surfaces [25], are believed to sense these interactions as mechano-physiological stimuli and induce a programmed (cellular) response, resulting in a change in a specific cell susceptibility, among others [4]. Accordingly, the present work was undertaken to investigate the significance of yeast cell attachment as a means of enhancing yeast cell resistance to osmotic stress. The possibility that yeasts colonizing solid substrata are equipped with a specific protection is supported by previous findings that attached yeasts manifest compositional changes in their cell surface structures, thereby providing an increased resistance to agents damaging the cell wall or plasma membrane [7, 10, 11]. In this context, the covalent attachment of yeast cells via glutaraldehyde amino spacers to a physiologically inert support has been shown as a suitable method to simulate the conditions of natural (multipoint) cellular bioadhesions [17]. Therefore, this technique was used to investigate interrelationships between the intracellular levels of trehalose, fatty acids, and ergosterol, and the sensitivity of suspended or attached yeast cells to defined osmotic upshifts.

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Materials and Methods

Yeast Strain and Culture Conditions

The *Saccharomyces cerevisiae* A-11 used in this study was a wild-type supplied from the culture collection of the Prague Institute of Chemical Technology. The sample was grown aerobically on a rotary shaker (90 rpm) at 28°C in a (water/glycerol) MWM (glucose) medium according to the method of Raynal *et al.* [22] (initial a_w=0.990, pH 5.5, osmotic presure: 1.38 MPa). The cells in the logarithmic phase were separated by centrifugation (5,500 ×g, 10 min). The cell suspension for immobilization was prepared by washing the harvested cells twice in a 50 mM citrate-phosphate buffer (pH 4.7).

Cell Attachment Procedure

The epoxide derivative of the microporous copolymer of 2-hydroxyethyl methacrylate, available as Separon P 1000 (Laboratory Instruments, Prague, Czech Republic), was used as the support after modification with a diamine-ε-aminocaproyl spacer arm [12]. The spacer arms of the resulting ε-aminocaproyl-NH_2-Separon were further extended with monomeric glutaraldehyde according to the method of Jirků [6].

Osmotic Shifts

The hyperosmotic shock was performed by an immediate mixing of the culture medium with pure glycerol whose amount was determined according to Norrish's equation

[20] to obtain a final a_w=0.900, 0.800, and 0.700. The control was prepared by the same ratio to dilute the culture medium with a water-glycerol solution (a_w=0.990). The suspended or attached cell populations were allowed to equilibrate for 20 min under the culture conditions (28°C, 90 rpm) before any measurement was initiated under osmotic stress.

Analytical Methods

Trehalose was extracted with cold 0.5 M trichloroacetic acid and determined according to Lewis *et al.* [16]. Sterols and fatty acids were extracted by the procedure of Bligh and Dyer [1].The fatty acids profiles were analyzed by gas chromatography [27], using a Carlo Erba Fractovap 2450 (Milan, Italy); the ergosterol was quantified by HPLC [23] on a TSP 3500 apparatus (Thermo Separation Products, Riviera Beach, FL, U.S.A.). The free and bound cell dry weights were determined on the basis of the nitrogen content determined by the Kjeldahls method [15].

Electron Microscopy

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

RESULTS AND DISCUSSION

Cell attachment simulates natural (specific) mechanisms that involve stereochemical complementarity between the cell surface "adhesin" and the substrate receptor [17]. A monolayer formation of sparsely and covalently attached yeast cells (Fig. 1) interacts with reactive amino acids in accessible cell surface proteins which bind with free aldehyde groups coupled to the support via a spacer arm [20]. Fungal cells attached in this manner grow and divide without any significant leakage of progeny into the medium due to an immobilization of daughter cells during their growth [7, 9], depending on the extent and homogeneity of the support modification [12]. This method is very suitable for the use as a model biofilm system that is readily accessible to solutes, thereby eliminating transport phenomena as factors influencing biofilm susceptibility [4].

Figure 2 shows the effect of reductions in water activity (a_w) on the growth of the suspended and attached yeasts, indicating that attached yeast cells can tolerate a growth inhibiting (osmotic) effect. The levels of trehalose and ergosterol as well as the fatty acid profile were analyzed to

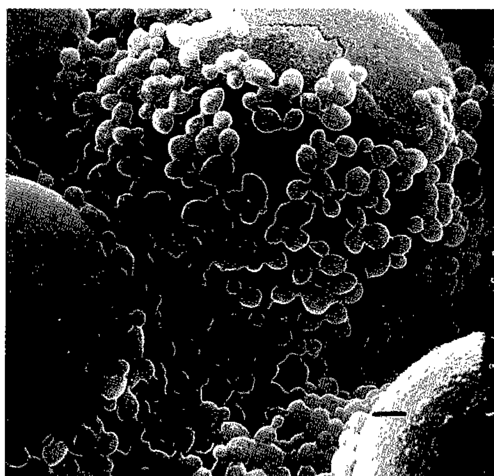

Fig. 1. Scanning electron micrograph of covalently attached and growing *S. cerevisiae* cells. Bar equals 1 μm.

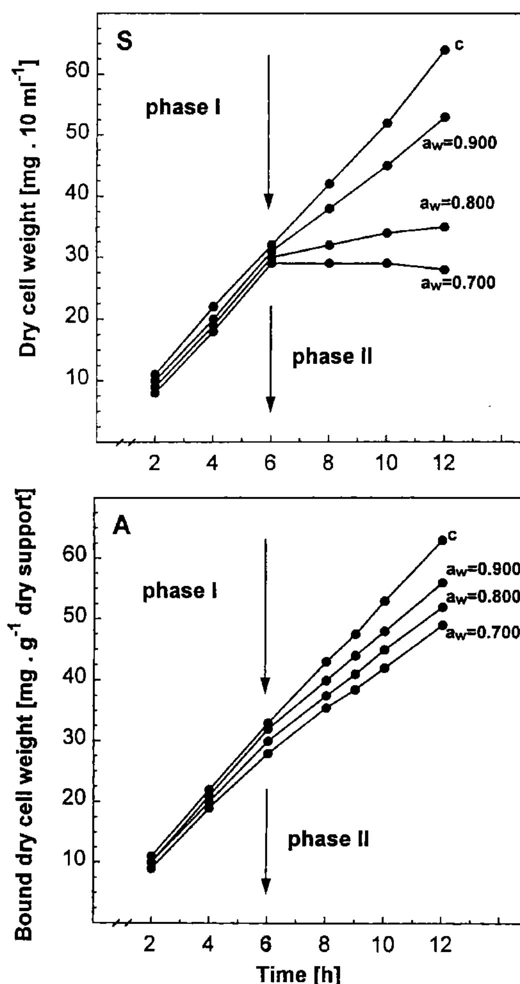

Fig. 2. Growth inhibition of suspended (S) and attached (A) *S. cerevisiae* cells exposed to low hydrated media.
(↓) glycerol added; (c) control (see Materials and Methods); phase I, II (see Table 1).

Table 1. Content of trehalose and lipid components in *S. cerevisiae* cells exposed and not exposed to the effect of cell attachment and osmotic upshifts.

Cultivation time (h)	Trehalose (% dcw, w/w)		Fatty acids (%)[a] 16:0		16:1		18:0		18:1		Ergosterol (% dcw, w/w)	
Phase I[b]	S	A	S	A	S	A	S	A	S	A	S	A[c]
2	0.35	0.15	8	21	20	11	13	24	26	13	0.07	0.30
4	0.33	0.13	8	22	22	9	15	25	31	11	0.012	0.33
6	0.32	0.15	9	24	24	12	15	26	21	10	0.014	0.31
Phase II[b]												
8	2.5	0.16	17	23	17	10	19	26	19	10	0.15	0.33
10	2.1	0.14	18	21	16	11	20	26	22	10	0.21	0.32
12	2.2	0.15	16	25	14	11	22	23	18	12	0.21	0.30

[a]% of total; [b]see Fig. 1; [c]S, suspended cells; A, attached cells.
*Values are the means of duplicate determinations; standard errors did not exceed 4.2%.

determine whether the attached cells exhibited an osmotic tolerance by compositional changes which were usually demonstrated by single cell organisms in response to an environmental stress, i.e. a trehalose accumulation and an alteration in the plasma membrane composition [24, 26]. The attached (more osmo-resistant) yeast populations showed a decrease in trehalose level and in the proportion of unsaturated fatty acids, whereas an increase in the proportion of saturated fatty acids and in the content of ergosterol was evident. The suspended cells exposed to osmotic stress showed an almost opposite pattern of changes in the concentrations of these compounds analyzed (Table 1). Accordingly, the altered stability in the attached cells would seem to suggest that their development is neither stimulated by transiently acting stimuli in the cellular microenvironment, nor affected by the effect of osmotic stress. Therefore, these results indicate a strong effect of the cell attachment over osmotic stress and also the fact that the mechanism enhancing the tolerance of the attached cells to osmotic stress is not due to the accumulation of trehalose (the protective role of trehalose has not been experimentally proven, since resistant *Candida utilis* cells retain osmotic tolerance even after the depletion of intracellular trehalose [21]). Since the plasma membrane has been implicated as the primary sensor of environmental stress [18, 28], cell attachment possibly altered cell membranes that suppress this function, thereby preventing the emission of signals that induce the synthesis of trehalose. However, at the same time, the change in the compositions of fatty acids and ergosterol accumulation in the attached cells may also stabilize the osmotically sensitive (membrane) functions [26]. The possibility that attached cells can specifically manifest a high-osmolarity glycerol response [3] by transport of extracellular glycerol (see Material and Methods) into the attached cells is not supported by any data that there is an active transport of glycerol into *Saccharomyces cerevisiae* cells [2].

The experimental approach described in this study confirmed that reduced biofilm susceptibility to osmotic stress was not a transport artifact or a phenomenon induced by a signal molecule accumulation, as detected in more complex bacterial biofilms [25]. Rather, the osmosensing of the attached yeasts appears to be modulated by a potent permanently acting signal in the microenvironment of the attached cells. The suggestion that cell-support (physical) contact is such a permanent signal which switches on an evolutionarily conserved "attachment genome" is intriguing, and certainly warrants further research similar to the investigation of the contact behavior of mammalian cells in some ways [13, 14]. Our present discussion on bacterial surface sensing at the gene level, as well as studies of physical and metabolic changes on attachment of microbial cells to a surface, all support this idea. In practical terms, it is well recognized that yeast cells are (in a water medium) better able to survive inhibitory osmotic conditions when attached than when free.

Acknowledgment

The financial support of the Grant Agency of the Czech Academy of Sciences (Grant No. A 4020802) and the technical assistance of Ms. H. Matoušková are gratefully acknowledged.

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