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EFFECT OF TREHALOSE AND OTHER COMPOUNDS ON THE RESISTANCE TO DESICCATION BY *Candida utilis* CELLS

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RESUMEN

Efecto de la trehalosa y otros compuestos sobre la resistencia a la desecación de *Candida utilis*

Al igual que otras levaduras, *Candida utilis* acumula trehalosa intracelularmente como material de reserva. Las células en fase estacionaria, muestran una mayor resistencia a la pérdida de viabilidad por desecación que las células en fase logarítmica cuando se someten a liofilización. Esta observación se correlaciona con el hecho de que las células en fase estacionaria contienen más trehalosa interna que las células en fase logarítmica.

El número de células viables puede ser incrementado por adición de trehalosa exógena inmediatamente antes del tratamiento de desecación. Estos resultados sugieren que la trehalosa desarrolla un papel protector además de servir como fuente de energía. La comparación del efecto protector de la trehalosa con el de otros compuestos sugiere que dicho papel no es exclusivo de este azúcar, ni parece estar relacionado con su estructura o su carácter no reductor. Otros disacáridos y glicerol pueden ser incluso más efectivos en la prevención de pérdida de viabilidad por desecación. No obstante, si se tiene en cuenta que la trehalosa es el único disacándo que se acumula endógenamente, la significación fisiológica de este efecto puede ser relevante.

Palabras clave: Trehalosa, desecación, *Candida utilis.*

SUMMARY

As other yeasts, *Candida utilis* intracellularly accumulates trehalose as reserve carbohydrate. Stationary-phase cells show higher resistance to the loss of viability by desiccation than log-phase cells when subjected to a lyophilization protocol. This finding correlates with the fact that resting cells contain higher levels of endogenous trehalose than exponentially growing cells.

The number of resulting viable cells can be increased by addition of exogenous trehalose inmediately before the desiccation treatment. These results suggest a protective role for trehalose in addition to the previously assumed of serving as energy source. The comparison of the protective effect of trehalose on cell death by dehydration to that developped by other compounds indicates that such a role is neither exclusive of this sugar nor dependent of its nonreducing character. Other disaccharides and glycerol can be even more effective in preventing loss of viability by desiccation. However, taking into account that trehalose is the only disaccharide endogenously accumulated, the physiological significance of this effect appears to be relevant.

Key words: Trehalose, desiccation, *Candida utilis.*

INTRODUCTION

Trehalose is a nonreducing disaccharide which accumulates inside the cells of some prokaryotic and eukaryotic microorganisms (CHANG & TREVITHICK, 1972; KELLY & CATLEY, 1976; ARGUELLES *et al.*, 1985; MARTIN et *al.*, 1986). This sugar can be intracellularly mobilized in a wide variety of physiological processes that have been well cha-

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racterized (THEVELEIN & JONES, 1983; THEVELEIN *et al.*, 1983; VAN ASSCHE *et al.*, 1978).

The yeast C. *utilis* also contains variable amounts of trehalose as a function of the balance between the activity of two enzymes involved in its metabolism: trehalose synthase and trehalase (ARGÜELLES *et al.*, 1985, 1986). Exponentially growing cells on media containing glucose show low levels of endogenous trehalose whereas higher amounts of this sugar are present in stationary-phase cells (ARGÜELLES et al., 1985; ARGUELLES & GACTO, 1985).

Since many anhydrobiotic microorganisms accumulate large amounts of trehalose it has been suggested that this sugar might play a common role by acting as a substitute for water during dehydration. Recently, evidences have been found in some systems allowing to stablish a correspondence between the ability for survival in extreme conditions (desiccation, temperature shock..., etc.) and the trehalose content (MARTIN *et al.*, 1986; HOTTIGER *et al.*, 1987; MCBRIDE & ENSING, 1987a). According to this, it has been suggested that trehalose, in addition to represent an available energy supply, could serve to maintain the functional integrity of some cell components located at the membrane level (CROWE *et al.*, 1984a, 1984b; MARTIN et al., 1986).

Although the above conclusions have been fairly stablished in several systems, the evidences on the protective role of trehalose against dehydration are rather scarce in yeasts and fungi. Only recently several papers have focused on this topic (HOTTIGER *et al.*, 1987; MCBRIDE & ENSING, 1987a; GADD *et al.*, 1987), in coincidence with the developpment of the present work.

On the basis of previous studies (ARGÜELLES & GACTO, 1985; ARGÜELLES *et al.*, 1985, 1986) we have chosen the cells of C. *utilis* as a system to study the effect of trehalose on cellular desiccation. In the present report we describe the effect of this sugar and other compounds on the viability of vegetative cells of this yeast subjected to extreme conditions of dehydration.

MATERIALS AND METHODS

YEAST STRAIN AND CULTURE CONDITIONS

C. utilis ATCC 60459 (CECT 1061) was grown in liquid Winge's medium containing 2% glucose (w/v) and 0.3% (w/v) yeast extract (Oxoid). The cultures were incubated at 30° C in an orbital shaker and the growth was followed by measuring changes in optical density at 600 nm. Cell number was determined with a haemocytometer.

DEHYDRATION STUDIES

Cells were removed from log-phase (O.D. = 1–3) or stationary cultures (O.D. = 11–12) by centrifugation at 3000 xg for 10 min and washed four times at 4° C with sterile distilled water. The washed cells were finally resuspended in sterile distilled water at an standard optical density for each experiment so that the initial cell concentration was similar in all samples within the same experiment.

Identical aliquots (5 ml) of the above normalized suspensions were centrifuged and the resulting sediments resuspended in 1 ml of cold distilled water containing 200 mM trehalose, maltose, sucrose, lactose, cellobiose or glycerol. In other series, trehalose at various concentrations (2-200 mM) was present. Parallel controls without exogenously added compounds were also performed. The cell suspensions were quickly frozen at –700 C and maintained for 4 hours before lyophilization in a Virtis 6201 apparatus.

After the dehydration treatment, cell survival was independently analyzed in both solid and liquid medium by measuring the viability in control samples and in samples supplemented with exogenous compounds. To this purpose, lyophilized samples were suspended in 5 ml of sterile distilled water and serial dilutions were carried out. Aliquots (0.1 ml) of each dilution were plated by triplicate on solid Winge's medium. The plates were incubated for 36 hours at 30° C and the colony counting was expressed as number of viable cells. In parallel experiments, 1 ml from each original suspension containing the lyophilized cell samples was inoculated into 200 ml of liquid Winge's medium and cultures were incubated as described before.

RESULTS

The dehydration by lyophilization is a very dastric procedure which promotes a high mortality in vegetative cells. However, the relative protective effect of trehalose can be established even under such a drastic circunstances. The effect of the addition of various concentrations of trehalose on the viability of exponentially growing cells of C. *utilis* subjected to dehydration treatment is summarized in table 1. The data are expresed as percentage of viability with respect to nondehydrated cell samples by using colony counting on solid medium as criterion of survival. The results clearly show that in control samples, that were dehydrated in the absence of added trehalose, the viability was extremely low whereas the presence of 200 mM trehalose increased the survival 3500 times. High levels of intracellular trehalose have been described in yeast cells accounting for up to 23% of the dry weight of the cells (LILLIE & PRINGLE, 1980).

TABLE 1. Protective effect of trehalose on the survival of dehydrated cells of *Candida utilis*.

Efecto protector de la trehalosa sobre la supervivencia de células deshidratadas de *Candida utilis*.

ADDITION	% VIABILITY
None	0.0001 ± 0.00007
Trehalose 2 mM	0.0003 ± 0.00011
Trehalose 20 mM	0.0220 ± 0.00880
Trehalose 200 mM	0.3500 ± 0.11200

Cells from exponentially growing cultures were used. The percentage of viability was referred to the viable cells before the dehydration treatment by using colony counting on solid media. Values are the mean of three independent determinations ± standard deviation.

The results of the effect of similar treatments on the survival of stationary cells also showed the same trend, although this type of cells appear to be relatively more resistant than log-phase cells to the effect of the dehydration treatment. Typical experimental values obtained in such conditions indicated a percentage of viability equivalent to 0.01 and 1.08 for control samples and for samples dehydrated in the presence of 200 mM trehalose, respectively.

On the other hand, the percent survival is dependent on the nature of the compound added as protective agent. Several sugars, structurally related to the disaccharide trehalose, and glycerol were used to compare the resulting survival. Table 2 shows that, at a common concentration of 200 mM, sucrose lacks the ability to protect the cells whereas glycerol or cellobiose are effective protective agents able to increase cell viability by a factor of around 4600 and 5200 times, respectively. Thus, under the followed experimental conditions, other compounds are comparatively more efficient than trehalose in maintaining cell survival.

TABLE 2. Effect of the addition of several compounds on the survival of dehydrated cells of *Candida utilis*.

Efecto de la adición de varios compuestos sobre la supervivencia de células deshidratadas de *Candida utilis*.

ADDITION	% VIABILITY
None	0.010 ± 0.005
Sucrose	0.013 ± 0.008
Trehalose	1.080 ± 0.203
Lactose	2.410 ± 0.321
Maltose	4.370 ± 0.311
Glycerol	46.170 ± 6.231
Cellobiose	52.800 ± 8.130

Cells from stationary cultures were used. All compounds were present at 200 mM concentration. The percentage of viability was calculated as indicated in table I.

In addition to the above evidences, a serie of experiments was conducted by repeating the previous type of assays but by analysing the growth ability in liquid medium of the dehydrated cells instead of the colony forming capacity on solid media. The results obtained were in cualitative agreement with those previously indicated. In all cases, the stabilized growth rate of the surviving treated cells was similar, irrespective of the absence or presence of added compounds during dehydration. However, the time to reach the exponential phase in the inoculated cultures was temporarily delayed as a function of the absence or presence of the compounds in the treatment of the cells employed as inoculum. This fact was taken as criterion of protection for survival.

Figure 1 shows the growth in cultures originated from log-phase or stationary-phase cells dehydrated in the presence or absence of exogenously added trehalose. The results indicate that in both cases the delay to reach the stabilized growth rate was shorter when the cells were dehydrated in the presence of trehalose than in its absence. Moreover, it was again clear that stationary-phase cells were more resistent to the dehydration than log-phase cells. These results correlate with those obtained on solid media and might be interpreted as due to the higher content of endogenous trehalose in stationary-phase cells (ARGÜELLES & GACTO, 1985).

The exent of the delay to reach the stabilized growth rate was somewhat inversely proportional to the amount of trehalose present during the dehydration treatment (fig. 2), which demostrates the validity of the shortening of this period as a criterion of protection for survival used in liquid medium. The effect of other compounds was also determined in a similar way by measuring viability on liquid medium. Figure 3 gives on liquid medium evidence compatible with the type of results obtained on solid medium shown in table 2. Taken together, these data allow to establish similar cualitative conclusions as to the relative protective action of the assayed compounds.

DISCUSSION

The accumulation of trehalose by microorga-

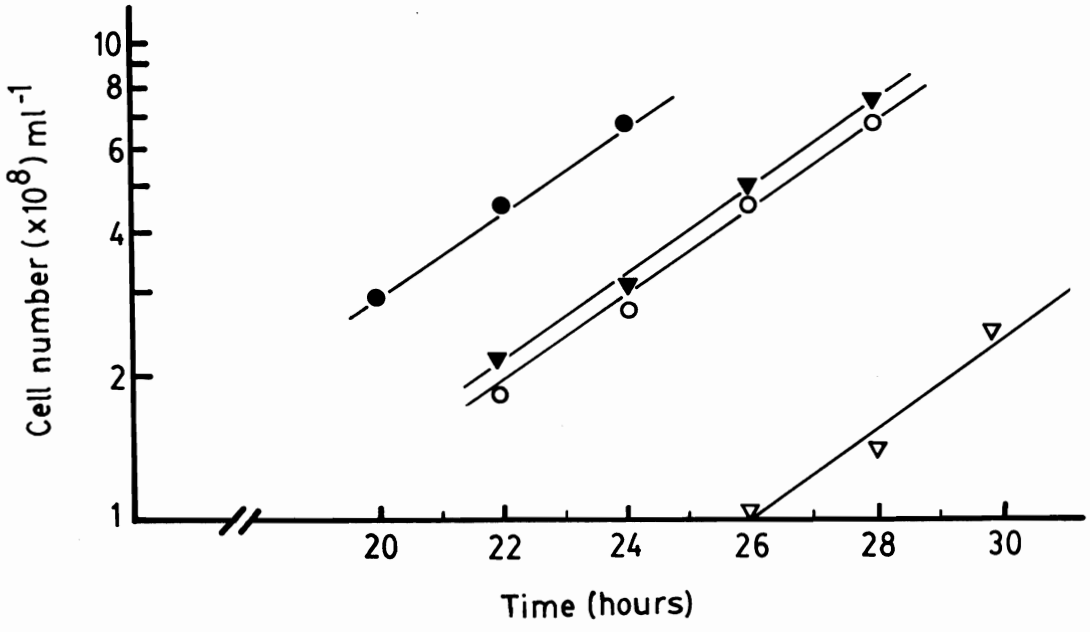

FIGURE 1. Growth of *Candida utilis* cells dehydrated in absence (open symbols) or presence (closed symbols) of 200 mM trehalose. The same number of treated cells was used as inoculum in all cases (see Materials and Methods).
(▽) log-phase cells dehydrated without trehalose.
(▼) log-phase cells dehydrated with trehalose.
(○) stationary-phase cells dehydrated without trehalose.
(●) stationary-phase cells dehydrated with trehalose.

Crecimiento de células de *Candida utilis* deshidratadas en ausencia (símbolos abiertos) o presencia (símbolos cerrados) de trehalosa 200 mM. En todos los casos se utilizó como inóculo el mismo número de células tratadas (ver Materiales y Métodos).
(▽) células exponenciales deshidratadas sin trehalosa.
(▼) células exponenciales deshidratadas con trehalosa.
(○) células estacionarias deshidratadas sin trehalosa.
(●) células estacionarias deshidratadas con trehalosa.

nisms is a phenomenon of wide occurrence in nature which has been interpreted as an strategy to guarantee the availability of energy and carbon source under various circumstances (PANEK, 1963; THEVELEIN, 1984). However, this endogenous sugar might play additional roles. In particular, some evidences have pointed to a protective role against heat and dehydration (MARTIN *et al., 1986;* MCBRIDE & ENSING, 1987a; VAN LAERE *et al.*, 1987; HOTTIGER *et al.*, 1987). VAN LAERE *et al.* (1987) have suggested that this could be even the sole function of trehalose in spores of *Phycomyces blakesleeanus* since, during germination, the stored sugar is quickly converted to glycerol that is released into the medium.

Although the function of trehalose as energy reserve in yeast cells has been clearly demostratred (BARTON *et al.*, 1982; LILLIE & PRINGLE, 1980; ARGÜELLES *et al.*, 1985; ARNOLD & MCLELLAN, 1975), our results with vegetative cells of *C. utilis* also suggest an additional function for this sugar. The viability of the cells subjected to dehydration increases in the presence of extracellular trehalose. This protective effect against dehydration can be demonstrated under two different conditions of determining cell viability. In either solid or liquid medium, the qualitative effect was similar although cuantitative results were not comparable because the two different conditions to estimate viability probably reflect differential ways to express cell damage and recovery.

It could be argued that the action of the trehalose during dehydration is due to an active utilization of the sugar by the cells so that some metabolic advantage for survival would develop in these cells as compared to control cells, dehydrated in the absence of the sugar. However, such interpretation can not be maintained since *C. utilis* is unable to transport trehalose from the outside and therefore to use this ex-

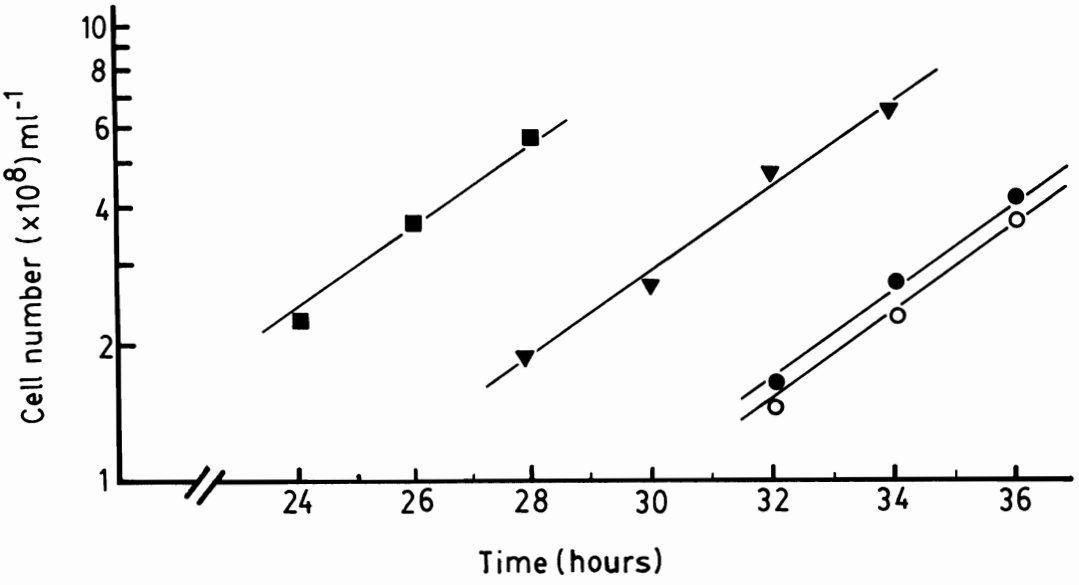

FIGURE 2. Growth of log-phase cells of *Candida utilis* dehydrated in the absence (○) or presence of 2 mM (●), 20 mM (▼), or 200 mM (■) trehalose. The same number of treated cells was used as inoculum in all cases (see Materials and Methods).

Crecimiento de células exponenciales de *Candida utilis* en ausencia (○) o presencia de trehalosa 2 mM (●), 20 mM (▼), o 200 mM (■). En todos los casos se utilizó como inóculo el mismo número de células tratadas (ver Materiales y Métodos).

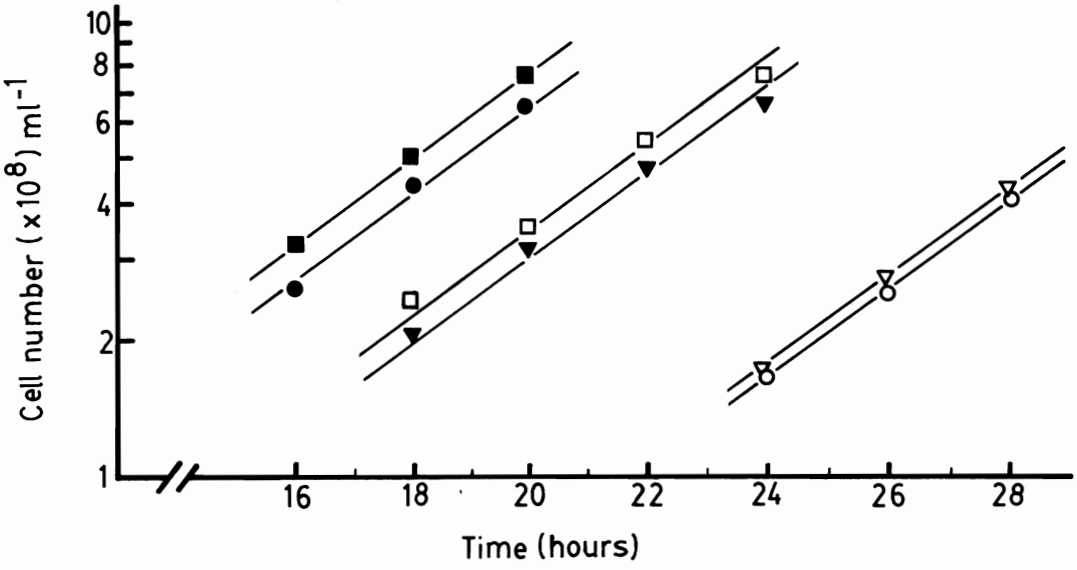

FIGURE 3. Growth of stationary-phase cells of *Candida utilis* dehydrated in the absence (○) or presence of various compounds (▽ sucrose; ▼ lactose; □ maltose; ● glycerol; ■ cellobiose. All compounds were at a final concentration of 200 mM.

Crecimiento de células estacionarias de *Candida utilis* en ausencia (○) o presencia de varios compuestos (▽ sacarosa; ▼ lactosa; □ maltosa; ● glicerol; ■ celobiosa). Todos los compuestos se utilizaron a una concentración final de 200 mM.

ternal sugar as carbon and energy source (ARGÜELLES *et al.*, 1985).

In addition, even if trehalose was transported into the cells, the amount of trehalose passed with the inoculum to the media used for stimating viability would be negligible (less than 1 mM) as compared to glucose (2%) whose presence, in any case, would repress its active utilization. Thus, the differences shown in colony forming ability or growth delay, taken in this study as criteria of differential viability, do not represent side effects due to initial metabolic differences between the cells subjeted to desiccation with or without this external sugar available.

As far as resistance to desiccation is concerned, exogenously added trehalose simulates «in vivo» conditions in which trehalose acts internally (see, for example, fig. 1: log-phase cells supplemented with trehalose behave as stationary-phase cells without added trehalose during the dehydration procedure). This observation, together with the unability of *C. utilis* cells to transport trehalose from the outside, suggests that the protective role is mainly a physical membrane effect. Thus, confirming previous findings that suggested a target at the membrane level (HOTTIGER *et al.*, 1987; MARTIN *et al.*, 1986; MCBRIDE & ENSING, 1987a, 1987b; GADD *et al.*, 1987), trehalose appears to be operative as protective agent both when it is outside or inside the cell. It is tempting to speculate that during dehydration trehalose could help to maintain the functional integrity of some cell components by acting as a substitute for water throughout its interaction with the polar portion of membrane phospholipids (CROWE *et al.*, 1984c; RUDOLPH & CROWE, 1985). This action would stabilize and preserve the activity of key enzyme systems bound to the membrane structture (MARTIN *et al.*, 1986).

The nonreducing character of trehalose has been considered to be an important feature for serving as protective agent because potentially harmful reactions with cell proteins can be avoided (MCBRIDE & ENSING, 1987a). Nevertheless, our results indicate that the protective effect is not exclusive of trehalose. The presence of a reducing disaccharide (such as cellobiose) or compounds that are not estructurally related to trehalose (such as glycerol) in the external medium during dehydration may increase the survival of the yeast cells even much more effectively than trehalose. The basis for the differential effects on cell viability carried out by the others compounds assayed and their significance are not clear. However, the physiological implications of the effect of trehalose appears to be relevant because, contrariwise to the others compounds, this sugar is the only disaccharide known to occur inside yeast cells and to accumulate from intermediate precursor metabolites (THEVELEIN, 1984).

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