ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 6, pp. 639–643. © Pleiades Publishing, Inc., 2006.
Published in Russian in Mikrobiologiya, 2006, Vol. 75, No. 6, pp. 737–741.

EXPERIMENTAL ARTICLES

Comparative Analysis of Glycogen and Trehalose Accumulation in Methylotrophic and Nonmethylotrophic Yeasts[1]

S. Türkel

Uludag University, Faculty of Arts and Sciences, Department of Biology, 16059, Bursa, Turkey

Received December 28, 2005

Abstract—Trehalose and glycogen accumulate in certain yeast species when they are exposed to unfavorable growth conditions. Accumulations of these reserve carbohydrates in yeasts provide resistance to stress conditions. The results of this study indicate that certain *Pichia* species do not accumulate high levels of glycogen and trehalose under normal growth conditions. However, depending on the *Pichia* species, both saccharides accumulate at high levels when the *Pichia* cells are exposed to unfavorable or stress-inducing growth conditions. Growth in glycerol or methanol medium mostly led to trehalose accumulation in *Pichia* species tested in this study. It was shown that the metabolic pathways for glycogen and trehalose biosynthesis are present in *Pichia* species. However, it appears that the biosynthesis of trehalose and glycogen may be regulated in different manners in *Pichia* species than in the yeast *S. cerevisiae*.

DOI: 10.1134/S0026261706060038

Key words: *Pichia*, yeast, trehalose, glycogen, stress response.

INTRODUCTION

Methylotrophic yeasts have an outstanding potential for various industrial applications. At present, *Pichia anomala* and *Pichia angusta* are being used for the large-scale production of biologically active proteins [1]. Certain strains of *P. anomala* are also used as biocontrol yeast to protect against mold spoilage [2].

Glycogen and trehalose are the major storage carbohydrates in *Saccharomyces cerevisiae* [3]. Depending on the growth conditions, these carbohydrates can accumulate to up to 25% of the cell dry mass. Trehalose accumulates in response to unfavorable growth conditions such as high temperature, high osmolarity, and nutrient limitations [3–7]. The major function of trehalose is to protect cell membranes and cellular proteins from denaturation [7]. The enzyme complex that catalyzes the biosynthesis of trehalose is also involved in the regulation of the glycolytic pathway in *S. cerevisiae* [8, 9].

There is no detailed information on the biosynthesis and the functions of trehalose and glycogen in methylotrophic yeasts. In this study, accumulations of glycogen and trehalose were investigated in three different species of the *Pichia* genus to explore the levels of storage carbohydrates of the methylotrophic yeasts that are cultivated under different growth conditions.

[1] The text was submitted by the author in English.

MATERIALS AND METHODS

Yeast strains. Methylotrophic yeasts *Pichia anomala, Pichia farinosa*, and *Pichia angusta* were obtained from the DBVPG collection (University of Perugia, Italy). *Saccharomyces cerevisiae* strain (MC996A), which is used as a reference strain in this study, was obtained from E. Boles (Heinrich Heine Universitat Düsseldorf, Germany).

Determination of trehalose and glycogen. In order to determine the time-course dependent accumulation of trehalose and glycogen, the yeast strains were precultured in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) up to the stationary stage (60 h) first [10]. Then, these cultures were used to inoculate the yeast strains to the fresh YPD mediums. The initial cell densities of the yeast cultures were adjusted to OD_{600} = 0.2. The yeast strains were cultivated in a 30°C orbital shaker (140 rev/min) for 60 h. Yeast samples (50–60 mg wet mass) were removed every 6 h. The trehalose and glycogen contents of the samples were determined by the method of Parrou and Francois [11].

Furthermore, to test the effects of non-fermentable carbon sources on the glycogen and trehalose accumulation, yeast strains were also cultivated in YP medium supplemented with 1% methanol or 2% glycerol up to the stationary stage and then glycogen and trehalose contents were analyzed. Concentrations of glycogen and trehalose were expressed as mg of glucose equivalent per g of wet mass (mg/g) of the yeast cells [11]. Cultivation of the yeast strains was done in duplicates

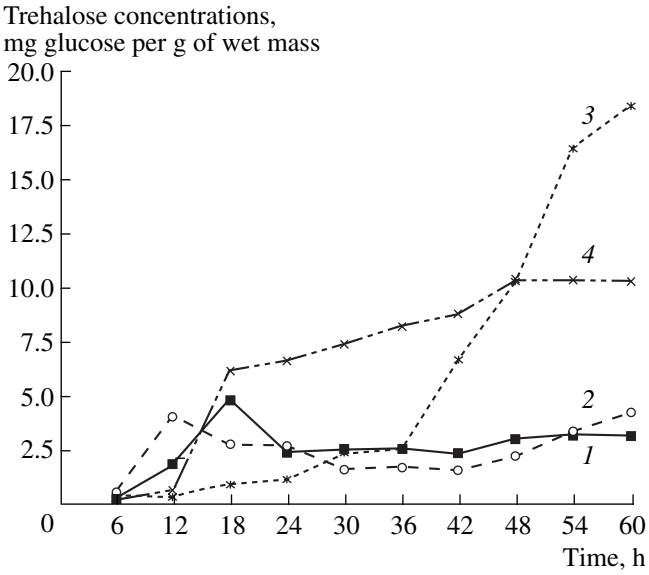

Fig. 1. Trehalose accumulation patterns in the yeast strains in a time-course-dependent manner. *1—P. anomala*; *2—P. farinosa*; *3—P. angusta*; *4—S. cerevisiae*.

and the experiments were repeated three times. Hence, the glycogen and trehalose contents given in the tables and figures are the average values of the six independent measurements.

Application of stress conditions. In order to analyze the effects of heat stress on the trehalose and glycogen accumulation, yeast strains were precultured in YPD medium in duplicates (20 ml) at 25°C with constant shaking up to OD_{600} = 0.5. Then portions of the yeast cultures (10 ml) were transferred to a 37°C water bath for heat shock [5]. The cells were incubated for 2 h at 37°C, and then harvested for glycogen and trehalose determination as described [11].

For nitrogen starvation, 10 ml of the logarithmically growing yeast cells (OD_{600} = 0.8) were harvested, washed twice with sterile distilled water, then resuspended in 10 ml of YNB medium without ammonium sulfate and the amino acids were supplemented with 2% dextrose [6]. Yeast cells were incubated at 30°C with constant shaking (140 rev/min) for 3 h in an orbital shaker. Then the glycogen and trehalose contents of the yeast cells were determined as explained [11].

RESULTS

Time-course analysis of trehalose and glycogen accumulation in *Pichia* species. In order to determine the exact timeframe at which trehalose and glycogen start to accumulate and to test if there is any fluctuation in the accumulation patterns of these reserve carbohydrates among the *Pichia* species, the trehalose and glycogen contents of the yeast strains were analyzed in a time-course-dependent manner. The growth curves of each strain indicated that the *Pichia* species reached higher cell densities than *S. cerevisiae* at the end of the 60-h growth period (data not shown). It is clear that the yeast strains enter the stationary stage at the end of the 30-h incubation period in YPD medium.

The degradation of previously accumulated trehalose took place very rapidly when the stationary-stage yeast cells were inoculated into fresh medium (Fig. 1). After inoculation to a fresh YPD medium, the trehalose contents of all four yeast species decreased dramatically to nearly zero within six hours. Accumulation of trehalose starts at the late logarithmic stage in *S. cerevisiae* and it remains at high levels (8–10 mg/g) through the stationary phase (Fig. 1).

Unlike in *S. cerevisiae*, trehalose accumulation in *P. farinosa* initiates at an earlier stage but remains at low levels. The trehalose accumulation pattern in *P. anomala* shows similarity to those of *P. farinosa* and also remains at low levels during the entire stationary stage. Moreover, the trehalose contents of *P. farinosa* and *P. anomala* decrease significantly (up to 50% compared to their levels at the mid-to-late logarithmic stage) once they enter the stationary stage (Fig. 1). Trehalose accumulation in *P. angusta* occurs in a very different manner. The trehalose amount remains at low levels (1–2 mg/g wet mass) until the mid-stationary phase (Fig. 1). But, after 36 h, trehalose suddenly begins to accumulate in *P. angusta* cells and reaches a very high level (18 mg/g) during the late stationary stage.

The degradation of reserve glycogen also took place very rapidly in *P. angusta* and in *S. cerevisiae* when they were inoculated into fresh YPD medium. However, glycogen degradation in *P. anomala* did not occur during the first six hours of the growth period in fresh medium (Fig. 2). In fact, the glycogen content of *P. anomala* increased slightly during the early stage of the logarithmic growth phase. Glycogen accumulation started at the end of the logarithmic stage in *S. cerevisiae* as expected. Its level gradually increases (up to 6.39 mg glucose equivalent/g wet mass of yeast) through the stationary phase (Fig. 2). Unlike *S. cerevisiae*, glycogen accumulation in *P. angusta* initiates at the late stationary stage (at 42 h). However, the glycogen content of *P. angusta* increases very rapidly and reaches much higher levels (up to 10 mg/g) then in *S. cerevisiae*. The glycogen content of *P. farinosa* remains at low levels during the entire growth period. This result indicates that there is no clear accumulation of glycogen in *P. farinosa* when it is cultivated in YPD medium. A low level of glycogen accumulation (2.29 mg/g) was determined in *P. anomala* after 60 h of growth in YPD medium.

Effect of carbon sources on trehalose and glycogen accumulation. In order to analyze the effects of nonfermentable carbon sources on the trehalose and glycogen contents in *Pichia* species, these yeasts were also grown in methanol or glycerol containing medium up to the stationary stage. Especially in *P. anomala*,

glycogen and trehalose accumulation is very low in methanol-grown cells. But the growth of *P. anomala* in glycerol medium induced high levels (9.30 mg/g wet mass) of trehalose accumulation. The glycogen level in *P. anomala* and *P. farinosa* were also very low in glycerol-grown cells. However, the trehalose content of *P. farinosa* increased to 5.82 mg/g wet mass when it was grown in methanol. The growth of *P. farinosa* in glycerol medium also activated the trehalose accumulation in this yeast strain (Table 1).

The growth of *P. angusta* in glycerol-containing medium resulted in high levels of accumulation of glycogen and trehalose—measured as 4.91 and 12.34 mg/g wet mass of yeast cells, respectively. In addition, the growth of *P. angusta* in methanol medium also led to high level accumulation of trehalose (up to 12 mg/g) (Table 1). Low levels of glycogen accumulations were also observed in methanol-grown *P. angusta* cells. Both the glycogen and trehalose contents of *S. cerevisiae* cells increased dramatically when grown in glycerol.

Effects heat stress and nitrogen starvation on the reserve carbohydrate accumulation in *Pichia* species. The glycogen amount was measured as 2.56 mg/g wet mass in logarithmic stage *P. anomala* cells when grown in YPD medium. However, the glycogen level of *P. anomala* decreased by approximately 40% (from 2.56 to 1.63 mg/g wet mass) after heat stress (Table 2). Trehalose biosynthesis in *P. anomala* was activated by heat stress and determined as 6.34 mg/g wet mass of *P. anomala* after 2 h of heat stress (Table 2). This was severalfold higher than the trehalose amount of the stationary-stage cells of *P. anomala* (Fig. 2).

Interestingly, neither glycogen nor trehalose biosynthesis was induced at significant levels by heat stress in *P. angusta*. The glycogen and trehalose amounts remained at very low levels (0.4–1.03 mg/g wet mass) before and after heat stress in *P. angusta* at the logarithmic stage. However, trehalose synthesis in *P. farinosa* was induced by heat stress and resulted in the accumulation of (from 0.06 mg/g to 2.84 mg/g wet mass) moderate levels of trehalose (Table 2). As expected, heat stress also activated trehalose biosynthesis in *S. cerevisiae* and resulted in 5.10 mg/g of trehalose accumulation.

Nitrogen starvation led to a high amount of trehalose and glycogen accumulation in *P. anomala*. When compared to normally grown logarithmic stage *P. anomala* cells, glycogen contents increased approximately fivefold (from 2.56 to 13.66 mg/g wet mass) upon nitrogen starvation in this methylotrophic yeast (Table 2). Moreover, nitrogen starvation also induced a significant level of trehalose accumulation (6.69 mg/g) in *P. anomala*. However, the levels of trehalose and glycogen remained at their base levels in *P. farinosa* after nitrogen starvation (Table 2). Hence, it appears that nitrogen starvation does not trigger a high level of accumulation of glycogen and trehalose in *P. farinosa*.

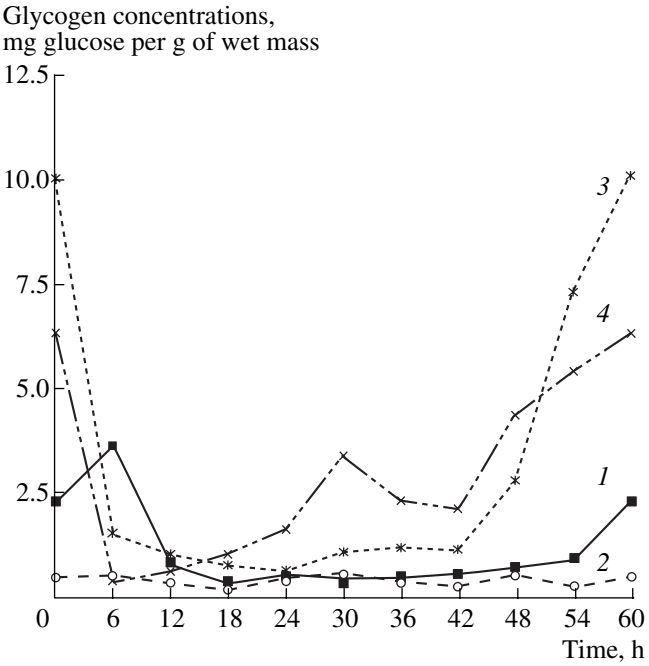

Fig. 2. Glycogen accumulation patterns in the yeast strains in a time-course-dependent manner. *1—P. anomala*; *2—P. farinosa*; *3—P. angusta*; *4—S. cerevisiae*.

Nitrogen starvation induced glycogen and trehalose accumulation in *P. angusta*. After 3 h of incubation in the growth medium without nitrogen, the glycogen and trehalose contents of *P. angusta* cells were determined as 4.24 and 2.20 mg/g, respectively (Table 2). In *S. cerevisiae*, nitrogen starvation resulted in a high level of glycogen and trehalose accumulations as expected (Table 2).

DISCUSSION

Trehalose and glycogen accumulate in *S. cerevisiae* when it is subjected to unfavorable growth conditions [3–7]. However, the results presented in this study indi-

Table 1. Trehalose and glycogen contents of the various *Pichia* species and *S. cerevisiae* grown in methanol or glycerol medium up to the stationary stage

Yeasts	Carbon sources	Glycogen*	Trehalose
P. anomala	Methanol, 1%	0.24	1.46
	Glycerol, 2%	0.43	9.30
P. farinosa	Methanol, 1%	0.60	5.82
	Glycerol, 2%	0.70	7.10
P. angusta	Methanol, 1%	1.83	12.13
	Glycerol, 2%	4.91	12.34
S. cerevisiae	Glycerol, 2%	11.72	9.97

* Glycogen and trehalose concenrtations were expressed as mg glucose per gram of wet mass of the yeast cells.

Table 2. The effects of heat stress and nitrogen starvation on trehalose and glycogen contents of different *Pichia species*

Yeasts	Glycogen*			Trehalose		
	Normal growth	Heat stress	Nitrogen starvation	Normal growth	Heat stress	Nitrogen starvation
P. anomala	2.56	1.63	13.66	0.17	6.34	6.69
P. farinosa	0.18	0.28	1.56	0.06	2.84	1.85
P. angusta	0.87	0.42	4.24	0.06	1.03	2.20
S. cerevisiae	0.41	1.10	9.97	0.03	5.10	5.46

* Glycogen and trehalose concentrations were expressed as mg glucose per gram of wet mass of the yeast cells.

cate that the accumulations of the reserve carbohydrates trehalose and glycogen occur in a different manner in certain methylotrophic yeasts as compared to in bakers' yeast *S. cerevisiae*.

It is clear that the strains of *P. anomala* and *P. farinosa* species do not accumulate high levels of trehalose when they grown in glucose-containing medium. Accumulation of trehalose and glycogen begins at the late stationary stage in *P. angusta*. Hence, it is conceivable that glucose depletion in the growth medium is not a signal for trehalose accumulation in *P. angusta*. It appears that, in addition to glucose depletion, other metabolic signals are required to initiate the accumulation of trehalose in this *Pichia* species.

Methanol can be used as a carbon and energy source by methylotrophic yeasts. It has been reported that the major products of the methanol metabolism are trehalose and glycerol in the methylotrophic yeast *Hansenula polymorpha* (*P. angusta*) [12]. Hence, the cultivation of *P. farinosa* and *P. angusta* in methanol resulted in a high level of accumulation of trehalose rather than the glycogen. But this was not the case in *P. anomala*. Hence, it can be suggested that in certain methylotrophic yeasts like *P. anomala*, the major end product of methanol catabolism may not be trehalose [12, 13].

When heat stress is applied to the logarithmically growing *Pichia* cells, like *S. cerevisiae* cells, high levels of trehalose accumulations were detected in *P. anomala* and *P. farinosa* cells. Interestingly, heat stress led to a significant decrease in the glycogen content of *P. anomala* cells. This may result from the stress-induced recycling of glycogen [5]. Heat stress did not lead to trehalose accumulation in *P. angusta*. But apparently, nitrogen starvation induced biosynthesis and the accumulation of trehalose and glycogen in this *Pichia* species, suggesting that heat stress and nitrogen starvation do not have an overlapping signal transduction pathway in the activation of trehalose and glycogen biosynthesis in *P. angusta*.

Pichia fermentans accumulate trehalose when grown in glucose [14]. But when this *Pichia* species depletes free glucose in the growth medium, intracellular trehalose is rapidly degraded to glucose by highly active trehalose phosphorylase [14]. A similar situation may occur in *P. anomala* and *P. farinosa* when they are grown in glucose. However, trehalose phosphorylase may not be active when these *Pichia* species are exposed to heat stress or nitrogen starvation. This situation may lead to a high level of accumulation of trehalose in *P. anomala* and *P. farinosa* during heat stress or nitrogen starvation.

Recently, a metabolite profile of the strain of *P. anomala* has been investigated after growth in oxygen-limiting conditions [13]. The results of that study showed that trehalose, arabitol, and glycerol accumulate in the *P. anomala* strain in response to oxygen limitation. It is conceivable that glycerol and arabitol may accumulate more than trehalose in certain *Pichia* species such as *P. farinosa*. Trehalose and glycogen accumulations do not seem to be general phenomena in all yeasts. Trehalose and glycogen biosynthesis is not induced in *Candida albicans* in response to stress conditions [15]. Previous reports indicate that sterol glycosides and cerebrosides accumulate under stress inducing conditions in certain *Pichia* species and in other nonconventional yeasts [16].

It is clear that trehalose and glycogen biosynthesis pathways are present in *Pichia* species, but the results of this study indicate that reserve carbohydrate metabolism may operate in a completely different manner in various *Pichia* species than in *S. cerevisiae* cells.

ACKNOWLEDGMENTS

This study was supported by a grant from The Scientific and Technical Research Council of Turkey (TüBITAK, TBAG-AY/265).

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