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The involvement of trehalose in yeast stress tolerance

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SUMMARY

A total of 12 yeast strains from various genera were examined for their ability to produce ethanol in the presence of high concentrations of glucose. From these studies, the yeasts *Torulaspora delbrueckii* and *Zygosaccharomyces rouxii* were observed to be the most osmotolerant. These osmotolerant yeast strains were also observed to possess high concentrations of intracellular trehalose. Futhermore, these strains were found to be tolerant to long-term storage at −20 °C and to storage at 4 °C in beer containing 5% (v/v) ethanol. Cells containing high trehalose levels at the time of freezing or cold storage exhibited the highest cell viabilities. Trehalose concentration was observed to increase during growth on glucose, reaching a maximum after 24–48 h. Increasing the incubation temperature from 21 to 40 °C also resulted in an increase in intracellular trehalose content. These results suggest that trehalose plays a role in enhancing yeast survival under environmentally stressful conditions.

INTRODUCTION

There are a number of factors which can affect or influence yeast fermentation rates. These include cell density, cell viability, fermentation temperature, media composition, fermentable carbohydrate composition, osmotic pressure and ethanol concentration effects [2,5,19]. The fermentation of sugars in brewing is generally carried out by employing yeasts that produce 4–9% (v/v) ethanol. Over the years there has been increasing interest in high-gravity brewing. However, high substrate concentrations have been shown to inhibit both yeast growth and fermentation as a result of high osmotic pressure and low water activity [1,7,10]. The protection of yeast under such adverse environmental conditions is a major requirement.

Trehalose is a non-reducing disaccharide consisting of two glucose moieties linked together by an α-1,1-glycosidic bond. The intracellular concentration of trehalose has been suggested to play an important role in the ability of many organisms to tolerate adverse environmental conditions [20,22]. For example, trehalose has been shown to be an important factor in stabilizing dry membranes in anhydrobiotic organisms and has been proposed to serve as a protectant against severe dehydration and desiccation stress [3,6,8,11]. Furthermore, it has been suggested that trehalose also plays a protective role in osmoregulation [22], protecting cells in conditions of nutrient limitation and starvation [6,12] and enhances cell resistance to external conditions such as high and low temperatures [6,20,22]. It is conceivable, therefore, that high trehalose levels in yeast may be associated with increased osmotolerance, thermotolerance and ethanol tolerance. Such an increase in yeast stress tolerance will result in enhanced fermentation ability. This manuscript reports on the relationship between trehalose concentration and yeast viability under these environmentally stressful conditions.

MATERIALS AND METHODS

Yeast strains and growth medium

The 12 yeast strains employed in this study are listed in Table 1 and were from the John Labatt Limited Culture Collection. The yeast cells were subcultured in PYN medium which consisted of: peptone, 3.5 g; yeast extract, 3.0 g; KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $(NH_4)_2SO_4$, 1.0 g; and glucose, 100 g; all dissolved in 1 l of distilled water and adjusted to pH 5.6.

Fermentation conditions

Fermentations were conducted in PYN medium containing 50% (w/v) glucose. The glucose was added to the PYN broth prior to autoclaving. Fermentations were carried out at 30 °C in 300-ml Erlenmeyer flasks containing 100 ml of medium, with constant agitation of 150 rpm. The yeast inoculum employed was 3.5 g wet weight cells/l with cell viabilities of greater than 90% in all cases. The cells were pregrown for 16 h in PYN

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medium containing 10% glucose. Ethanol was determined using a Carle AGC series 100 gas chromatograph as described elsewhere [4].

Yeast freezing

Cells were grown in 100 ml of PYN medium containing 5% glucose at 30 °C. After 24 h, 30 ml of cell suspension was centrifuged at 4000 × *g* for 10 min at 4 °C and washed twice with ice-cold sterile distilled water. The pellets were suspended in 15 ml PYN medium containing 5% glucose and frozen in 5 ml portions at −20 °C for up to 12 weeks with cell viabilities determined every 4 weeks.

Yeast storage in beer

Cells were grown for 24 or 144 h in 16 degree plato brewery wort. The cells were centrifuged at 4000 × *g* for 10 min at 4 °C, washed twice with ice-cold sterile distilled water and suspended in beer containing 5% (v/v) ethanol at a concentration of 10 g wet weight of cells/20 ml. The suspension was stored at 4 °C for 30 days at which time the viability was determined.

Viability determination

Percent cell viability was determined by plating four representative samples per sample time on PYN agar plates containing 2% glucose and incubating at 30 °C for 3 days. Cells were serially diluted in sterile distilled water and 0.1 ml cell suspension surface-plate to give approximately 300 colonies per plate. Percent viability was calculated by the number of colonies at a specific time point divided by the number of colonies at the time of freezing or cold storage.

Trehalose assay

Yeast strains were grown for 24 h in PYN medium containing 5% glucose at 30 °C. The cells (20 ml suspension) were washed twice with ice-cold sterile distilled water and centrifuged at 4000 × *g* for 10 min at 4 °C. The trehalose was extracted from the cells with 4 ml of ice-cold 0.5 M trichloroacetic acid by agitation for 20 min at 4 °C followed by centrifugation at 4000 × *g* for 10 min at 4 °C [21]. This procedure was repeated three times. The supernatants from the extractions were pooled and trehalose determined by the anthrone method of Trevelyan and Harrison [21]. For dry weight determinations, 10-ml cell suspensions were washed twice with distilled water at 21 °C, suspended in 5 ml of distilled water and dried in an aluminum dish at 100 °C for 4 h.

RESULTS AND DISCUSSION

A total of 12 yeast strains from various genera were examined for their ability to produce ethanol in the presence of high concentrations of glucose. The strains producing the highest levels of ethanol were considered to be osmotolerant [4,5]. The results presented in Table 1 indicate that the yeast strains *Torulaspora delbrueckii* 236, *Zygosaccharomyces rouxii* 233 and fusion product 1648, a strain obtained from the fusion of these two strains, were the most osmotolerant. These strains produced the highest levels of ethanol from 50% glucose (w/v) after 48 h of fermentation compared to the other strains.

The intracellular trehalose concentration in the various yeast strains was then assayed to determine if there is any correlation between trehalose content and osmotolerance. Table 2 indicates that the yeast strains

TABLE 1

Ethanol production from 50% glucose in PYN medium by various strains

Yeast strain	Labatt code	Ethanol (g/l)[a]
S. cerevisiae	3001	4.0
S. rouxii	233	30.1
T. delbrueckii	236	40.3
S. cerevisiae	67	6.2
S. cerevisiae	267	5.2
S. cerevisiae	254	2.5
S. cerevisiae	275	5.4
K. marxianus	1510	0.7
S. cerevisiae	299	3.9
S. carlsbergensis	1488	0.4
S. cerevisiae	1489	0.3
Fusion Product (233 × 236)	1648	15.0

[a] Ethanol produced after 48 h fermentation.

TABLE 2

Initial trehalose concentration and cell viability after storage at −20 °C

Yeast strain	Trehalose (μg/mg dry weight)	% Viability		
		4 weeks	8 weeks	12 weeks
233	21.9	59	52	37
236	26.2	70	54	46
67	8.3	3	−ve[a]	−ve
254	7.2	3	2	−ve
267	4.8	5	4	−ve
275	6.8	3	3	−ve
299	4.7	5	1	−ve
1488	8.3	−ve	−ve	−ve
1489	11.4	−ve	−ve	−ve
1648	25.4	69	62	31

[a] −ve: no viable cells detected.

shown to be osmotolerant in Table 1 contained the highest levels of intracellular trehalose. In addition, these yeast strains were examined for their ability to tolerate long-term frozen storage. After 12 weeks of frozen storage at − 20 °C, the yeast strains containing the largest levels of trehalose at the time of freezing exhibited the highest cell viabilities. The yeast strains *T. delbrueckii* 236, *Z. rouxii* 233 and fusion product 1648 possessed greater than 30% cell viability after 12 weeks of frozen storage. In strains with low intracellular trehalose, no viable cells were detected after 12 weeks of frozen storage. In a separate study Oda et al. [14] selected 11 yeast strains suitable for use in frozen dough. The selected yeast strains possessed the ability to accumulate trehalose intracellularly. The increased viability of these yeast cells in frozen dough was suggested to be related to the higher trehalose content [14]. Furthermore, a strain of *T. delbrueckii* has been developed as a bakery yeast which is tolerant to high sugar concentrations and to freeze-thawing in dough [15,18].

Trehalose and glycogen can be accumulated in yeast in similar amounts, but the mode of accumulation is quite different. Glycogen is formed only during the exponential phase of growth of the yeast and is subsequently depleted. On the other hand, trehalose levels have been reported to be high in stationary phase cells and extremely low during rapid growth [20,22]. The intracellular trehalose content of a number of yeast strains was monitored during growth on PYN media containing 5% glucose. As can be seen in Fig. 1A with the osmotolerant *Z. rouxii* strain 233, the intracellular concentration of trehalose increased during growth reaching a maximum after 24–48 h. Associated with this increase in trehalose concentration was an increase in yeast survival after 30 days frozen storage at − 20 °C. Similar results were observed with *T. delbrueckii* 236 and fusion product strain 1648 (data not shown). On the other hand, there was no increase in trehalose concentration in *Saccharomyces cerevisiae* strain 67 (Fig. 1B). Furthermore, there were virtually no viable cells remaining after 30 days of frozen storage. This strain was shown in Table 1 to be osmosensitive, unable to ferment 50% glucose. Similar results were also observed with the other osmosensitive yeast strains (data not shown). These results strongly suggest a correlation between the intracellular trehalose concentration and the ability of the yeast to survive long-term frozen storage. A similar conclusion was made by Gadd et al. [6] in determining the role of trehalose in dehydration resistance of *S. cerevisiae*. They observed that high levels of intracellular trehalose in stationary phase yeast cells resulted in an increase in resistance to dehydration. On the other hand, exponential-phase cells which contained low levels of trehalose exhibited negligible dehydration resistance.

Other investigators have shown that trehalose metabolism is influenced by heat stress [8,9,22]. A large increase in trehalose synthesis was observed when yeasts are heated to 40 °C [9]. It was found that trehalose content and thermotolerance of cells were closely correlated during heat shock and during recovery, suggesting that trehalose may act as a thermoprotectant [8,9]. The effect of temperature on trehalose accumulation was investigated in *T. delbrueckii* strain 236 and *S. cerevisiae* strain 67 (Fig. 2). Increasing the incubation temperature from 21 °C to 40 °C resulted in a large increase in trehalose content in the osmotolerant strain 236 after 45–60 min incubation (Fig. 2A). On the other hand, there was only a slight increase in trehalose content in the osmosensitive strain 67 after 60 min treatment at 40 °C (Fig. 2B). These results support the suggestion that heat treatment in tolerant strains can result in an increase in trehalose accumulation. Heat-induced trehalose accumulation protects growing yeast cells against heat and desic-

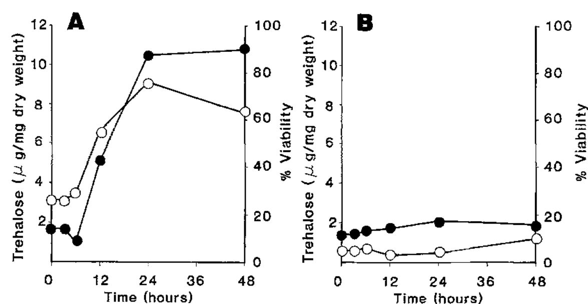

Fig. 1. Intracellular trehalose concentration during growth and its effect on cell viability. *Z. rouxii* 233 (**A**) and *S. cerevisiae* 67 (**B**) were grown on 5% glucose for various lengths of time, trehalose analysed and viability determined after 30 days storage at − 20 °C. (●) indicates trehalose concentration and (○) percent viability.

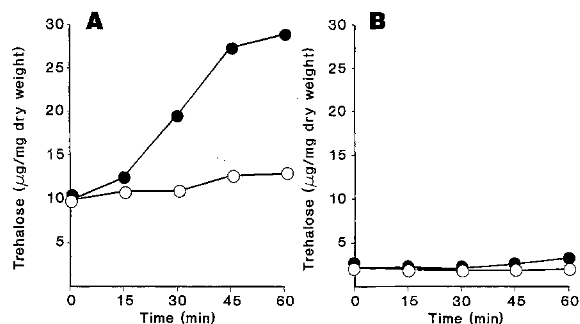

Fig. 2. Effect of temperature on intracellular trehalose concentration. *T. delbrueckii* 236 (**A**) and *S. cerevisiae* 67 (**B**) were grown on 5% glucose for 12 h at 21 °C. Half of the suspension was incubated at 21 °C (○) and the other half incubated at 40 °C (●). Trehalose was determined every 15 min for 60 min.

cation stress [8]. The increase in heat tolerance is probably due to the stabilizing effect of trehalose on membranes [3,8,9]. This property may be of beneficial importance to the baker's yeast industry. An increase in trehalose accumulation has also been recently demonstrated in a catabolite derepressed mutant yeast strain [13]. This accumulation of trehalose may be related to the constitutive utilization of maltose in this organism [16].

The role of trehalose in the maintenance of yeast cell viability during typical brewing storage conditions was investigated. To simulate brewery yeast storage conditions, yeast cells were grown in 16° plato wort and stored in beer containing 5% (v/v) ethanol at 4 °C for 30 days. Table 3 shows the initial trehalose concentration and percentage cell viability after storage. As expected, the yeast cells contained a much higher intracellular trehalose content after 144 h of growth versus after only 24 h of growth. Furthermore, yeasts with higher trehalose content resulted in greater survival after 30 days of cold storage. This was even noted for the ale-brewing strain 3001, which had a large increase in trehalose content when grown for 144 h corresponding to a higher cell viability as compared to cells grown for only 24 h. Recently, Sall et al. [17] have suggested that fermentation performance and viability of yeast stored under industrial conditions is independent of glycogen or trehalose content. These investigators, however, did not correlate initial trehalose content with subsequent yeast performance and viability after storage, but only monitored the trehalose content during storage. Therefore, trehalose concentration may be an important determinant in yeast fermentation performance and the maintenance of cell viability.

In summary, the results presented in this manuscript demonstrate that yeast strains possessing the ability to ferment in the presence of high concentrations of glucose (i.e., osmotolerant) contain high concentrations of trehalose. These yeast strains were also shown to be tolerant to long-term frozen storage at −20 °C and to storage at 4 °C in 5% (v/v) ethanol beer. Cells containing high trehalose levels exhibited the greatest cell survival rates after storage. Increasing the incubation temperature also resulted in an increase in intracellular trehalose content.

TABLE 3

Initial trehalose concentration and percent viability after storage at 4 °C in beer

Yeast strain	Trehalose concentration at time of storage (μg/mg dry weight)		% Viability after 30 days	
	A	B	A	B
233	3.6	7.1	23	90
236	1.7	22.1	22	99
1648	2.5	25.3	26	99
3001	1.1	11.4	14	50

Yeasts were grown for (A) 24 h or (B) 144 h in 16° Plato wort, trehalose determined and cells suspended in beer containing 5% (v/v) ethanol at 4 °C for 30 days.

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