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Analysis of the Effects of Hyperosmotic Stress on the Derepression of Invertase Activities and the Growth of Different Baker's Yeast Strains

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Abstract: The growth of baker's yeast *Saccharomyces cerevisiae* in medium containing sucrose requires a high level of extracellular invertase enzyme activity. However, the expression of invertase is under the strict control of glucose repression in *S. cerevisiae*. In addition, invertase enzyme activity is also affected by physiological stress conditions that baker's yeast is exposed to during the various stages of industrial level production and downstream processing. We analyzed the effect of hyperosmotic stress on the derepression of invertase activities of a haploid laboratory yeast strain and three different industrial baker's yeast strains. Our results indicated that hyperosmotic stress interferes with the derepression of invertase activity in the yeast. The invertase activities of the yeast strains remained essentially at a repressed level in the presence of 1M NaCl or 1M KCl in the growth medium. However, the presence of low amounts of NaCl in the growth medium (0.2M) increased the invertase activities of yeast strains up to 40-50%. We also found that industrial yeast strains are more sensitive to hyperosmotic stress than the laboratory strains of *S. cerevisiae* used in this study.

Key Words: Invertase, *SUC2*, *S. cerevisiae*, hyperosmotic stress, glucose repression

Farklı Maya Suşlarının Üremelerine ve İvertaz Aktivitelerinin Derepresyonuna Hiperosmotik Stresin Etkilerinin Analizi

Özet: *Saccharomyces cerevisiae*'nin sukroz içeren ortamda üremesi için fazla miktarda hücre dışı invertaz enzim aktivitesi olması gerekir. Fakat, invertaz enziminin sentezi de çok sıkı bir şekilde glukoz baskılanması altındadır. Buna ek olarak, invertaz enzim aktivitesi *S. cerevisiae*'nin endüstriyel üretimi ve üretim sonrası işlemleri sırasında bulunduğu ortamların fizyolojik stres şartlarından da etkilenmektedir. Bu çalışmada hiperosmotik stresin invertaz aktivitesi derepresyonuna etkileri *S. cerevisiae*'nin haploid laboratuvar suşu ve üç farklı endüstriyel *S. cerevisiae* suşu kullanılarak araştırıldı. Elde ettiğimiz sonuçlar hiperosmotik stresin mayadaki invertaz aktivitesinin derepresyonunu durdurduğunu gösterdi. Bu çalışmada kullanılan maya suşlarının invertaz aktivitelerinin üreme ortamında 1M NaCl veya 1M KCl bulunduğu glukoz baskılanmasının olduğu miktarlarda kaldığı görüldü. Fakat düşük miktardaki NaCl'nin (0.2M) ortamda bulunması invertaz aktivitesini % 40-50 kadar arttırdı. Ayrıca, bu çalışmada kullanılan endüstriyel maya suşlarının laboratuvar suşlarına göre osmotik strese karşı çok fazla duyarlı oldukları bulundu.

Anahtar Sözcükler: İvertaz, *SUC2*, *S. cerevisiae*, hiperosmotik stres, glukoz baskılanması

Introduction

Sucrose and raffinose are hydrolyzed by the invertase enzyme of yeast *S. cerevisiae*. There are two different forms of invertase. Extracellular invertase is highly glycosylated while intracellular invertase is not glycosylated (1). The physiological significance of intracellular invertase is not known. Both intracellular and extracellular invertases are expressed from the *SUC2* gene (2).

Expression of the *SUC2* gene is regulated by multiple transcription factors in response to various physiological and environmental signals. The transcription of *SUC2* is repressed by Hxk2p and Mig1p complex in the presence of high levels of glucose (2%w/v) in the growth medium of *S. cerevisiae* (3,4). Nucleosome positioning over the promoter region of the *SUC2* gene is also required for the formation of repressed chromatin structure (5,6). In the presence of low glucose, sucrose or non-fermentable

carbon source in the growth media, transcription of the *SUC2* gene becomes derepressed (7). Mig1p is phosphorylated by protein kinase and dissociates from *SUC2* promoter (8). Hxk2p also dissociates from *SUC2* promoter under derepressed growth conditions (9).

Expression of *SUC2* gene and the invertase activity of *S. cerevisiae* is also modulated by certain physiological conditions. Transcription of *SUC2* is repressed by various physiological stress conditions such as hyperosmotic stress and oxidative stress (10). The secretion of invertase also ceases transiently in response to elevated temperatures (11).

Hyperosmotic stress activates the high osmolarity glycerol (HOG) signal transduction pathway in yeast (12). The components of the HOG pathway are well characterized in the laboratory strains of *S. cerevisiae* (13). The genome wide effect of hyperosmotic stress on gene expression has been recently analyzed in *S. cerevisiae* (14,15). Different sets of genes are activated in order to adjust the cellular metabolism to hyperosmotic growth conditions (16). Yeast cells accumulate glycerol as an osmoprotectant solute in response to osmotic stress (17). It is known that elevated intracellular glycerol occurs with the increased fermentation ability of yeast cells (18).

However, strains of *S. cerevisiae* used in the baking and fermentation industry are different from genetically well characterized laboratory strains. It was reported that most of the industrial *S. cerevisiae* strains are aneuploid (19). Hence, it is expected that the responses of industrial yeast strains to extracellular signals, like high glucose concentrations, high osmolarity and high temperature, could be different from the genetically stable haploid or diploid laboratory strains. This is because changes in gene numbers due to polyploidy or gene duplications may also change the operation of various metabolic pathways in industrial yeast strains. Therefore, it is reasonable to expect that these strains may have an altered response to the above mentioned stress conditions. High osmolarity and high temperature are the most common types of physiological stresses for baker's yeast (20). These types of stresses significantly affect the growth and survival rate, fermentation rate and leavening ability of industrial yeast strains (20, 21). Hence, stress tolerant yeast strains are more efficient than stress sensitive ones in biomass production, fermentation and leavening ability under specific growth conditions (22).

The aim of this study was to investigate the effects of hyperosmotic stress on the growth and the invertase activities of different baker's yeast strains. Hence the growth patterns and the invertase activities of the laboratory strains and industrial baker's yeast strains were analyzed under normal and hyperosmotic stress conditions.

Materials and Methods

Yeast Strains and Growth Conditions

The genotype of yeast strains used in this study are: YST124 (MATa, his3 Δ 1, leu2 Δ 0, met15 Δ 0, ura3 Δ 0); YST125 (MATa/a, his3 Δ 1/his3 Δ 1, leu2 Δ 0/leu2 Δ 0, MET15/met15 Δ 0, ura3 Δ 0/ura3 Δ 0); and YST129, (MATa, leu2-3/112, ura3-1, trp1-1, his3-11/15, ade2-1, can1-100, hog1 Δ ::TRP1, GAL, *SUC2*). Three different strains of industrial baker's yeast (IBY1-3) were isolated from compressed yeast samples produced and marketed by different companies in Turkey. Yeast strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) with constant shaking (100 rpm) at 30°C in an incubator shaker. Glucose repressed and derepressed yeast strains were prepared as described previously (23). Yeast strains were also grown in YPGL (1% yeast extract, 2% peptone, 2% glycerol, 2% lactate) for long-term derepressed growth conditions with constant shaking (100 rpm) at 30°C in an incubator shaker.

Determination of Invertase Activities

The secreted invertase activities of yeast strains were determined using whole cells as described previously with the following modifications (24). First, yeast cells were grown either in YPD or in YPGL media until the early-logarithmic stage (OD_{600} :0.7-0.8). Then, yeast cells were harvested and washed twice with cold, sterile distilled water. After that, yeast cells were resuspended in a proper volume of YP medium supplemented with glucose for derepressed (0.05% W/V) and repressed (2% W/V) growth conditions, respectively. Yeast cells were further incubated under these conditions for 1.5 – 2 h with constant shaking (100 rpm) at 30°C. Then, the cells were harvested and invertase activities were determined as described (24). The released glucose was measured with glucose oxidase assay using 5-10 μ l of reaction mixture (25). One unit of invertase activity is the amount of enzyme that catalyzes the deliberation of 1 μ mol glucose per minute per 100 mg dry weight of yeast cells.

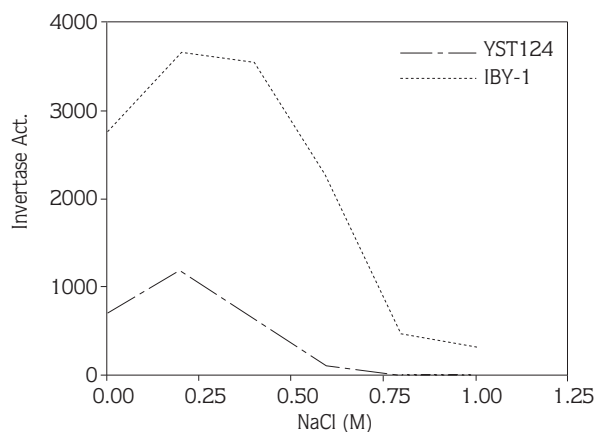


Figure 1. Testing of the effects of various levels of osmotic stress on the invertase activity of genetically stable haploid laboratory strain (YST124) and industrial baker's yeast (IBY1).

In order to analyze the effect of hyperosmotic stress on the invertase activity of baker's yeast, a haploid yeast strain (YST124) and one of the industrial yeast strains (IBY-1) were grown in YPD media until the early logarithmic stage. Then, their aliquots were shifted to derepressed growth conditions, while at the same time increasing concentrations of sterile NaCl (0.2-1M) were also included in the growth medium. Yeast strains were grown under these conditions for 1.5-2 h and then harvested for invertase assays.

Apart from NaCl, the effects of hyperosmotic stress on the secreted invertase activities of the yeast strains were also measured by adding different salts, such as KCl and sorbitol to 1M final concentrations. For this set of experiments, yeast strains were grown in 25 ml of YPD medium under glucose repressed growth conditions until the early logarithmic stage. Then, the yeast cells were washed twice with sterile distilled water and then divided

into five equal aliquots. The first part of the yeast strains was grown under repressed growth conditions while the second part was grown as derepressed yeast cells. The third, fourth and fifth part were also grown as derepressed yeast cells, but at the same time hyperosmotic stress was applied by adding sterile NaCl, KCl, and sorbitol respectively, to 1M final concentrations and grown for 1.5-2 h under these conditions. The yeast strains were harvested at the end of the incubation periods and secreted invertase activities were determined as explained previously. Each experiment was repeated three times.

Analysis of Hyperosmotic Stress Tolerance Efficiencies

The effect of hyperosmotic stress on the growth and survival of yeast strains was analyzed by the dilution plating test method (26). In order to analyze the effects of hyperosmotic stress on survival rates, yeast strains were grown in YPD medium until the logarithmic stage (OD_{600} :0.9-1.0). Then serial dilutions of each yeast strains were prepared either in YPD or in YPD supplemented with 0.8 or 1.4 M NaCl. After that, decreasing amounts of yeast cells (from 10^3 to 2-5 cells) were spotted either on YPD plates or on YPD plates supplemented with various concentrations of NaCl as indicated in Figure 2. The plates of yeast strains were incubated at 30°C for two days.

Results and Discussion

Comparative Analysis of the Invertase Activities of Laboratory and Industrial Yeast Strains

Invertase is required for the hydrolysis of sucrose and raffinose in the growth media of *S. cerevisiae*. The

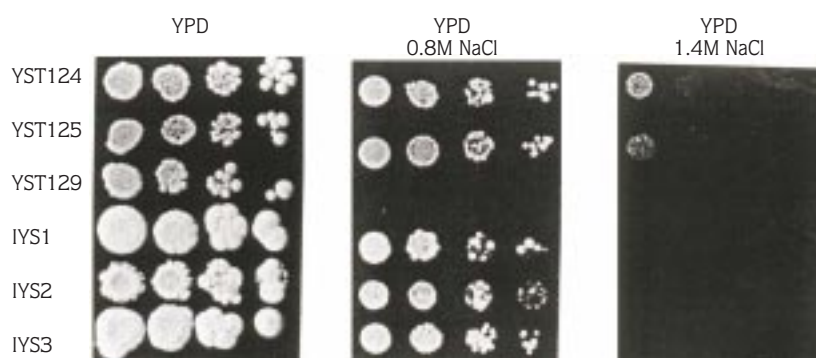


Figure 2. Analysis of the osmotic stress tolerances of laboratory strains and industrial baker's yeast strains. Yeast cells serially diluted and spotted (10^3 , 10^2 , 10^1 and 2-5 cells for each spot, respectively) on the YPD or YPD plates containing 0.8M or 1.4 M NaCl. IYS; industrial baker's yeast strains. YST124, YST125 and YST129 are the laboratory strain of *S. cerevisiae*.

invertase activity of different yeast strains shows a large degree of variation due to differences in the copy number of the *SUC2* gene that expresses the intracellular and secreted invertase (19). Hence, invertase activities of haploid laboratory strain and various industrial baker's yeast strains were analyzed. Yeast strains were grown under glucose repressed growth conditions first and then shifted to derepressed conditions as described. As expected, all the industrial yeast strains have very high levels of invertase activity under glucose derepressed growth conditions. The invertase activities of industrial yeast strains (IBY 1-3) are 4 to 5 fold higher than the genetically well characterized haploid (YST124) yeast strain (Table 1). The invertase activities of industrial yeast strains were approximately 3000 units, while the haploid laboratory strain yielded only 725 units of invertase activity. However, invertase expression from the *SUC2* gene of industrial yeast strains was still subjected to glucose repression. Growth of industrial yeast strains under glucose repressed conditions resulted in approximately 10 to 20 fold lower invertase activities (138-285 units). Invertase expression in laboratory strain YST124 was completely repressed in the presence of 2% glucose in the growth media and yielded only 1-2 units. Nonetheless, glucose repressed level invertase activities of industrial yeast strains was still 100 fold higher than the repressed level invertase activity of haploid yeast strain (Table 1).

Invertase activities of the yeast strains that are grown under long-term derepressed growth conditions were

also analyzed. The yeast strains were grown in YPGL media until the early logarithmic stage and then parts of them shifted to glucose repressed growth conditions. Surprisingly, the effects of glucose repression on the invertase activities of the industrial yeast strains that were grown in long-term derepressed conditions were negligible. The addition of the high concentrations of glucose on the growth medium of these industrial yeast strains did not result strong glucose repression. All the industrial yeast strains still yielded 1500 – 2000 units of invertase activity (Table 1). However, the invertase activity of the haploid yeast strain was still subjected to very strong glucose repression. The addition of glucose at repressing concentrations (2% W/V) to the growth medium of long-term derepressed haploid yeast strain YST124 resulted in an approximate 5 fold repression of invertase activity (Table 1).

Industrial yeast strains are specifically selected for high invertase activities (18,19). Multiple factors may contribute to the higher invertase activity of industrial baker's yeast. In addition to the high copy number of *SUC2* gene and higher specific activity of invertase, industrial strains may have a more efficient secretion pathway for extracellular invertase. They may also accumulate higher levels of extracellular invertase. Our results indicated that glucose repression is not fully functional in these strains. Hence, it is conceivable that high levels of invertase activity in industrial baker's yeast is a results of multiple reasons.

Table 1. Analysis of the invertase enzyme activities of haploid, and different industrial yeast strains.

Strains	Invertase Activities ^a			
	YPD		YPGL	
	R ^b	DR	R	DR
YST 124	1	725	206	925
IBY-1	285	2756	2061	3134
IBY-2	138	2942	1688	2675
IBY-3	206	3709	2180	3100

^a Invertase activities are given in μmoles of glucose liberated/min/100 mg of dry weight.

^b R and DR represents the repressed (2% glucose) and derepressed (0.05% glucose) growth conditions.

Table 2. Analysis of the invertase enzyme activities of industrial yeast strains under hyperosmotic growth conditions.

Strains	Invertase Activities ^a			
	No Stress	1M NaCl	1M KCl	1M Sorbitol
YST124	725	2	8	98
IBY-1	2756	442	998	1900
IBY-2	2942	416	825	803
IBY-3	3704	423	713	1487

^a Invertase activities are given in μmoles of glucose liberated/min/100 mg of dry weight.

Analysis of the effect of hyperosmotic stress on the derepression of invertase activities of yeast strains

We have shown that the invertase activities of industrial yeast strains are 4-5 fold higher than the genetically stable haploid yeast strain depending on the growth conditions. Previously it was shown that the invertase secretion and transcription of the *SUC2* gene is down regulated under the various stress conditions in genetically stable laboratory strains of *S. cerevisiae* (10,11). Therefore, we wanted to analyze the effect of hyperosmotic stress on the derepression of invertase enzyme activities of different yeast strains. First, we analyzed the increasing concentrations of NaCl on the invertase activities of haploid yeast strain (YST124) and one of the industrial baker's yeast (IBY-1). Hence, these strains were grown in YPD medium as glucose repressed until the logarithmic stage and then shifted to derepressed and hyperosmotic stress inducing growth conditions.

Analysis of the invertase activities of the yeast strains that were exposed to hyperosmotic stress indicated that industrial yeast strains were also sensitive to hyperosmotic stress after 0.4M of NaCl in the growth medium (Figure 1). Unexpectedly, low levels of hyperosmotic stress (0.2M NaCl) resulted in significant increases (up to 40-50%) in the derepression of invertase activity both in haploid and in industrial baker's yeast (Figure 1). But, increasing concentrations of NaCl (from 0.4M to 1M) lead to the gradual decrease in the derepressed level invertase activity of both yeast strains. High levels of hyperosmotic stress (0.8M-1M NaCl) decreased the invertase activities of yeast strains to their repressed levels, which is 1-2 units in haploid yeast strain and 300 units in industrial yeast strains (Table 1 and Figure 1).

Although NaCl is commonly used for triggering hyperosmotic stress pathways, it also applies ionic stress on the yeast cells. Hence, we wanted to test the effects of other osmotic stress inducing substances on the secreted invertase activities of yeast strains. KCl, sorbitol and sucrose were used as alternative osmotic stress inducers in our analysis. The presence of 1M KCl, sorbitol or high concentrations of sucrose in the derepressed growth media decreased the invertase activity of haploid yeast strain down to 8 units and 98 units, respectively (Table 2). In a similar way, hyperosmotic stress induced by 1M

KCl, sorbitol or high sucrose also resulted in significant decreases in the invertase activities of industrial baker's yeasts. While the effect of KCl on the invertase activities of these yeast strains was 3-5 fold, the effect of sorbitol was less than two fold on the invertase activities of industrial yeast strains (Table 2). The presence of 20-30% sucrose in the growth media also reduced the invertase activity at significant levels (data not shown). These results indicated that the derepression of invertase expression in industrial baker's yeast strains analyzed in this study is interfered hyperosmotic stress. The effect of NaCl or KCl on the derepression was much higher than that of sorbitol or sucrose. The reason for this may rely on the fact that salts also act as ionic stress inducers when they are present in the growth media.

In previous studies it was shown that hyperosmotic stress represses the transcription of the *SUC2* gene up to 50% in haploid laboratory strain of *S. cerevisiae* (10). But, the effect of hyperosmotic stress on the secreted invertase activity of yeast strains is much higher than its effect on the transcription of the *SUC2* gene. These results suggest that hyperosmotic stress also acts on the post-transcriptional or post-translational stages of *SUC2* gene expression. It is conceivable that hyperosmotic stress also interferes with the secretion or accumulation of invertase in *S. cerevisiae*.

Analysis of the hyperosmotic stress tolerances of industrial yeast strains

Yeast cells are exposed to various types of stress in their growth habitat (20). Hyperosmotic stress results in the accumulation of intracellular glycerol and also increases fermentation capacity (17,27). Hence, we have analyzed the growth efficiencies of industrial yeast strains together with haploid, diploid and Δ hog1 mutant yeasts in hyperosmotic growth medium. We have used dilution-plating method, which was previously devised for this purpose (26). Yeast cells were spotted on normal YPD, and also on 0.8M and 1.4M NaCl containing YPD growth medium. Yeast cells were incubated two days at 30°C and then the growth patterns of each strain were analyzed. It was clearly seen that hog1 mutant yeast strain (YST129) is very sensitive to hyperosmotic stress. It did not grow on hyperosmotic stress inducing media as expected (Figure 2). However, we found that industrial yeast strains analyzed in this study are also sensitive to elevated levels of hyperosmotic stress since they cannot grow on 1.4M NaCl containing growth medium. In all

concentrations of industrial yeast strains, we could not detect any growth in high levels of hyperosmotic media. But genetically stable haploid or diploid yeast strains (YST124 and YST125) were not affected at a significant level by moderate or high levels of hyperosmotic stress.

Recently, Bell et al. (22) also analyzed the hyperosmotic stress tolerances of more than 30 different baker's and wine yeasts. They reported that strains sensitive to osmotic stress have a low CO₂ production capacity (relative gassing activity) while the osmotic stress resistant yeast strains show higher CO₂ production capacity. The invertase activity of industrial yeast strains used in this study is much higher than the laboratory strain. However, these industrial strains are very sensitive to hyperosmotic stress.

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