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The Effect of Hyperosmotic Stress and Nitrogen Starvation on Growth and β-Galactosidase Synthesis in *Kluyveromyces lactis* and *Kluyveromyces marxianus*

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Abstract: *Kluyveromyces lactis* and *Kluyveromyces marxianus* are industrial yeasts widely used in the production of the β-galactosidase enzyme. Biosynthesis of β-galactosidase is controlled by glucose repression. In this study it was demonstrated that the derepression of β-galactosidase biosynthesis in these yeast strains is inhibited by high osmotic stress. It was found that the β-galactosidase activity of *K. lactis* and *K. marxianus* remained approximately at the repressed level when these yeast cells were subjected to NaCl-, KCl-, or sucrose-induced high osmotic stress. Derepression of β-galactosidase biosynthesis seems to be more sensitive to high osmotic stress in *K. marxianus* than in *K. lactis*. In addition, it was shown that nitrogen starvation resulted in a significant decrease in the level of β-galactosidase biosynthesis in *K. lactis*, while nitrogen starvation led to a 2-fold increase in β-galactosidase biosynthesis in *K. marxianus*. Results of this study indicated that high levels of NaCl, but not sucrose, inhibited the growth of *K. lactis* and *K. marxianus*.

Key Words: *Kluyveromyces*, β-galactosidase, osmotic stress, nitrogen regulation, glucose repression

Yüksek Ozmotik Stres ve Azot Açlığının *Kluyveromyces lactis* ve *Kluyveromyces marxianus*'ta Üreme Hızı ve β-Galaktozidaz Sentezine Etkileri

Özet: *Kluyveromyces lactis* ve *Kluyveromyces marxianus* β-galaktozidaz enzimi üretiminde yaygın olarak kullanılan endüstriyel mayalardır. β-galaktozidaz'ın biyosentezi glukoz baskılaması ile kontrol edilir. Bu çalışmada β-galaktozidaz biyozentezi derepresyonunun yüksek ozmotik stress tarafından inhibe edildiği gösterildi. *K. lactis* ve *K. marxianus*'ta β-galactozidaz aktivitelerinin bu maya türleri NaCl, KCl veya sukroz tarafından oluşturulan yüksek ozmotik strese uğradıklarında β-galaktozidaz aktivitelerinin yaklaşık olarak baskılanmış seviyede olduğu bulundu. *K. marxianus*'da β-galaktozidaz biyosentezinin ozmotik strese *K. lactis*'den daha hassas olduğu görülmektedir. Buna ek olarak, azot açlığının *K. lactis*'de β-galaktozidaz biyosentezinde önemli seviyede azalmaya neden olurken, *K. marxianus*'da 2 kat artışa yol açtığı gösterildi. Bu çalışmanın sonuçları yüksek seviyede sukroz'un değil NaCl'nin *K. lactis* ve *K. marxianus*'da üremeyi inhibe ettiğini gösterdi.

Anahtar Sözcükler: *Kluyveromyces*, β-galactosidaz, ozmotic stres, azot düzenlemesi, glukoz baskılaması

Introduction

Kluyveromyces lactis and *Kluyveromyces marxianus* are lactose-metabolizing yeasts widely used in the production of the β-galactosidase enzyme (EC 3.2.1.23) (1,2). β-Galactosidase is used in the food industry to modify the lactose content of dairy products (3,4). In addition, β-galactosidase catalyzes the enzymatic synthesis of galacto-oligosaccharides, which are used to stimulate the growth of prebiotic bacteria in intestinal flora (5). The β-galactosidase enzyme is encoded by the *LAC4* gene in *K. lactis* (6). Its expression is regulated by glucose repression and derepression mechanisms at the transcriptional level (7.8). *K. lactis* is also used for the production of recombinant proteins (3,9).

Growth conditions have a great impact on the biosynthesis of industrial enzymes by the relevant microorganisms (10,11). It is known that biosynthesis of β-galactosidase is affected by air pressure in the growth chamber, and by the growth stage of *K. marxianus* and *K. lactis* (12-14). The presence of large quantities of sugars or salts in the growth medium interferes with the derepression of invertase synthesis in *K. lactis* and in certain industrial yeast species (10,15). Likewise, nitrogen starvation and oxidative stresses also affect several metabolic pathways in industrial yeasts (16,17).

The effects of hyperosmotic stress and nitrogen starvation on the overall metabolism of *S. cerevisiae* are well known. The components of the signal transduction pathways that respond to these environmental stresses have been identified in *S. cerevisiae* (18,19). *Sho*1, which is involved in the sensing of hyperosmotic stress, has been cloned from *K. lactis* (20); but how osmotic stress and nitrogen starvation affect the growth and biosynthesis of β-galactosidase in *K. lactis* and *K. marxianus* has not as yet been analyzed. Hence, the aim of the present study was to investigate the effects of high osmotic stress and nitrogen starvation on the growth and derepression of β-galactosidase biosynthesis in *K. lactis* and *K. marxianus*.

Materials and Methods

Strains and Culture Conditions

Kluyveromyces lactis (DBVPG 6731) and *Kluyveromyces marxianus* (DBVPG 6072) were obtained from the industrial yeast collection of Universita di Perugia (Italy). Yeast cells first were grown in 10 ml of YP medium (1% yeast extract, 2% peptone) supplemented with 4% glucose on an incubator shaker (120 rev/min) at 28 ºC to the early logarithmic stage. Then a portion of the yeast cells (5 ml) was harvested and washed twice with ice-cold sterile distilled water, then was resuspended in 5 ml of YP medium supplemented with 2% lactose, and further incubated at 28 ºC in an incubator shaker (120 rev/min) for 3 h to derepress β-galactosidase biosynthesis (7,14).

In order to test the effects of hyperosmotic stress on the derepression of β-galactosidase biosynthesis, derepressed yeast cells were prepared as above, but at the same time one of the osmolytes (NaCl, KCl, or sucrose) was also added to the growth medium at the different concentrations indicated in the related tables (15,21). Yeast cells were grown for an additional 3 h and harvested to measure β-galactosidase activity.

In order to test the effects of nitrogen starvation on the derepression of β-galactosidase biosynthesis in *K. lactis* and *K. marxianus*, yeast cells were first cultivated in 25 ml of YNB minimal medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 4% glucose) to the logarithmic stage. Yeast cells were harvested and washed twice with 25 ml of ice-cold sterile distilled water. Yeast cells were resuspended in 25 ml of yeast nitrogen base without amino acids or nitrogen. Then nitrogen and carbon sources were added to aliquots of the yeast cultures, as indicated in Table 3 (22). Yeast cells were grown for an additional 3 h at 30 °C in an incubator shaker and then harvested for determination of β-galactosidase activity.

Determination of β-galactosidase Activity

Yeast cells were harvested at the end of their growth period, washed with cold, sterile distilled water, and then resuspended in 200 µl of buffer solution (7,8). Yeast cells were permeabilized with 20 µl of 0.1% SDS and 20 µl of chloroform (23). β-Galactosidase activity of the permeabilized yeast cells was determined in triplicate at 30 °C using O-nitro-phenyl-β-D-galactoside in Tris/HCl buffer (7,24). Enzyme activity was expressed in Miller units (24). Standard deviations in all assays were < 15%.

Determination of Growth Rates

Yeast cells were first pre-cultured in YP or YNB medium supplemented with 4% glucose or 2% lactose to the stationary stage. Then yeast cells were inoculated into fresh media that contained different osmolytes or different nitrogen sources, as indicated in Table 4. Initial cell density of the yeast cultures was adjusted to A_{600}:0.2. Yeast cultures were incubated in an incubator shaker at 30 °C and samples were taken every 2 h. Duplication times of the yeast strains were calculated from the growth curves that were obtained by plotting A_{600} values of the samples versus time points. All experiments were performed in triplicate and repeated twice. Hence, the numbers given in the tables are the mean values of 9 independent experiments. Standard deviations were < 10% in triplicate assays.

Results and Discussion

Osmotic Stress Interferes with the Derepression of β-galactosidase biosynthesis in *K. lactis* and *K. marxianus*

β-Galactosidase biosynthesis is regulated by glucose repression and derepression mechanisms. Hence, β-galactosidase activity in *K. lactis* and *K. marxianus* cells remained at low levels when they were grown in glucose medium (Table 1). However, when the yeast cells were shifted to lactose medium, biosynthesis of β-galactosidase increased 10-fold in *K. lactis* and 122-fold in *K. marxianus*. In order to test the effects of hyperosmotic stress on the derepression of β-galactosidase, increasing quantities of NaCl (0.2-0.8 M) were also added when the yeast cells were shifted to derepressed (2% lactose) growth medium. Low-level osmotic stress (0.2 M NaCl) did not have any significant effect on the derepression of β-galactosidase biosynthesis in *K. lactis*. However, the presence of 0.8 M NaCl in the growth medium resulted in a 7-fold decrease in the derepression of β-galactosidase in *K. lactis* (Table 1).

The effect of hyperosmotic stress on the derepression of β-galactosidase biosynthesis in *K. marxianus* was much greater than in *K. lactis*. Low-level osmotic stress (0.2 M NaCl) led to a 2-fold decrease in the derepression of β-galactosidase biosynthesis in *K. marxianus* (Table 1). Furthermore, β-galactosidase biosynthesis was not derepressed in *K. marxianus* in the presence of 0.8 M NaCl in the growth medium and remained at the repressed level (Table 1).

High osmotic stress changed the transcription profile of approximately 5% of *S. cerevisiae* genes (19,21). The membrane protein involved in sensing high osmotic stress was cloned from *K. lactis* (20). Transcription factors *MSN2* and *MSN4*, which are involved in the general stress response pathway, are also present in the *K. lactis* genome (25). It is conceivable that a similar type of response will occur in the gene expression profiles in *K. lactis* or *K. marxianus* upon exposure to high osmotic stress.

In order to test whether the inhibition of β-galactosidase biosynthesis is specific for the sodium ions or not, we also tested the effects of 0.8 M KCl and high levels of sucrose on the derepression of β-galactosidase biosynthesis in *K. lactis* and *K. marxianus*. High-level KCl resulted in a similar type of effect on the derepression of β-galactosidase biosynthesis as with 0.8 M NaCl (Table 2). The biosynthesis of β-galactosidase was not derepressed and remained at repressed levels in *K. lactis* and *K. marxianus* in the presence of 0.8 M KCl in the growth medium (Table 2). Surprisingly, high osmotic stress generated by 10% sucrose did not have any inhibitory effect on the derepression of β-galactosidase biosynthesis in *K. lactis* (Table 2). However, the presence of 10% sucrose in the growth medium of *K. marxianus* led to complete inhibition of β-galactosidase derepression in this yeast species (Table 2).

It was shown that high salt content prevents the binding of transcriptional activators to their binding sites on the promoter regions in *S. cerevisiae* (26). Thus, shifting *K. lactis* or *K. marxianus* cells to high salt medium may interfere with the transcription of the *LAC4* gene. Contrary to *K. marxianus*, our results also showed that the derepression of β-galactosidase biosynthesis in *K. lactis* was sensitive to ionic stress only, since its derepression occurred at normal levels in the presence of 10% sucrose.

Table 1. Hyperosmotic stress interferes with the derepression of β-galactosidase biosynthesis in *K. lactis* and *K. marxianus*.

Yeast Strains	β-galactosidase Activity[a] (± SD)				
	R[b]	DR	DR+ 0.2 M NaCl	DR+ 0.4 M NaCl	DR+ 0.8 M NaCl
K. lactis	41 ± 3	552 ± 29	420 ± 51	346 ± 21	75 ± 10
K. marxianus	3 ± 1	366 ± 26	174 ± 16	35 ± 4	2 ± 1

[a]β-galactosidase activity is given in Miller units.
[b]R: Repressed (4% glucose).
DR: Derepressed (2% lactose) growth conditions.
SD: Standard deviation.

Table 2. Differential effects of high levels of osmolytes on β-galactosidase derepression in *K. lactis* and *K. marxianus*.

Yeast Strains	β-galactosidase Activity[a] (± SD)			
	R[b]	DR	DR+ 0.8 M KCl	DR+ 10% Sucrose
K. lactis	41 ±3	552 ± 29	67 ±10	414 ±48
K. marxianus	3 ±1	366 ±26	2 ± 1	2 ± 1

[a]β-galactosidase activity is given in Miller units.
[b]R: Repressed (4% glucose).
DR: Derepressed (2% lactose) growth conditions.
SD: Standard deviation.

The Effect of Nitrogen Starvation on β-galactosidase Biosynthesis

In order to test the effects of nitrogen starvation on the derepression of β-galactosidase biosynthesis, *K. lactis* and *K. marxianus* cells were pre-cultured in YNB minimal medium supplemented with 4% glucose. When the yeast strains were shifted to lactose medium, β-galactosidase biosynthesis was derepressed and high levels of activity were observed in both strains (Table 3). Lack of nitrogen sources in the growth medium resulted in a 2-fold decrease in the derepression of β-galactosidase biosynthesis in *K. lactis* (Table 3), but the presence of low quantities of nitrogen (0.05% ammonia) or a poor nitrogen source, like 0.1% proline, in the growth medium did not have any effect on the derepression of β-galactosidase biosynthesis (Table 3). β-Galactosidase biosynthesis was derepressed nearly to the normal level (545-555 Miller units) in *K. lactis* that were cultivated in a low nitrogen or proline medium.

When *K. marxianus* cells were shifted to YNB lactose medium, β-galactosidase biosynthesis was rapidly derepressed and yielded 107 Miller units of activity (Table 3). However, nitrogen starvation resulted in unusual regulation of β-galactosidase biosynthesis in *K. marxianus*. When *K. marxianus* cells were transferred to minimal lactose medium that did not contain any metabolizable nitrogen sources, derepression of β-galactosidase was further activated (Table 3). In a similar manner, derepression of β-galactosidase biosynthesis was approximately 2-fold higher in minimal lactose medium supplemented with small quantities of ammonium or proline (Table 3).

Table 3. The effects of nitrogen starvation on β-galactosidase biosynthesis in *K. lactis* and *K. marxianus*.

Yeast Strains	β-galactosidase Activity[a] (± SD)				
	R[b]+ 0.5% NH_4	DR+ 0.5% NH_4^+	DR+ No NH_4^+	DR+ 0.05% NH_4^+	DR+ 0.1% Proline
K. lactis	14 ± 2	609 ± 4	295 ± 18	545 ± 63	555 ± 18
K. marxianus	7 ± 1	107 ± 15	160 ± 11	172 ± 16	220 ± 13

[a]β-galactosidase activity is given in Miller units.
[b]R: Repressed (4% glucose).
DR: Derepressed (2% lactose) growth conditions.
SD: Standard deviation.

Nitrogen regulation of target genes is carried out by the URE2/GLN3 system in *S. cerevisiae* (27). It is known that different sets of genes are either repressed or activated by ammonia (18,28). It was shown that invertase biosynthesis and ethanol production are under the control of nitrogen regulation in *S. cerevisiae* (16-17, 29). Currently, it is not known if a URE2/GLN3-type of nitrogen regulation system is also present in *K. lactis* or *K. marxianus*.

The Effect of Osmotic Stress on the Growth of *K. lactis* and *K. marxianus*

Osmotic stress had diverse effects on the yeast cells. We tested the effects of high levels of NaCl or sucrose on the growth of *K. lactis* and *K. marxianus* species. Specific growth rates of *K. lactis* and *K. marxianus* cells were 0.32 and 0.35 in YP medium supplemented with 4% glucose, respectively (Table 4). Growth on YP lactose medium resulted in higher growth rates (0.40 and 0.39) than on the glucose medium, both in *K. lactis* and *K. marxianus*. However, when 0.8 M NaCl was added to the growth media that contained glucose and lactose, the specific growth rate of *K. lactis* decreased to 0.08 and 0.12, respectively. Moreover, the growth of *K. marxianus* immediately stopped when NaCl was added to the growth medium. Although high levels of sucrose inhibited the biosynthesis of β-galactosidase, it did not have a significant effect on the growth of *K. marxianus* (Table 4). Nonetheless, the specific growth rate of *K. lactis* decreased slightly when 10% sucrose was added to the YP lactose growth medium.

Table 4. The effects of osmotic stress on the growth of *K. lactis* and *K. marxianus*.

Growth Media	Specific Growth Rate[a]	
	K. lactis	*K. marxianus*
YP+ 4% glucose	0.32	0.35
YP+ 2% lactose	0.40	0.39
YP+ 10% sucrose	0.35	0.36
YP+ 4% glucose + 0.8 M NaCl	0.08	No Growth
YP+ 2% lactose 0.8 M NaCl	0.12	No Growth
YP+ 2% lactose 10% sucrose	0.34	0.39
YN+ 2% lactose (0.5% $NH4^+$)	0.22	0.27
YN+ 2% lactose (0.1% proline)	0.21	0.33

[a]Specific growth rate (μ): ln2/doubling time (h).

Tolerance to high salt content-induced osmotic stress requires the biosynthesis and accumulation of glycerol and the effective efflux of salt ions from yeast cells (19). Sucrose, on the other hand, generates only high osmotic stress, not ionic stress. It appears that both *K. lactis* and *K. marxianus* were able to tolerate high osmotic stress, probably due to high-level accumulation of intracellular glycerol. An ATP-dependent sodium transporter has been identified in *S. cerevisiae* (29,30). In addition to glycerol biosynthesis, these sodium pumps are required for salt tolerance in *S. cerevisiae* (28,31). The lack of efficient sodium or potassium efflux in *K. lactis* and *K. marxianus* may result in the accumulation of these ions, which, in turn, inhibit the growth of these yeast strains.

Results of this research showed that the derepression of β-galactosidase biosynthesis was affected by high osmotic stress and was sensitive to nitrogen starvation in *K. lactis*. Unlike *K. lactis*, β-galactosidase biosynthesis was more sensitive to high osmotic stress, but not nitrogen starvation in *K. marxianus*. In addition, it appears that ionic stress, not osmotic stress, inhibited the growth of *K. lactis* and *K. marxianus*. The type of nitrogen source, which was ammonium sulfate or proline, in the growth medium did not affect the specific growth rate of *K. lactis*; however, the growth of *K. marxianus* in proline medium resulted in shorter duplication times and, hence, higher specific growth rates. Thus, high-level β-galactosidase activity might be the cause of the higher specific growth rate of *K. marxianus* grown in proline medium.

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