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ORIGINAL ARTICLE

Effect of osmotic stress on the derepression of invertase synthesis in nonconventional yeasts

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Abstract

Aims: The aim of this study was to analyse the effect of osmotic stress on the biosynthesis of invertase enzyme in nonconventional yeasts.
Methods and Results: Invertase activities of the nonconventional yeast species belonging to *Kluyveromyces*, *Schwanniomyces* and *Pichia* genus were measured either in the presence or in the absence of various amounts of NaCl. The effect of hyperosmotic stress on the glucose consumption of *Saccharomyces cerevisiae* and *Pichia anomala* were also compared. Like *S. cerevisiae*, derepression of invertase synthesis in *Kluyveromyces lactis*, *Schwanniomyces occidentalis* and *Pichia jadinii* is inhibited by hyperosmotic stress. However, derepression of invertase synthesis in *P. anomala* is not affected by hyperosmotic stress. In addition, low levels of osmotic stress activated invertase synthesis three- to fourfold in *P. anomala* and *K. lactis*.
Conclusions: This study shows that low levels of osmotic stress induces the invertase synthesis at very high levels in *P. anomala* and *K. lactis*. Glucose consumption was not influenced at significant levels by the hyperosmotic stress in *P. anomala*.
Significance and Impact of the Study: This study shows the activation of invertase synthesis by low levels of osmotic stress in *P. anomala* and *K. lactis*.

Introduction

The invertase enzyme (E.C.3.2.1.26) catalyses the hydrolysis of sucrose and raffinose in *Saccharomyces cerevisiae* (Carlson and Botstein 1982). Its biosynthesis was strictly controlled by glucose repression in many different yeast species (Gancedo 1998). In addition to its industrial use as a purified enzyme, invertase has a significant role in the growth of yeast species in sucrose or raffinose containing growth media such as various molasses (Park and Sato 1982; Zech and Gorisch 1995). Apart from the glucose repression, biosynthesis of invertase is also affected by the hyperosmotic stress resulting from the high amount of sugar or salts in the growth medium. It is known that the decrease in the invertase biosynthesis due to the hyperosmotic stress leads to a significant decrease in the growth rate and relative gassing ability of the industrial strains of *S. cerevisiae* (Park and Sato 1982; Myers *et al.* 1997). Hence, osmotolerant yeast strains that produce high amounts of invertase under hyperosmotic stress may have a wide range of application fields in industry (Attfield 1997; Olsson and Nielsen 2000).

Nonconventional yeasts are receiving increasing attentions because of their metabolic and growth characteristics (Wolf 1996; van Dijk *et al.* 2000). Yeasts belonging to the genera of *Pichia*, *Kluyveromyces* and *Schwanniomyces* are especially important for their various industrial applications (Wolf 1996). Certain yeast species from these genera also synthesize invertase. Genes that encode invertase enzyme have been cloned from *Pichia anomala*, *Kluyveromyces lactis*, *Schwanniomyces occidentalis* and *Pichia jadinii*. Expression of genes encoding invertase in these species was also regulated by glucose repression and derepression mechanisms (Klein *et al.* 1989; Perez *et al.* 1996; Chavez *et al.* 1998; Georis *et al.* 1999). Catalytic activities of invertase enzymes show variations among the yeast species. For example, K_m value of invertase synthesized from *P. anomala* is lower than the K_m of *S. cerevisiae*

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invertase (Rodriguez *et al.* 1995). Glucose repression is not so effective on the *KlINV1* gene of *K. lactis* and *INV1* genes of *Candida utilis* (Georis *et al.* 1999; Belcarz *et al.* 2002). Hence, it is clear that there are significant differences in the expression patterns and activities of invertase enzymes among various nonconventional yeasts.

It is known that hyperosmotic stress interferes with the derepression of *SUC2* gene and reduces the biosynthesis of invertase and also glucose uptake in *S. cerevisiae* (Türkel 1999, 2000; Brandao *et al.* 2002; Türkel and Turgut 2002). In this study, we have analysed the effects of hyperosmotic stress on the biosynthesis of invertase enzymes from different nonconventional yeasts in comparison with *S. cerevisiae*. In addition, the effect of hyperosmotic stress on the glucose consumption was also analysed in *P. anomala* in comparison with *S. cerevisiae*.

Materials and methods

Yeast strains and growth conditions

Saccharomyces cerevisiae strain used in this study was W303-1A (MATa, ura3-1; leu2-3 112; trp1-1; his3-11, 15; ade2-1; can1-100). The wild-type strains of the nonconventional yeasts are obtained from DBVPG collection (University of Perugia, Italy). These yeasts are: *P. anomala* (DBVPG nos 3511, 4357 and 6781), *K. lactis* (DBVPG no. 6112), *Sch. occidentalis* (DBVPG No: 6723) and *P. jadinii* (DBVPG no. 6160). Yeast strains except *P. jadinii* were cultivated in YPAD medium (1% yeast extract, 2% peptone, 20 mg l^{-1} adenine, 2% glucose) with constant shaking (120 rev min^{-1}) in 25°C orbital shaker. *Pichia jadinii* was cultivated in YMD medium (5 g l^{-1} yeast extract, 3 g l^{-1} malt extract, 5 g l^{-1} bactopeptone, 50 g l^{-1} glucose) as described (Granström *et al.* 2000). Glucose repressed and derepressed yeast cells were prepared as described previously (Celenza and Carlson 1984).

Determination of invertase activities

First, yeast strains were grown to early logarithmic stage (OD_{600}: 0·7) in 10 ml of YPAD or YMD medium in duplicates. Then a portion of yeast cultures (5 ml) was harvested and washed twice with cold, sterile distilled water. After that, yeast cells were resuspended in 5 ml of YPA or YM medium supplemented with low glucose (0·05% w/v). Yeast cultures were further grown for 2 h for the derepression of invertase synthesis in orbital shaker and then harvested for invertase assay (Celenza and Carlson 1984).

To analyse the effect of hyperosmotic stress on the derepression of invertase biosynthesis, the yeast strains were grown in YPAD or YMD media in duplicates until the early logarithmic phase, harvested and then shifted to derepressing growth conditions. At the same time one of the hyperosmotic stress inducing chemical (0·2 mol l^{-1} NaCl, 0·8 mol l^{-1} NaCl or 0·8 mol l^{-1} KCl) was also included in the growth medium. Yeast strains were grown under these conditions for 2 h and then harvested for invertase assays. Secreted invertase activities of yeast strains were determined using whole cells as described (Rothe and Lehle 1998). The released glucose was measured with glucose oxidase–peroxidase assay using 10–20 μl of reaction mixture (Goldstein and Lampen 1975; Rothe and Lehle 1998). One unit of invertase activity is the amount of enzyme that releases 1 μmol of glucose per minute per 100 mg of dry weight of yeast cells. Each experiment was repeated three times. Hence the invertase activities were given as the mean values of at least six independent experiments.

Determination of glucose consumption efficiencies

Glucose consumption was determined with the decrease in the glucose concentration in the growth medium of the yeast cells (Wieczorke *et al.* 1999). First, yeast cells were grown up to the mid-logarithmic phase in YPAD medium. The yeast cells were harvested and washed twice with sterile distilled water. Then the yeast strains were resuspended in YPA medium containing 0·05% (21 μmol l^{-1}) glucose either in the presence or in the absence of 0·8 mol l^{-1} NaCl. The decrease in the extracellular glucose concentration was determined enzymatically at 30-min time intervals with glucose oxidase–peroxidase assay (Goldstein and Lampen 1975).

Results

Analysis of the effects of high osmotic stress on the invertase biosynthesis in nonconventional yeasts

Invertase biosynthesis remained at very low levels when the yeast species grown in the high glucose medium (Table 1). As expected, invertase biosynthesis became derepressed and gave very high levels of invertase activity when the yeast cells transferred to the low glucose medium (Table 1).

Invertase biosynthesis in *S. cerevisiae* remained at very low levels in the presence of 0·8 mol l^{-1} of NaCl in the growth medium (Table 1). In a similar way, invertase biosynthesis in *K. lactis*, *Sch. occidentalis* and in *P. jadinii* was not derepressed in the presence of 0·8 mol l^{-1} of NaCl in the growth medium. Invertase activities remained at their repressed levels in these nonconventional yeast strains (Table 1). However, invertase biosynthesis in *P. anomala* was not affected by the hyperosmotic stress at

Table 1 Derepression of invertase biosynthesis in nonconventional yeasts under hyperosmotic stress conditions

	Invertase activities*		
Yeast strains	R	DR	DR + 0·8 mol l^{-1} NaCl
Saccharomyces cerevisiae	11	974	72
Pichia anomala	12	247	216
Kluyveromyces lactis	14	112	28
Schwanniomyces occidentalis	30	258	11
Pichia jadinii	164	1176	141

R, repressed (2% glucose); Dr, derepressed (0·05% glucose) growth conditions.
*Invertase activities were given in μmol of glucose deliberated per min per 100 mg of dry weight.

Table 3 Effects of low level osmotic stress on invertase biosynthesis in *Saccharomyces cerevisiae* and in different nonconventional yeasts

	Invertase activities*		
Yeast strains	R	Dr	Dr + 0·2 mol l^{-1} NaCl
Saccharomyces cerevisiae	11	974	1518
Pichia anomala	12	247	744
Kluyveromyces lactis	14	112	499
Schwanniomyces occidentalis	30	258	223
Pichia jadinii	164	1176	1136

R, repressed (2% glucose); Dr, derepressed (0·05% glucose) growth conditions.
*Invertase activities were given in μmol of glucose deliberated per min per 100 mg of dry weight.

significant level. Invertase biosynthesis was derepressed to its normal level (216 units) in the presence of 0·8 mol l^{-1} NaCl. In addition to NaCl, the effects of 0·8 mol l^{-1} of KCl were also tested on the derepression of invertase biosynthesis in above-mentioned yeast strains. Except *P. anomala*, 0·8 mol l^{-1} of KCl also had similar negative effect on the derepression of invertase biosynthesis in these yeast strains (data not shown).

To show that the lack of hyperosmotic stress effect on the invertase biosynthesis in *P. anomala* is not a strain-specific phenomenon, we have determined the invertase activities of two other *P. anomala* strains in the presence of 0·8 mol l^{-1} of NaCl or 0·8 mol l^{-1} of KCl. Derepression of invertase biosynthesis in other strains of *P. anomala* was not affected by 0·8 mol l^{-1} of NaCl. Moreover, invertase synthesis is derepressed to their normal levels in the presence of 0·8 mol l^{-1} KCl in all three of *P. anomala* species (Table 2). As expected, derepression of invertase synthesis was inhibited by 0·8 mol l^{-1} of KCl in *S. cerevisiae* (Table 2).

Table 2 Comparative analysis of the effects of high osmotic stress on the derepression of invertase synthesis in *Saccharomyces cerevisiae* and various strains of *Pichia anomala*

	Invertase activities*			
Yeast strains	R	Dr	Dr + 0·8 mol l^{-1} NaCl	Dr + 0·8 mol l^{-1} KCl
S. cerevisiae	11	974	75	103
P. anomala (DBVPG 3511)	12	247	216	336
P. anomala (DBVPG 4357)	32	400	374	542
P. anomala (DBVPG 6781)	9	414	363	527

R, repressed (2% glucose); Dr, derepressed (0·05% glucose) growth conditions.
*Invertase activities were given in μmol of glucose deliberated per min per 100 mg of dry weight.

Low levels of osmotic stress activates the invertase biosynthesis in *P. anomala* and *K. lactis*

The effect of low-level osmotic stress on the invertase biosynthesis was also analysed in the given nonconventional yeasts. Yeast strains were cultivated in the presence of 0·2 mol l^{-1} NaCl under derepressed growth conditions. Unlike hyperosmotic stress, in most of the strains tested, low level osmotic stress did not affect derepression of invertase biosynthesis (Table 3). Apart from *Sch. occidentalis*, invertase biosynthesis in these yeast strains was further activated above the derepressed levels in the presence of 0·2 mol l^{-1} of NaCl. The invertase biosynthesis was activated threefold in the presence of low levels of osmotic stress in *P. anomala* and 4·3-fold in *K. lactis* (Table 3). But, the derepression of invertase biosynthesis was not influenced by the low level of osmotic stress in *P. jadinii*. However, moderate level increase (56%) occurred in the derepression of invertase biosynthesis in *S. cerevisiae* when it was cultivated in the presence of low-level osmotic stress.

Effect of hyperosmotic stress on glucose consumption in *P. anomala*

Hyperosmotic stress reduces the glucose consumption rate and represses the transcriptional activation of certain *HXT* genes in *S. cerevisiae* (Türkel 1999; Brandao *et al.* 2002). Effects of hyperosmotic stress on the glucose consumption of *S. cerevisiae* and *P. anomala* were also compared. While the extracellular glucose concentration decreased *c.* 50% (from 21 to 10·3 μmol l^{-1}) in the growth medium of *P. anomala* at the end of 60 min, it decreased only 22% and remained nearly at its initial levels (16·3 μmol l^{-1}) in the presence of 0·8 mol l^{-1} NaCl in the culture medium of *S. cerevisiae* (Fig. 1). Glucose consumption efficiencies of *P. anomala* and *S. cerevisiae* were very similar under normal growth conditions. The extracellular glucose concentration

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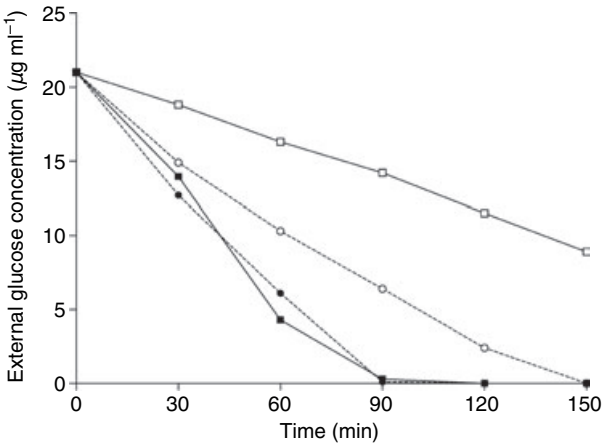

Figure 1 Comparative analysis of the effect of hyperosmotic stress on glucose consumption in *Pichia anomala* and *Saccharomyces cerevisiae*. The decrease in the external glucose concentration in the growth medium of the yeast strains was monitored *vs* time as indicated. Yeast cells were cultivated either as derepressed (full signs) or derepressed plus 0·8 mol l^{-1} of NaCl (open signs); *S. cerevisiae* (straight lines), *P. anomala* (dashed lines).

decreased *c.* 63% (from 21 to 7·6 μmol l^{-1}) in the growth medium of both strains after 60-min incubation (Fig. 1). At the end of 2·5 h of incubation period in the presence of 0·8 mol l^{-1} NaCl, glucose consumption by *P. anomala* was 100% while it was only 50% in *S. cerevisiae*.

Discussion

Growth conditions of microbial cultures may have a large effect on the gene expression. Osmotic stress inducing compounds such as high salts also have a dramatic effect on the regulation of many genes in *S. cerevisiae*. Osmotic stress induces the high osmolarity glycerol (HOG) pathway that results in the activation or repression of several genes in *S. cerevisiae* (Rep *et al.* 2000). Previously, it was reported that high osmotic stress interferes with the derepression of invertase synthesis in *S. cerevisiae* (Türkel 2000). The involvement of cell integrity pathway through the protein kinase C in the derepression of invertase synthesis suggests the presence of cross-regulatory interactions between the osmotic stress and glucose signalling in the laboratory strains of *S. cerevisiae* (Brandao *et al.* 2002; Salgado *et al.* 2002).

Our results indicated that hyperosmotic stress does not affect the derepression of invertase biosynthesis in *P. anomala*. This difference might result from the differences in the responses of *S. cerevisiae* and *P. anomala* towards the sodium or potassium ions. There is no specific sodium uptake and efflux system in *S. cerevisiae* and high amounts of sodium was exported through Ena1p, i.e. a P-type ATPase that functions in the sodium and lithium efflux. But there is a specific sodium pump in halotolerant yeasts like *P. anomala* (Prista *et al.* 1997; Thome-Ortiz *et al.* 1998).

Low levels of osmotic stress stimulated the derepression of invertase biosynthesis at significant levels in *P. anomala* and *K. lactis*. It is unlikely that the sodium ion alone improves the catalytic aspects of the invertase enzyme in *P. anomala*. Because, low levels of potassium also show the same stimulatory effect. Hence, it is conceivable that low levels of osmotic stress generated by sodium or potassium activates the signal transduction mechanisms that eventually activates *INV1* gene transcription in *P. anomala*. We have identified stress response element-binding sites (5′-CCCCT-3′ sequence) for the transcription factors Msn2p/Msn4p on the promoter region of the *SUC2* gene of *S. cerevisiae* (Schmitt and McEntee 1996). It was shown that the overproduction of Msn2p suppresses Snf1p requirement for the derepression of *SUC2* gene in *S. cerevisiae*. Hence, it is possible that the low level of osmotic stress might further activate the transcription of *SUC2* and *INV1* genes in a stress response element-dependent manner.

In addition to the derepression of invertase biosynthesis, previous studies also suggested that the glucose uptake was downregulated by hyperosmotic stress in *S. cerevisiae* (Brandao *et al.* 2002). Contrary to *S. cerevisiae*, there is no strong inhibition of glucose consumption in *P. anomala* by hyperosmotic stress. This may result from the presence of efficient sodium efflux in the *P. anomala* cells.

Components and the operation of high-osmolarity glycerol signal transduction pathway at molecular level in *P. anomala* have not been analysed yet. But, it is clear that high osmolarity does not have any inhibitory effect on the derepression of invertase biosynthesis in *P. anomala*. It was suggested that the overall metabolism of the halotolerant yeast *Debarryomyces hansenii* was resistant to high amounts of salts in the growth medium (Prista *et al.* 1997). This observation may be true for the invertase biosynthesis in *P. anomala*. Nonetheless, in addition to the repression of *SUC2* gene expression, high levels of osmolytes may have an inhibitory effect on the invertase enzyme activity in *S. cerevisiae* as it is not an osmotolerant yeast species.

Recently, it was shown that hyperosmotic stress causes rapid dissociation of transcription factors from chromatin in *S. cerevisiae* (Proft and Struhl 2004). Therefore, based on this new evidence, we suggest that salt stress interferes with the derepression of invertase biosynthesis by preventing the binding of transcriptional activators to promoter regions of invertase genes in *S. cerevisiae* and in other yeast species that are sensitive to high osmotic stress.

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