DETERMINATION OF INVERTASE ENZYME ACTIVITY AND RELATED GENE CONTROL DEPENDING ON THE STRESS CONDITIONS IN DIFFERENT YEAST SPECIES

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ABSTRACT

DETERMINATION OF INVERTASE ENZYME ACTIVITY AND RELATED GENE CONTROL DEPENDING ON THE STRESS CONDITIONS IN DIFFERENT YEAST SPECIES

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Biomass increase resulting from the increased growth rate is largely depends on the carbon source in the growth media. Growth of yeast species especially *Saccharomyces cerevisiae* is closely related to the uptake and effective utilization of carbon sources in growth media. Utilization of sucrose is solely depends on the extra cellular enzyme invertase which is

expressed from *SUC2* gene in *Saccharomyces cerevisiae* and *INV1* gene of *Pichia anomala*.

In this research *Pichia farinosa*, *Pichia anomala*, *Pichia jadini*, *Candida milleri* and *Saccharomycese cerevisiae* yeast species were grown on different carbon sources, and their invertase activities were determined at different stress conditions. *Saccharomyces. cerevisiae* were used as a control in our research.

Pichia farinosa did not show invertase activity in all stages of growth and at different temperatures and pHs.

The enzyme activity of *Pichia anomala* was controlled by glucose repression, in both logarithmic and stationary stages of growth. At different temperatures and pHs, invertase activity of enzyme was still controlled by glucose signalling.

Pichia jadini and *Candida milleri* showed the highest invertase activity that was not affected by glucose repression and stresses at 2% glucose.

Our results showed that temperatures between 25°C and 30°C were an optimum temperature and pHs between 5.0 and 6.0 were a suitable pH for growth and maximum invertase activity of *Pichia anomala, Pichia jadini* and *Candida milleri.*

Invertase enzyme activities of *Pichia anomala* and *Saccharomyces cerevisiae* at short-term derepression condition was greater than long-term derepression condition.

Key Words: *Pichia* sp., *Candida* sp., Invertase, Heat Stress, Acidity, Glucose Repression

ÖZET

DEĞİŞİK MAYA TÜRLERİNDE STRES ŞARTLARINDA İNVERTAZ ENZİM AKTİVİTESİNİN VE İLGİLİ GEN KONTROLÜNÜN BELİRLENMESİ

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Üreme hızı sonucu ortaya çıkan biyolojik kütledeki artış çoğunlukla ortamdaki karbon kaynağına bağlı olarak farklılık gösterir. *Saccharomyces cerevisiae* maya türünde, ortamda bulunan değişik karbon kaynaklarının alınması ve kullanılması üreme ile yakın ilişkilidir. Sukrozun kullanılması, *Saccharomyces cerevisiae* maya türünde *SUC2* geni ve *Pichia anomala*

maya türünde *INV1* geni tarafından kodlanan hücredışı invertaz aktivitesine bağlıdır.

Bu araştırmada *Pichia farinosa*, *Pichia anomala*, *Pichia jadini*, *Candida milleri* ve *Saccharomyces cerevisiae* maya türleri farklı karbon kaynaklarında üretilerek stres şartlarındaki invertaz aktiviteleri belirlendi. *Saccharomyces cerevisiae* araştırmamızda kontrol maya türü olarak kullanıldı.

Pichia farinosa üreme aşamalarının hiçbirinde invertaz aktivitesi göstermedi. Ayrıca bu maya türünde değişik sıcaklık ve pH değerlerinde de invertaz enzim aktivitesine rastlanmadı.

Pichia anomala maya türünün invertaz aktivitesinin, logaritmik ve durağan üreme safhalarında, glikoz baskılaması ile kontrol edildiği görüldü. Değişik sıcaklıklarda ve pH değerlerinde enzim aktivitesinin yine glikoz baskılaması tarafından kontrol edildiği gözlendi.

Pichia jadini ve *Candida milleri* maya türleri en yüksek invertaz aktivitesini göstermiş olup, bu enzim aktivitesinin glikoz baskılaması ve stres şartlarından etkilenmediği görüldü.

Elde ettiğimiz sonuçlar *Pichia anomala, Pichia jadini* ve *Candida milleri* maya türleri için 25°C ile 30°C arasındaki sıcaklık değerlerinin, ve 5.0 ile 6.0 arasındaki pH değerlerinin enzim aktivitesi için optimum şartlar olduğunu gösterdi.

Pichia anomala ve *Saccharomyces cerevisiae* maya türlerinde, invertaz aktivitesinin kısa-dönem derepresyon şartlarında uzun-dönem derepresyon şartlarından daha fazla olduğu gözlendi.

Anahtar Kelimeler: *Pichia* sp., *Candida* sp., İnvertaz, Isı Stresi, Asidite, Glikoz Baskılaması.

To My Family and Friends

&

To My Grandfather

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1. INTRODUCTION

1. 1. Overview of Yeast Species

Yeast is a good model system to understand metabolic regulation of eukaryotic organisms because of its suitable characteristics such as rapid growth, ease of replica plating, well-defined genetic system, non-pathogenic characteristics and highly versatile DNA (Deoxyribo Nucleic Acid) transformation system for biological studies. Yeast cells respond to the quality and quantity of carbon and nitrogen sources in the environment both by adjusting their transcriptional and metabolic profiles to made optimum use of the available nutrients and by selecting a developmental program such as budding, pseudohyphal differentiation, quiescence or sporulation that maximizes their potential for survival under the existing nutrient conditions (Schneper *et al.,* 2004). For yeasts, like for many other microorganisms, glucose is preferred carbon and energy source. Therefore glucose is an important primary messenger molecule, signaling optimal growth conditions to the cellular machinery (Rolland *et al.* 2002).

Baker's yeast refers to the strains of *Saccharomyces cerevisiae* used in the baking industry. The strains are different from the laboratory strains in their DNA content and chromosomal polymorphism; most of the industrial

strains are aneuploids (Codon *et al.,* 1998). Baker's yeast is required to have several characteristics such as high leavening ability, osmotolerance, freeze and chemical tolerance, mellibiose utilization, good storage ability, and non-agglomeration (Walker, 1998).

Non-conventional yeasts, belonging to genera other than *Saccharomyces*, are receiving increasing attention as subjects for biochemical and molecular studies because of easy procedures for allowing their genetic manipulations (Creg *et al.,* 1985; Gleeson *et al.,* 1986; Gleeson and Sudbery, 1988; Nicaud *et al.,* 1989). *Pichia* genus contains the largest number of species and consequently includes some of the more attractive organisms from both the academic and industrial points of view (Barnett *et al.,* 1990). Some *Pichia* sp. has been shown to be excellent hosts for the expression of heterologous genes, with production yields equivalent to or higher than those obtained with *Saccharomyces* sp. (Buckholz and Gleeson, 1991).

Saccharomyces cerevisiae can grow on variety of fermentable and non-fermentable carbon sources. Accordingly, on addition of glucose to cells grown in a non-fermentable carbon source, yeast cells undergo, a major metabolic and transcriptional reprogramming: numerous enzymes are either phosphorylated, targeted for degradation or both, and approximately 30% of all genes significantly change their expression levels (Schneper *et al.,* 2004).

Glucose may affect enzyme levels by causing a decrease in their translation rate, or on increase in the degradation rate of the protein. In turn mRNA (messenger Ribo Nucleic Acid) levels would depend both on the rate

of transcription of the corresponding gene and on the stability of the mRNA. The main effect of glucose takes place at the transcription level (Gancedo, 1998; Carlson, 1987).

Enzyme levels are regulated at the stage of gene transcription (repression and induction), mRNA stability, translation and protein stability while enzyme activities are regulated post-translationally by allosteric and covalent activation and inhibition most of these processes are affected either directly or indirectly by specific glucose sensing and signal transduction pathways. There are two classes of genes that are responsible from glucose signaling. One of them repressed by glucose, which encodes proteins involved in respiration, gluconeogenesis and glycoxylate cycle. Another class encodes proteins that are involved in the uptake and metabolization steps of alternative carbon sources such as the sucrose, galactose and maltose and genes play a role in utilization of ethanol, lactate and glycerol (Rolland *et al.,* 2002).

1.2. Glucose Sensing, Signaling and Phosphorylation

Controlled uptake and utilization of monosaccharides are key metabolic events for the growth of the yeast *Saccharomyces cerevisiae* in various environments. Both uptake and utilization of monosaccharides are regulated by extracellular and intracellular signals. Monosaccharide uptake, which is mediated by hexose transporter proteins, occurs by means of facilitated diffusion mechanism (Romano, 1982).

Twenty different *HXT* (Hexose transporter) related genes, *HXT1-HXT17, GAL2* (Galactose transporter 2)*, SNF3* (Sucrose non-fermenting 3) and *RGT2* have been identified in *Saccharomyces cerevisiae* but only a limited number of *HXT* genes (*HXT1-HXT7*) are required for regulated glucose uptake in laboratory conditions (Boles and Hollenberg, 1997; Reifenberg *et al.,* 1995). Cellular functions of the *HXT8-HXT17* genes in terms of their glucose or nutrient transport activities are not known yet. There are seven members of *HXT* genes metabolically significant in hexose transport. These are *GAL2* (for galactose transport), *HXT1, HXT2, HXT3, HXT 4*, *HXT6* and *HXT7* (for glucose, fructose and mannose transport). Two other members of the family, *SNF3* and *RGT2*, encode glucose sensors. It is presumed that binding of glucose to those cell membrane proteins produce signals that allow the cell to synthesize the transport proteins needed for glucose uptake (Özcan *et al.,* 1996; Özcan *et al.,* 1998).

In *Saccharomyces* cerevisiae, glucose transport is thought to be mediated by three kinetically distinct systems. One of them is a glucose-repressible high affinity system that operates in low glucose concentration. The other one is intermediate affinity system that works in intermediate glucose concentration. The last one is low affinity system, which operates in high glucose concentration. The *SNF3* gene is glucose repressible and is required to high affinity glucose transport. The *RGT2* is required for low affinity glucose transport, which operates at high concentrations of glucose. *HXT1* and *HXT3* encode low affinity transporter (K_m=50 to 100 mM), *HXT2* and *HXT4* encode intermediate affinity transporters (K_m≈10mM), and *HXT6*

and *HXT7* encode high affinity transporters (K_m=1 to 2 mM) (Reifenberg *et al.,* 1997; Kruckeberg and Bisson, 1999; Bisson *et al.,* 1987).

The Ras-cAMP pathway responds to environmental stimuli in addition to glucose, such as heat shock (Shin *et al.,* 1987). Cyclic AMP (cyclic adenosine mono phosphate-cAMP) levels affect cAMP-dependent protein kinase A (PKA) that in turn affects the activities and synthesis of a large number of gene products involved in metabolism and growth control (Broach and Deschenes, 1990). Cyclic AMP binds to the regulatory subunits of PKA and induces dissociation of the catalytic subunits (Toda *et al.,* 1987).

This pathway plays a key role in activating adenylyl cyclase, since both Ras and Cdc25 (cell division cycle 25) proteins are essential for basal adenylyl cyclase activity and cell viability (Newcomb *et al.,* 2003). Ras proteins, shuttling between GDP (Guanosine diphosphate) and GTP-bound (Guanosine triphosphate) forms, play diverse physiological roles by mediating signaling for receptor protein kinases and tyrosine kinase which is encoded by *RAS1* and *RAS2,* are associated with adenylate cyclase and regulate the level of cAMP (Toda *et al.,* 1985, Toda *et al.,* 1987).

Three different enzymes phosphorylate intracellular glucose. Hexokinase A or P-I (Hxk1p) phosphorylates glucose and fructose, but the rate of fructose phosphorylation is 2.5 times greater than that of glucose. Hexokinase B or P-II (Hxk2p) exhibits similar rates of phosphorylation on the two sugars. Glucokinase (Glk) does not act on fructose. All three of these enzymes act on mannose (Maitra and Lobo, 1971). Hexokinase 2 protein (Hxk2p) encoded by *HXK2* gene, initiates glucose metabolism and highly

expressed during growth in glucose. Hxk2p is found both in the nucleus and cytoplasm. The nuclear localization of Hxk2p is dependent on the phosphorylation status (Herrero *et al.,* 1998).

1. 3. Glucose Repression and Derepression

Wild type yeast cells, when grown on glucose, have lower levels of many enzymes needed to utilize alternate carbon sources. As the glucose in the medium is exhausted, the synthesis of these enzymes is derepressed. This phenomenon, called 'carbon catabolite repression' or 'glucose repression', in many cases has been demonstrated to act at the level of transcription (Ronne, 1995). The glucose repressible genes can be divided into three groups:

First *FBP1* (Fructose biphosphatase) and *PCK1 (PEB* Carboxykinase 1) genes, which encode Fructose biphosphatase enzyme and *PEB* carboxykinase enzyme unique to gluconeogenesis. These genes are strictly repressed by glucose to prevent gluconeogenesis from taking place simultaneously. The enzymes of the glycoxylate cycle, such as isocitrate lyase, are also required for growth on gluconeogenetic carbon sources and seem to be coordinately regulated with Fbp1p and Pck1p (Ronne, 1995; Francisca *et al.,* 1997).

The second group comprises genes encoding mitochondrial enzymes that are involved in the Krebs cycle and in respiration. The mitochondria metabolize non-fermentable carbon sources, but are dispensable during fermentative metabolism of glucose (Ronne, 1995; Ciriacy, 1978).

The third group of glucose-repressed genes encodes proteins that take up and metabolize other carbon sources. This includes the products of the *GAL, SUC* and *MAL* genes, which catalyze the initial steps in utilization of galactose, sucrose and maltose, respectively. The genes involved in utilization of ethanol, lactate and glycerol are similarly repressed by glucose (Ronne, 1995; Neigeborn and Carlson, 1984).

1. 4. Structure of *SUC2* Gene

A family of closely related *SUC* genes encodes the enzyme invertase, which hydrolysis extracellular sucrose and raffinose into its monosaccharides (Carlson, 1987). Raffinose is first cleaved by mellibiase then by invertase. This SUC gene family includes *SUC1, SUC2, SUC3, SUC4, SUC5* and *SUC7* genes (Carlson and Bonstein, 1983; Carlson *et al.,* 1985).

These polymeric *SUC* genes are localized in the telomeric regions of different chromosomes: *SUC1* on chromosome VII, SUC2 on chromosome IX, *SUC3* on chromosome II, *SUC4* on chromosome XIII, *SUC5* on chromosome IV and *SUC7* on chromosome VIII (Carlson and Bonstein, 1983; Naumov *et al.,* 1996).

Each *SUC* gene encodes two forms of α-fructosidase, invertase (EC 3.2.1.26, α -D-fructofuranoside-fructohydrolase). One is highly glycosylated and located in the periplasmic space, and the other one is nonglycosylated intracellular form. The extracellular invertase is required for the hydrolysis of sucrose into glucose and fructose. Even thought the function of

intracellular invertase is unknown; it might have a role in the sucrose metabolism or degradation of intracellular sucrose. Because, the direct transport of sucrose in to the cell has been reported (Santos *et al.,* 1982). Extracellular enzyme synthesis is regulated by glucose repression, but the synthesis of intracellular invertase is constitutive at low levels (Neigeborn and Carlson, 1984).

The secreted and intracellular forms of invertase are encoded by two differently regulated *SUC2* mRNAs (Carlson and Botstein, 1982). The secreted invertase is encoded by a 1.9kb mRNA and cytoplasmic enzyme is encoded by a 1.8kb mRNA. These two mRNAs differ at their 5'ends. Because, the extracellular enzyme's mRNA (1.9kb) includes a signal peptide coding sequence for transportation to out of cell (Carlson and Botstein, 1982; Carlson *et al.,* 1983).

1. 5. Regulation of *SUC2* Gene

SUC2 gene is regulated by repression-derepression mechanism depending on concentration of glucose out of the cell. Genes required for the derepression of *SUC2* gene in response to glucose deprivation have been identified in different research.

The mutation in *SNF1* gene (sucrose-non-fermenting 1) (also known as *CAT1* or *CCR1*) abolishes the derepression of secreted invertase synthesis but do not affect the synthesis of cytoplasmic invertase. *SNF1* gene encodes a protein serine/threonine kinase. The *SNF1* kinase is associated with other

proteins in a large complex. One of the components of this complex is *SNF4* (also known as *CAT3*). *SNF4* gene encodes a protein that is required for maximal *SNF1* kinase activity (Yang *et al.,* 1994; Celenza and Carlson, 1989). The Snf1 protein kinase and Snf4p subunit interact with Sip1/Sip2/Gal83 component in the kinase complex (Jiang and Carlson, 1997). The targets of yeast Snf1 protein complex are *MIG1* (Multicopy inhibitor of GAL genes 1), *SIP3* (*SNF1* interaction protein 3*)* and *SIP4 (SNF1* interaction protein 4*)* (Hardie *et al*., 1998).

MSN1, MSN2 MSN3 and *MSN4* (Multicopy Suppressor of Snf) encode transcriptional activators that affect genes regulated by the *SNF1* pathway. (Hubbard *et al.*, 1994; Trillman *et al.,* 1995).

SNF2 (*SWI2*) encodes an activator of *SUC2*. *SWI1* (Switch 1), *SWI2* (Switch 2), and *SWI3* (Switch 3), genes. *SWI2* is identical with *SNF2* (Peterson and Herskowit, 1992; Peterson *et al.,* 1994).

SNF3 gene encodes a putative glucose sensor. It is expressed under low glucose concentrations and required for the rapid induction of hexose transporters (*HXT*'s) in limiting substrate concentrations (Bisson *et al.,* 1993; Laurent *et al.,* 1990; Liang and Gaber, 1996).

The *SNF5* and *SNF6* gene products are also required for expression of *SUC2* gene (Estruch and Carlson, 1990; Hirschhorn *et al.,* 1992).

The nuclease-sensitive chromatin structure of the *SUC2* promoter disappeared in *snf2* and *snf5* mutant strains. However, both *SUC2* transcription and its characteristic chromatin structure are restored when one of the two sets of genes encoding histones H2A and H2B is deleted

(Hirschhorn *et al*., 1992). Snf/Swi destabilizes nucleosomes and then a second step is necessary, to either modify or remove histones from DNA, to allow transcription to occur (Figure 1) (Hartzog and Winston, 1997; Gavin and Simpson, 1997; Sundarsanam and Winston, 2000).

Five other genes required for derepression of the *SUC2* gene are *SNF7, SNF8, SNF9, SNF10* and *GAL11*. (Tu *et al.,* 1993; Yeghiayan *et al*., 1995; Vallier and Carlson, 1991).

DNA binding transcription factor Gcr1p (Glycolysis regulation-1) is required for the high level transcription of glycolytic genes (Clifton *et al*. 1978). Gcr1p is essential for the regulated transcription of *HXT4*, which encodes high affinity hexose transporter, Ty2-917 retrotransposon of *Saccharomyces cerevisiae* and repression of *SUC2* gene transcription (Türkel and Bisson 1999; Türkel *et al*. 1997; Türkel *et al.,* 2003). Gcr1p specifically interacts with CT box sequence (CTTCC) present in the upstream activation sequences (UAS) of glycolytic genes promoter regions (Baker, 1991). Gcr1p specifically binds to the promoter region of *SUC2* gene and controls the transcription of *SUC2* gene (Türkel *et al.,* 2003).

Several gene products (Hxk2p, Hex2p, Cat80p, Cid1p, Grr1p, Reg1p, Glc7p, Mig1p, Ssn6p and Tup1p) act negatively in glucose repression of disaccharide utilizing enzymes (Schuller and Entian, 1991). Nuclear localization of Hxk2p is necessary for glucose repression signaling of the *SUC2* gene. It was shown that Hxk2p is able to bind directly to *SUC2* promoter region (Herrero *et al.,* 1998; Randez-Gil *et al.,* 1998).

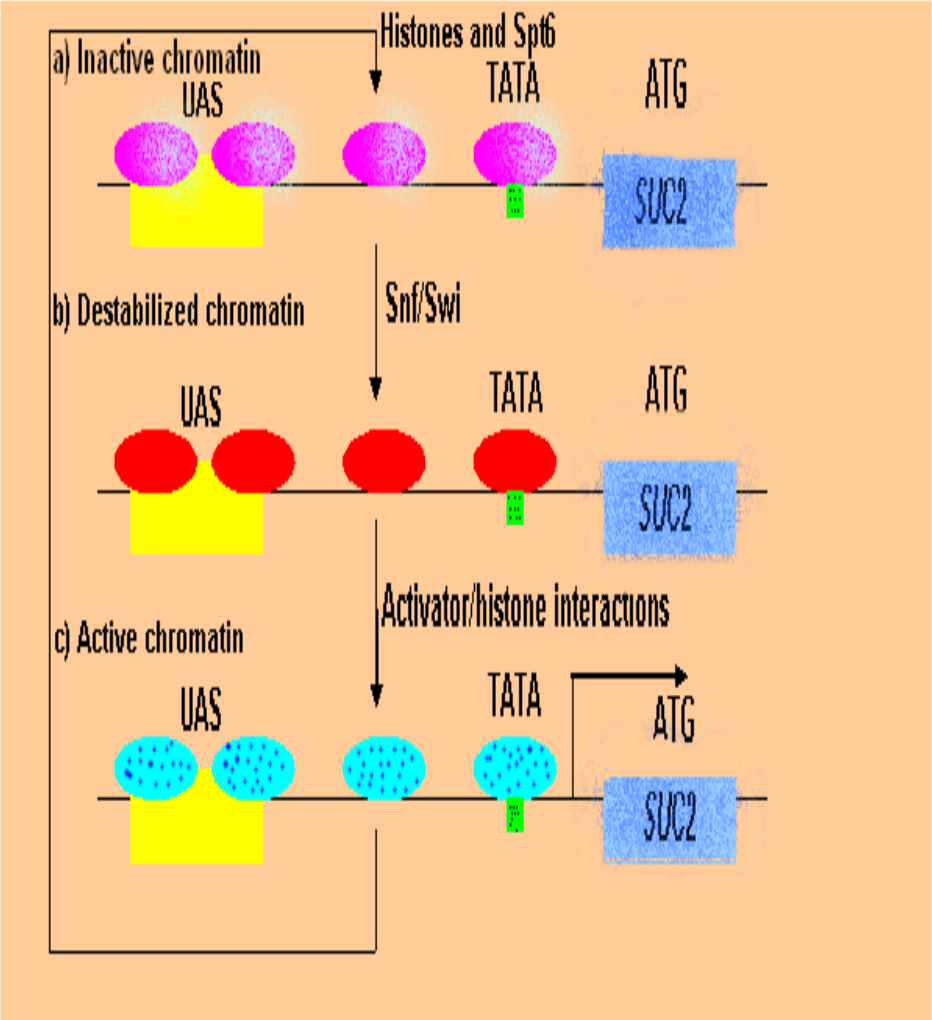


Figure 1: A multistepmodel for either inducing or overcoming nucleosome mediated repression at the yeast *SUC2* promoter (Hartzog and Winston, 1997).

Figure 1 (continued):

The repression of the *SUC2:*

a) The formation of nucleosomes over the promoter region causes the repression of *SUC2* gene. This repression requires the activity of Spt6, perhaps in nucleosome assembly. To overcome *SUC2* repression, at least two steps are required.

b) The Snf/Swi complex is required to remodel nucleosomes, destabilizing the chromatin; this step is blocked in snf/swi mutants.

c) The histones must then be either modified or removed; this step is blocked particularly in hta1 (H2A) mutants.

The pink ovals in (a) represent nucleosomes repressing transcription, the red ovals in (b) represents destabilized nucleosomes that still repress transcription and the blue ovals in (c) represent nucleosomes that have been either further modified or removed allowing transcription to occur.

Mig1p is the repressor protein that is responsible from glucose repression of many genes, including *GAL, SUC* and *MAL*. Mig1 is a Cys2 – His2 zinc finger containing protein that binds to promoters of glucose-repressed genes and recruits the general repressors Ssn6p-Tup1p. Hxk2p and Reg1p activate the Mig1p repressor (Lutfiyya and Johnston, 1996).

Mig1p, Ssn6p and Tup1p complex is negatively controlled by Snf1 protein kinase. Glucose induced changes in the localization of Mig1p is independent from the DNA binding ability of Mig1p. Addition of glucose that inactivates Snf1p, allows a rapid dephosphorylation of Mig1p by the Rep1-Glc7 phosphatase complex (Figure 2). Reg1p is phosphorylated in response to glucose limitation and that this phosphorylation requires Snf1p; moreover, Reg1p is dephosphorylated by Glc7p when glucose is added and Hxk2p has a role in regulating the phosphorylation state of Reg1p (Figure 3) (DeVit *et al.,* 1997; Östling and Ronne, 1998; Sanz *et al.,* 2000; Treitel *et al.,* 1998).

Other regulatory factors including Sko1p, Glc7p, Gal11p, Grr1p and Med8p are also required for the regulation of the *SUC2* transcription (Vallier and Carlson, 1991; Moreno *et al.,* 1999)

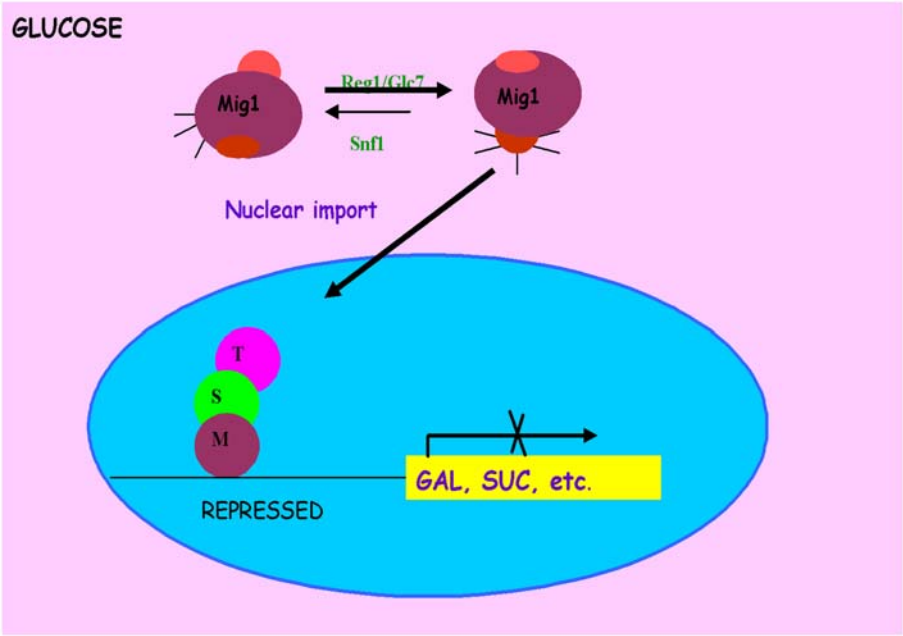


Figure 2: Phosphorylation of Mig1p in the presence of glucose (DeVit *et al.,* 1997).

Dephosphorylation of Mig1p induces nuclear import, either by activating a nuclear localization signal or by inactivating a nuclear export mechanism. When Mig1p enters the nucleus, it binds to its target genes (e.g. *SUC2*) and repress transcription of genes, which is not required for growth on glucose.

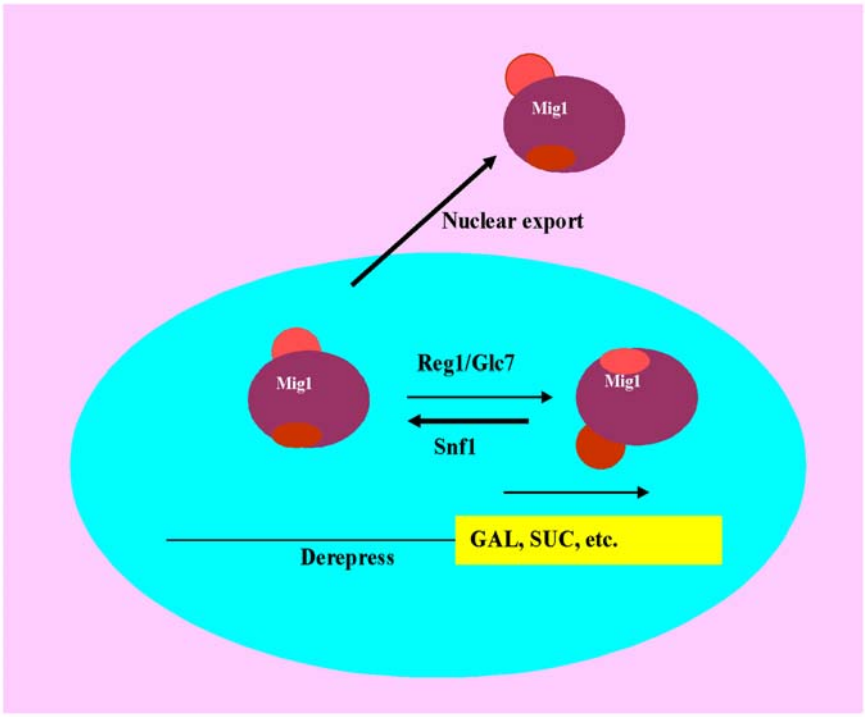


Figure 3: Dephosphorylation of Mig1p in the absence of glucose (DeVit *et al.,* 1997).

When glucose depleted Snf1 protein kinase is activated and then it phosphorylates Mig1p. Phosphorylation of Mig1p induces nuclear export and inhibits import, thus Mig1p is sequestered in the cytoplasm.

1. 6. Invertase Enzyme Activity in Different Yeast Species

A heterologous protein that has been expressed in a *Pichia* system is invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) from *Saccharomyces cerevisiae* (Tschopp *et al*., 1987). A genomic library from the yeast *Pichia anomala* has been constructed and employed to clone the gene encoding the sucrose-hydrolysing enzyme invertase by complementation of a sucrose non-fermenting mutant of *Saccharomyces cerevisiae*. The cloned gene, *INV1*, was sequenced and found to encode a polypeptide of 550 amino acids which contained a 22 amino-acid signal sequence and ten potential glycosylation sites. The amino-acid sequence shows significant identity with other yeast invertases and also with *Kluyveromyces marxianus* inulinase, a yeast β -fructofuranosidase that has different substrate specificity. The nucleotide sequences of the 5′ and 3′ non-coding regions were found to contain several consensus motifs probably involved in the initiation and termination of gene transcription (Perez *et al*., 1996)

The K_m values for sucrose and raffinose about 40 % lower than those reported for *Saccharomyces cerevisiae* invertase on the same substrates under similar assay condition indicating a higher affinity of *Pichia anomala* invertase for both sucrose and raffinose (Gascon *et al*., 1968).

Like *Saccharomyces cerevisiae*, the yeast *Candida utilis* can use sucrose as an alternative carbon source. The invertase from *Candida utilis* has been purified and characterized and synthesis of this enzyme has been

shown to decrease drastically when glucose concentration is higher than 1 % in the medium (Chavez *et el*., 1998). The *CuINV1* gene was cloned from a DNA genomic library and putative binding sites for the Mig1p repressor were found in the 5'-flanking sequence of the coding region These observations suggest that the *CuINV1* gene from the *Candida utilis* is repressed by a Mig1p homologue (Chavez *et al*., 1997).

Candida utilis, which is cultivated in liquid media enriched with saccharose, synthesizes two forms of invertase enzyme with a molecular mass of 300 kDa and 60 kDa. First enzyme presents both in periplasmic space and culture broth. The presence of the latter enzymatic form was detected in cells as well as in the liquid culture medium. Both invertase forms were purified and named as F-form (Fast; 60 kDa) and S-form (Slow; 300 kDa). The F-form of invertase was found to be nonglycosylated as opposed to the S-form of invertase (Belcarza *et al*., 2002).

The cloned *INV1* gene of *Candida utilis* was sequenced and found to encode a polypeptide of 533 amino acids that contain a 26 amino-acid signal peptide and 12 potential N-glycosylation sites. The nucleotide sequences of the 5' and 3' non-coding regions were found to contain motifs probably involved in initiation, regulation and termination of gene transcription. The amino-acid sequence shows significant identity with other yeast, bacterial and plant beta-fructofuranosidases. The *INV1* gene from *Candida utilis* was able to complement functionally the *SUC2* mutation of *Saccharomyces cerevisiae* (Chavez *et al*., 1997).

Candida utilis produces high yields of invertase whether the carbon source is sucrose, glucose, maltose, or xylose and still higher yields with lactic acid, glycerol, and ethyl alcohol. Approximately 20 to 30% of the total invertase of *C. utilis* is extracellular (Dworschack and Wickerham, 1961).

Invertase gene was also cloned and sequenced from the *Schwanniomyces occidentalis* (Klein *et al.,* 1989).

The industrial yeasts are generally superior in invertase production to the other yeast included in the survey (Turgut, 2000; Türkel *et al.,* 2002)

1. 7. Stress Conditions

Continuously changing environmental conditions are a source of stress for living organisms. Stress is also generated endogenously through metabolic activity. Adaptation to changes in the environment is of crucial importance for the survival of any living organism.

These adaptation mechanisms can be divided into three-phases:(i) Perception of an external signal, (ii) Intracellular transmission of this signal, (iii) leading to an adaptive response.

A wide variety of signal transduction machineries have been characterized that enable yeast cells to trigger the appropriate responses to external stress stimuli (Hohmann and Mager, 1997).

Thermal stress in yeast induces a heat shock response that is characterized by temporary arrest in the G_1 phase of the cell cycle at the regulatory step and transient decline in the abundance of most cellular

RNA's, while the expression of stress-responsive genes is induced (Mager and Ferriera, 1993). A major stress-responsive gene is the one coding for heat shock protein Hsp104, which has been implicated in resolubilazation of proteins and reactivation of mRNA splicing, after heat inactivation (Parsell *et al.,* 1994; Vagel *et al.,* 1995). Hsp104p necessary for survival at high temperatures (Sanchez and Lindquist, 1990).

The growth of the strain *S. bayanus* var. *uvarum* decreased by 25% between 30°C and 25°C then becomes stable for inferior temperatures whereas growth of the strain *S. cerevisiae* falls continually (loss of 13% and 40%, respectively, between 35 and 30°C and between 30°C and 25°C). The strain *S. bayanus var. uvarum* is less sensitive to temperature falls than the strain *S. cerevisiae*; this is in agreement with the classification of this species as cryophilic yeast (Serra *et al.,* 2005).

When budding yeast cells such as *Saccharomyces cerevisiae*, exposed to high osmolarity stress, they increase the intracellular osmolarity by upregulating glycerol synthesis, which is induced by activation of the HOG (high osmolarity glycerol response) pathway composed of Hog1p MAPK, Pbs2p MAPK kinase (MEK) and redundant MEK kinasess (MEKK) Ssk2p, Ssk22p and Ste11 (Boguslawski, 1992; Brewster *et al.,* 1993; Maeda *et al.,* 1995; Posas and Saito, 1997).

In the fission yeast *Schizosaccharomyces pombe,* a HOG1 homolog Spc1 was identified as a regulator of the osmotic response and cell cycle. In contrast to the budding yeast HOG pathway, which is activated only by osmostress, *Schizosaccharomyces pombe* Spc1 is activated by many different

forms of stress including high osmolarity, oxidative stress, heat shock, UV irradiation and nutritional limitation (Schüller *et al.,* 1994; Degols *et al.,* 1996; Shizaki and Russell, 1997).

Glycogen and trehalose are the major storage carbohydrates in *Saccharomyces cerevisia*e. Trehalose accumulated in response to unfavorable growth conditions such as high temperature, high osmolarity and nutrient limitations (Lillie and Pringle, 1980; Parrou *et al.,* 1997; Parrou *et al.,* 1999).

Exposure of cells of *Schizosaccharomyces pombe* to heat shock or osmotic up shift results in an increased level of neutral trehalase activity, which is responsible for hydrolysis of intracellular trehalose (Cansado *et al.,* 1998).

When the heat stress applied to the logarithmically growing *Pichia* cells, high levels of trehalose accumulations observed in *Pichia anomala* and *Pichia farinosa* (Turkel, 2006). In addition, heat stress caused to decrease in the glycogen contents of *Pichia anomala.* Trehalose accumulation was not recorded for *Pichia angusta* cells (Turkel, 2006).

Transcriptionally silent chromatin structure is dynamic and may change its conformation in response to external or internal stimuli. In general, increasing the temperature within the range of 23°C–37°C strengthens HM and telomeric silencing but reduces rDNA silencing. DNA at the silent HML locus becomes more and more negatively supercoiled as temperature increases in a Sir-dependent manner, which is indicative of enhanced silent chromatin. This enhancement of silent chromatin is not

dependent on silencers and therefore does not require *de novo* assembly of silent chromatin. MAP kinase-mediated Sir3p hyperphosphorylation, which plays a role in regulating silencing in response to certain stress conditions, is not involved in high temperature-induced strengthening of silencing. In addition, Pnc1p, a positive regulator of Sir2p activity, plays no role in thermal regulation of silencing. Therefore, growth temperature regulates transcriptional silencing by a novel mechanism (Serra *et al.,* 2005).

The effects heat stress are thought to result from the damage of proteins and permeabilization of membranes, particularly the plasma membrane leading to the dissipation of the transmembrane H^+ gradient and to a decrease in intracellular pH (Weitzel *et al.,* 1987; Piper, 1993). Acidic pH caused by the presence of permanent weak acids is the most common stress encountered by food borne pathogens and food-spoiling microorganisms during food preservation (Brown and Booth, 1991; Archer, 1996). Depletion of glucose, or other nutrients, induces a starvation response in yeast, which is part of the general stress response and renders cells resistant to various stress conditions (Hohmann and Mager, 1997). The ability of cells to grow and maintain viability in the presence of permanent weak acids at low pH reflects their capacity to maintain control over intracellular pH (pH_i) by excluding H^+. The pH_i depends on the activity of the H^+-ATPase in the plasma membrane and on the plasma membrane passive permeability to protons (Serrano, 1998; Carmelo *et al.,* 1997). At optimal concentrations, weak acids induce the activation of *Saccharomyces cerevisiae* plasma membrane ATPase *in vivo* and this response is vital in the

restoration of pH homeostasis during recovering from acid stress (Viegas and Sa-Correia, 1991; Coote *et al.,* 1994; Viegas and Sa-Correia, 1995; Carmelo *et al.,* 1997).

Intracellular pH (pH_i) in eukaryotic cells is strictly regulated. Eukaryotic cells control cytoplasmic pH at 7.0–7.4 by ion transport mechanisms and a high buffering capacity of the cytosol. First of all, most enzymes have an optimum pH for maximum activity. The activity of enzymes taking part in the cellular metabolism is pH-sensitive. In mammalian cells, DNA replication is extremely pH- sensitive (Coote *et al.,* 1991).

The contractile activity of acto-myosin has dramatically influenced by small change in pH. Microtubule assembly and disassembly is affected by pH with an increased disassembly at alkaline pH. Moreover, pH oscillations seem to be important in controlling the cell cycle and proliferative capacity of cells. The regulation of the pH_i of phagocytic cells is critical to their function and viability. Failure to maintain pH homeostasis results in decreased cellular enzyme activity, cellular migration, and microbial function (Viegas *et al.,* 1998).

Yeasts, like many microbes, encounter large variations in ambient pH in their natural environments. Microorganisms capable of growing over a wide pH range require a versatile and efficient pH homeostatic mechanism protecting intracellular processes against extremes of pH. Budding yeasts grow well at acidic pH as well as alkaline pH in the range of 2.5–8.5, and the kinetics of growth and fermentation are not affected between pH 3.5 and 6.0

because of the tight control of intracellular pH. Proton or ion transport proteins in plasma membranes and vacuole membrane might play roles in regulating intracellular pH.

However, it has not been described or investigated how the pump proteins are regulated, nor how cells sense extracellular pH, nor what signaling components are involved in pH regulation (Serrano, 2006).

As seen, there are significant differences in the expression patterns and activities of invertase enzymes among various nonconventional yeasts at different stress conditions. The aim of this study was to analyse the effect of heat stress and pH stress on the biosynthesis of invertase enzyme in nonconventional yeasts. Also, in this research invertase enzyme activity was determined after prolonged incubation of nonconventional yeasts in fermentablea nd nonfermentable carbon sources.

2. MATERIALS AND METHODS

2. 1 Yeast Strains

The yeast strains used in this study are given in the Table 1. The non-conventional yeast strains (*Pichia farinosa, Pichia anomala, Pichia jadinii,* and *Candida milleri*) are obtained from DBVPG collection (University of Perugia, Italy). *Saccharomyces cerevisiae* (YST124), used as the reference strain, is obtained from Euroscarf Collection (University of Frankfurt, Germany).

The non-conventional yeast strains are known as wild type. The genotype of *S. cerevisiae* strain (YST124) used in this study is *MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*.

Strains	Species Name and Genotype	Source
NCY 2	*Pichia farinosa* Wild Type	DGVPG-3626/CBS185
NCY 12	*Pichia anomala* Wild Type	DBVPG-3511
NCY 16	*Pichia jadinii (Type of Torula (Candida) utilis)* Wild Type	DBVPG-6160/CBS621
NCY 17	*Candida milleri* Wild Type	DBVPG-6754/CBS8195
YST 124	*Saccharomyces. cerevisiae* *MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0.*	Frankfurt Üniv.

Table 1: List of yeast strains used in this study.

2. 2 Growth Conditions

Yeast strains *Pichia farinosa, Pichia anomala* and *Saccharomyces cerevisiae* were cultivated in YPD medium (10g/lt Yeast Extract, 20g/lt Bacto-Peptone, 2% glucose (w/v)) or YPGL medium (10g/lt Yeast Extract, 20g/lt Bacto-Peptone, 2% glycerol (v/v) and 2% lactate (v/v)) with constant shaking (250 rpm) in 25°C and 30°C orbital shaker. *P. jadinii* and *C. milleri* were cultivated in YM medium containing 5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone as described (Granström et al. 2000). YM media either supplemented with 2% glucose (w/v) or 2% glycerol (v/v) and 2% lactate (v/v) as carbon source.

For growth of *Pichia* and *Candida* in minimal medium, yeast cells were cultivated in YNBD (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$) and 2% glucose (w/v)) or YNBGL (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$), 2% glycerol (v/v) and 2% lactate (w/v)).

For growth of *S. cerevisiae* in minimal medium, yeast cells were cultivated in YSD (Synthetic Complete Drop out Medium without Uracil, 0.5% Uracil (w/v), 2% glucose (w/v)) or YSGL (Synthetic Complete Medium without Uracil, 0.5% Uracil (w/v), 2% glycerol (v/v) and 2% lactate (v/v)) (Guthrie and Fink 1991).

Glucose repressed and derepressed yeast cells were prepared as described previously (Celenza and Carlson 1984). Yeast cells were grown in YP media supplemented with 2% glucose (w/v) until the logarithmic stage

(OD_{600}: 0.8-0.9). Then portion of the growing cultures harvested and washed twice with sterile ice-cold distilled water, and then yeast cells were resuspended in fresh YP, YNB or YS media supplemented with 0.05% glucose (w/v) for derepressed and 2% glucose for repressed growth conditions. Yeast cells were further incubated under these conditions for 2-3 hrs with constant shaking (250 rpm) at 30 °C for *S. cerevisiae* and at 25°C for other yeast species.

2. 3. Growth Characteristics and Doubling Times

In order to determine the growth characteristics and doubling times of all strains, they were grown in YP medium (20 g/l Bacto-peptone and 10 g/l Yeast Extract), in YM medium (5g/l yeast extract, 3 g/l malt extract, 5 g/l Bacto peptone), in YNB medium (1.6 g/l Yeast Nitrogen Base and 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) or in YS medium (Synthetic Complete Drop out Medium without Uracil, 0.5% Uracil (w/v)). At each growth test, 2% glucose (w/v) was used as a fermentable carbon source and, 2% glycerol (v/v) and 2% lactate (v/v) were used as non-fermentable carbon source. For plate tests, same growth conditions were used. After 2-3 days of growth at 25°C or 30°C temperature, plates were photographed.

Yeast cells were grown at 25°C and 30°C temperature and with constant shaking at 250 rpm until the logarithmic stage (OD_{600}: 0.8-0.9). 1ml sample from the growing cultures were taken with 30 minutes time intervals and OD_{600} were measured. Then, growth curves were prepared from these

values by plotting the OD_{600} readings versus time intervals. Duplication times for three different OD_{600} intervals (from 0.4 to 0.8, from 0.5 to 1.0 and from 0.6 to 1.2, respectively) were determined. Then, the mean of these three duplication times were calculated. For each strain and carbon source this was repeated for three times. Hence, one doubling time value results from the three different growth curves for each carbon source. Standard deviations in these experiments were less than 25 minutes.

2. 4. Invertase Enzyme Assay

The secreted invertase activities of the yeast cells were determined using whole cells as described previously with following modifications (Rothe and Lehle, 1998). First, yeast cells were grown in YP and YM media supplemented with 2% glucose (w/v) or with 2% glycerol (v/v) and 2% lactate (v/v) until the logarithmic stage (OD_{600}: 0.8-0.9). Then 5 ml of cultures were harvested and washed twice with sterile ice-cold water, and then yeast cells were resuspended in 5 ml of appropriate media supplemented with 0.05% glucose (w/v) for derepressed growth conditions or 2% glucose (w/v) for repressed growth conditions. Yeast cells were further incubated under these conditions for 2-3 hrs with constant shaking at 250 rpm. Cells were harvested and washed twice with the 5 ml buffer-A (50 mM sodium acetate, pH: 5) and resuspended in 200 µl of buffer-B (50mM sodium acetate pH: 5.2, 2mM PMSF). 50µl of yeast cell suspension was incubated with 200 µl of 200mM sucrose for 15 minutes at 37°C

temperature. The reaction was stopped by addition of 50 µl 1 M Tris/HCl pH 8.8. The glucose released was measured with glucose oxidase assay using 5-10-20 µl of reaction mixture (Goldstein and Lampen, 1975; Rothe and Lehle, 1998). Dry weight of strains was determined as described in Coons *et al*., 1995 and it is equal to 0.5mg/ml of logarithmically grown yeast cell used in this study (Coons *et al.,* 1995). One unit of invertase activity is the amount of enzyme that catalyzes the release of one µmol glucose per minute per 100 mg dry weight.

For each strain, this was repeated for three times. Hence, the invertase activities were given as the mean values of three independent experiments.

2. 5. Time Course Invertase Enzyme

First, yeast cells were grown in YP or YM media supplemented with 2% glucose (w/v) for short-term derepression and with 2% glycerol (v/v) and 2% lactate (v/v) for long-term derepression until the early-logarithmic stage (OD_{600}: 0.8-0.9). Yeast culture (100 ml) were divided into two potions, harvested and washed twice with sterile ice-cold water. Yeast cells resuspended in 50 ml of appropriate media (YP or YM) supplemented with 0.05% glucose (w/v) for derepressed growth and 2% glucose (w/v) for repressed growth conditions. Yeast cells were further incubated under these conditions with constant shaking at 250 rpm, and the invertase enzyme

activities were determined at 30 minutes time intervals until the stationary stage.

2. 6. Determination of Invertase Enzyme Activity at Different Temperatures

In order to analyze the effect of temperature on the invertase enzyme activity, yeast strains first grown in 60ml YP media supplemented with 2% glucose (w/v) until the logarithmic stage (OD_{600}: 0.8-0.9). Yeast cells were harvested by centrifugation at 3000 rpm for 5 minutes and washed twice with sterile ice-cold water. Then yeast cells were divided into two aliquots. One portion of the cells resuspended with YP or YM media supplemented with 2% glucose (w/v) for repressed growth condition and other portion resuspended with YP or YM media supplemented with 0.05% glucose (w/v) for derepressed condition. Then both repressed and derepressed cultures were separated into four parts, and each one incubated for three hours at 25 °C, 30 °C, 37 °C and 42 °C temperatures with constant shaking in an orbital shaker. Invertase enzyme activities were determined for yeast cells as described before.

2. 7. Determination of Invertase Enzyme Activity at Different pH's

In order to analyze the effect of pH on the invertase enzyme activity, yeast strains were grown in 60ml YP or YM media supplemented with 2% glucose (w/v) until the logarithmic stage (OD_{600}: 0.8-0.9). Yeast cells were harvested by centrifugation at 3000 rpm for 5 minutes and washed twice with sterile ice-cold water. Then yeast cells were divided into two portions. One portion of the cells resuspended with YP or YM media supplemented with 2% glucose (w/v) for repressed growth condition, and other portion resuspended with YP or YM media supplemented with 0.05% glucose (w/v) for derepressed condition. Then both repressed and derepressed cultures were separated into five aliquots. pH of each aliquot was adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0 by using 1M HCl and 1M NaOH. Yeast cells were incubated at 25°C and 30°C with constant shaking (250 rpm) in an orbital shaker for 3 hours. Invertase enzyme activities of yeast cells were determined as described before.

3.RESULTS AND DISCUSSION

3. 1. Testing the Growth Pattern of Yeast Strains

Growth patterns of yeast cells vary depending on the growth media and genetic background of related yeast species. Growth rate of yeast cells is generally higher in rich media (YP and YM) supplemented with fermentable sugars such as glucose. However, the growth rate of yeast cells in minimal media (YNB or YS) supplemented with non-fermentable carbon source such, as glycerol is very low.

In order to test the growth pattern, yeast strains were patched on the YPD plate (10g/lt Yeast Extract, 20g/lt Bacto-Peptone, 20 g/l Agar and 2% glucose (w/v)), YPGL plate (10g/lt Yeast Extract, 20g/lt Bacto-Peptone, 20 g/l Agar, 2% glycerol (v/v) and 2% lactate (v/v)), YMD plate (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone, 20 g/l Agar and 2% glucose (w/v)), YMGL plate (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone, 20 g/l Agar, 2% glycerol (v/v) and 2% lactate (v/v)), YNBD plate (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$), 20 g/l Agar and 2% glucose (w/v)), YNBGL plate(1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$), 20 g/l Agar, 2% glycerol (v/v) and 2% lactate (v/v)), YSD plate (Synthetic Complete Drop out Medium without Uracil, 0.5% Uracil (w/v), 20 g/l Agar and 2% glucose (w/v)) and YSGL

plate (Synthetic Complete Medium without Uracil, 0.5% Uracil (w/v), 20 g/l Agar, 2% glycerol (v/v) and 2% lactate (v/v)).

As it can be seen at Figure4, Figure 5, Figure 6, Figure 7 and Figure 8 each yeast strain showed differential growth on different carbon sources.

As shown Figure 4 and 5, *Pichia farinosa* (NCY2) and *Pichia anomala* (NCY12) yeast species showed 100% growth (++++) on both rich (YP and YM) and minimal (YNB) media supplemented with fermentable and non-fermentable carbon sources.

Pichia jadinii (NCY16) showed well growth (%100) on YPD, YPGL and YMD plates and 75% growth on YMGL plate. It showed 100% (++++) growth on YNB-D and YNB-GL media (Figure 6). *Candida milleri* (NCY17) showed 75% growth (+++) on YPD, YPGL and YMGL plates, but 100% (++++) growth on YMD plate. This yeast species showed 50% growth (++) on YNB-D and 25% (+) growth on YNB-GL plates (Figure 7). *Saccharomyces cerevisiae* (YST124) showed 100% growth (++++) on YPD and YPGL plates. Growth of *S. cerevisiae* on YMD and YMGL plates decreased 25% and showed 75%growth (++++) (Figure 8). *S. cerevisiae* yeast species did not grow on YNB media supplemented either with glucose or with glycerol and lactate. Because, YNB-D and YNB-GL media were not contain necessary amino acids (Histidine, Leucine, Methionine and Uracil) for growth of yeast cells. Therefore *S. cerevisiae* were grown in synthetic complete drop out medium that includes all amino acids except uracil, which was supplemented. As shown in Figure 8, it showed 100% growth (++++) and 75% growth (+++) on YS-D plate and YS-GL plates, respectively.

All yeast species showed well growth in YP media supplemented with fermentable and non-fermentable carbon sources.

In all experiments YS medium was used for the growth of *S. cerevisiae* instead of YNB medium, and YM medium was used for growth of *C. milleri* and *P. jadinii* instead of YP medium.

The amount of growth was indicated as followed:

++++: 100% growth

+++: 75% growth

++: 50% growth

+: 25% growth

+/-: 10% and less growth

-: 0% growth

Growth Medium	Growth Pattern	Amount of Growth
YP- 2% Dextrose		++++
YP- 2% Glycerol & 2% Lactate		++++
YM- 2% Dextrose		++++
YM- 2% Glycerol & 2% Lactate		++++
YNB- 2% Dextrose		++++
YNB- 2% Glycerol & 2% Lactate		++++

Figure 4 : Growth pattern of *Pichia farinosa* in rich and minimal media supplemented with different carbon sources.

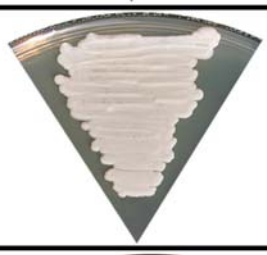
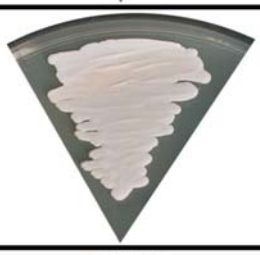
Growth Medium	Growth Pattern	Amount of Growth
YP- 2% Dextrose		++++
YP- 2% Glycerol & 2% Lactate		++++
YM- 2% Dextrose		++++
YM- 2% Glycerol & 2% Lactate		++++
YNB- 2% Dextrose		++++
YNB- 2% Glycerol & 2% Lactate		++++

Figure 5 : Growth pattern of *Pichia anomola* in rich and minimal media supplemented with different carbon sources.

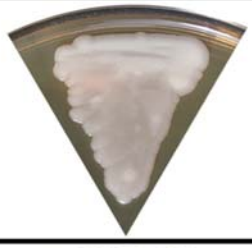
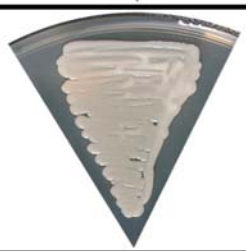
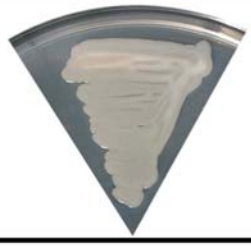
Growth Medium	Growth Pattern	Amount of Growth
YP- 2% Dextrose		++++
YP- 2% Glycerol & 2% Lactate		++++
YM- 2% Dextrose		++++
YM- 2% Glycerol & 2% Lactate		+++
YNB- 2% Dextrose		++++
YNB- 2% Glycerol & 2% Lactate		++++

Figure 6 : Growth pattern of *Pichia jadinii* in rich and minimal media supplemented with different carbon sources.

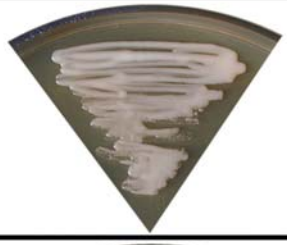
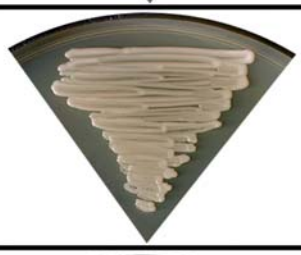
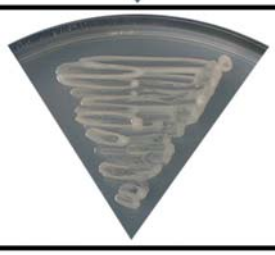
Growth Medium	Growth Pattern	Amount of Growth
YP- 2% Dextrose		+++
YP- 2% Glycerol & 2% Lactate		+++
YM- 2% Dextrose		++++
YM- 2% Glycerol & 2% Lactate		+++
YNB- 2% Dextrose		++
YNB- 2% Glycerol & 2% Lactate		+

Figure 7 : Growth pattern of *Candida milleri* in rich and minimal media supplemented with different carbon sources.

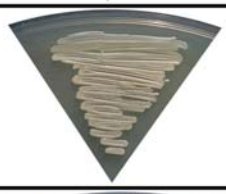
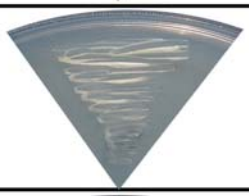
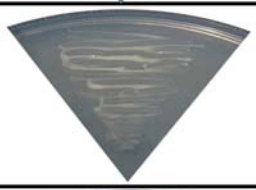
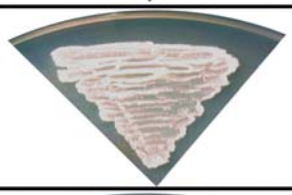
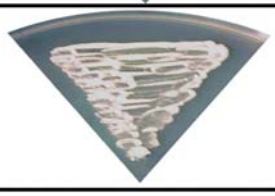
Growth Medium	Growth Pattern	Amount of Growth
YP- 2% Dextrose		++++
YP- 2% Glycerol		++++
YM- 2% Dextrose		+++
YM- 2% Glycerol & 2% Lactate		+++
YNB- 2% Dextrose		–
YNB- 2% Glycerol		–
YS- 2% Dextrose		++++
YS- 2% Glycerol & 2% Lactate		+++

Figure 8 : Growth pattern of *Saccharomyces cerevisiae* in rich and minimal media supplemented with different carbon sources.

3. 2. Doubling Times of Yeast Species at Different Carbon Sources

Although glucose is the preferred carbon source, yeast can grow on a variety of fermentable and non-fermentable carbon sources. Hence, the growth rates of the yeast species in these carbon sources were different and their doubling times were given in Table 2. Doubling times of logarithmically growing yeast species were calculated both in rich media (YP and YM) and minimal media (YNB and YS) supplemented with fermentable and non-fermentable carbon sources.

Doubling times of *Pichia farinosa* (NCY2) in both rich and minimal media were nearly the same. Doubling time of *P. farinosa* grown in YPD medium was nearly 3 hours (175.5 minutes) and in YPGL medium was 3.6 hours (220.5 minutes). When it was grown on minimal medium supplemented with glucose, the doubling time became 4.3 hours (255.5 minutes) and in YNB-GL medium it was 4.8 hours (291.2 minutes). *P. farinosa* grew slightly faster in rich medium than minimal medium in all carbon sources. As shown Figure 9, *P. farinosa* grew better in YPD and YPGL media than YNB-D and YNB-GL media. There was not a large difference between growth of *P. farinosa* in rich media and in minimal media.

Doubling times of *Pichia anomala* (NCY12) were similar to *P. farinosa*. Doubling times of *P. anomala* grown in rich media (104 minutes in YPD and 112.5 minutes in YPGL) was nearly same, 2 hours.

Yeast Strains	Doubling Time[a] (Minute)					
	YP-D[b]	YP-GL[c]	YPM-D[d]	YPM-GL[e]	YNBD[f] or YSD[g]	YNBGL[h] or YSGL[i]
Pichia farinosa	175.5	220.5	-	-	255.5	291.2
Pichia anomala	104	112.5	-	-	132.4	160.7
Pichia jadinii	-	-	102.3	128.6	80.4	150.0
Candida milleri	-	-	161.5	202.4	261.5	675.2
Saccharomyces cerevisiae	112.6	269.4	-	-	460.3	547.3

Table 2: Doubling times of yeast species growing in rich and minimal media supplemented with fermentable and non-fermentable carbon sources.

[(a)] Doubling times were given for logarithmic stage

[(b)] YPD; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glucose (w/v)

[(c)] YPGL; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

[(d)] YMD; YM (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone) supplemented with 2% glucose (w/v)

Table 2 (continued):

[e] YMGL; YM (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

[f] YNBD; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glucose (w/v)

[g] YNBGL; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

[h] YSD; YS (Synthetic Complete Drop out Medium without Uracil) supplemented with 0.5% Uracil (w/v) and 2% glucose (w/v)

[i] YSGL; YS (Synthetic Complete Drop out Medium without Uracil) supplemented with 0.5% uracil (w/v) and 2% glycerol (v/v) and 2% lactate (v/v)

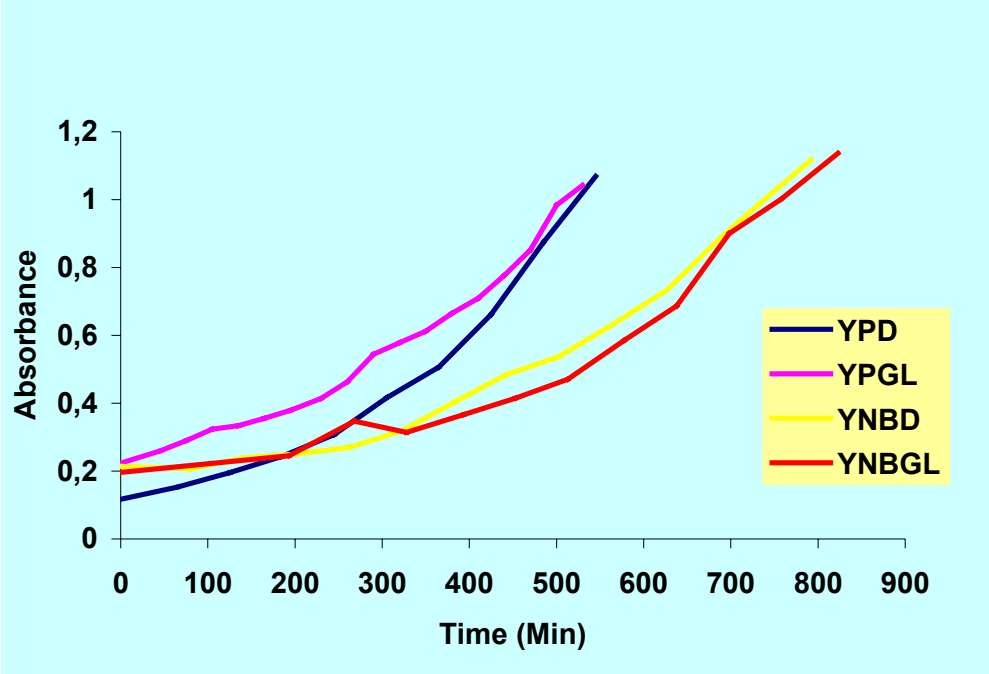


Figure 9: Growth rate of *Pichia farinosa* grown in rich (YP) and minimal (YNB) media with different carbon sources.

Abbreviations:

YPD; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glucose (w/v)

YPGL; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

YNBD; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glucose (w/v)

YNBGL; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

Doubling time in minimal medium supplemented with glucose was 2.2 hours (132.4 minutes) and in YNB-GL medium was 2.6 hours (160.7 minutes). Similar growth pattern was observed for *P. anomala* grown in rich and minimal media (Figure 10). But *P. anomala*, like *P. farinosa*, grew slightly faster in rich medium than minimal medium. *Pichia jadinii* (NCY16) grew very well in both rich (YMD and YMGL) and minimal media (YNB-D and YNB-GL). Doubling times of *P. jadinii* grown in YMD and YMGL were 102 minutes and 128 minutes, respectively. When *P. jadinii* were grown in YNB-D, the doubling time decreased to 80 minutes, and increased to 150 minutes in YNB-GL medium. As shown in Figure 11, the growth pattern of NCY16 in rich and minimal media was similar except YNB-D in which grew faster than other growth conditions.

Doubling time of *Candida milleri* (NCY 17) was 161.5 minutes in YMD, 202.4 minutes in YMGL, 261.5 minutes in YNBD and 675.2 minutes in YNBGL media. *C. milleri* showed the same growth pattern in YMD, YMGL and YNBD media (Figure 12). But the growth in YNBGL medium was very low.

When the laboratory strain of *Saccharomyces cerevisiae*, YST124, was grown in rich medium, doubling times became 112,6 minutes and 269.4 minutes for YPD and YPGL plates, respectively. Therefore *S. cerevisiae* grew better in fermentable carbon source than non-fermentable one. Doubling time of *S. cerevisiae* growing in minimal medium, YSD, increased to 460.3 minutes and in non-fermentable carbon source became 547.3 minutes.

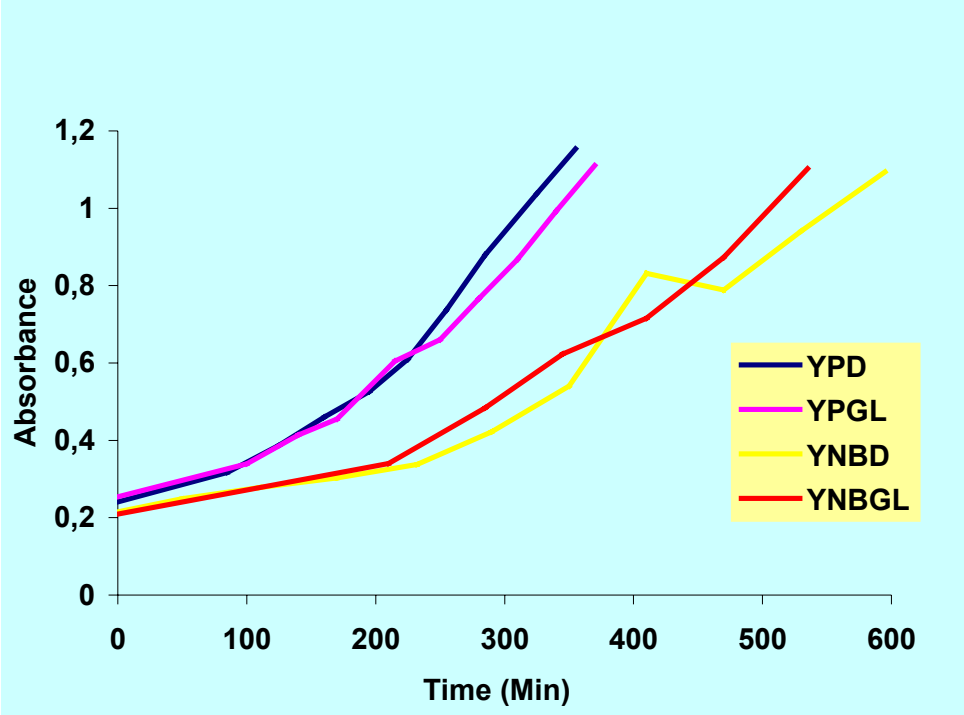


Figure 10: Growth rate of *Pichia anomala* grown in rich (YP) and minimal (YNB) media with different carbon sources.

Abbreviations:

YPD; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glucose (w/v)

YPGL; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

YNBD; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glucose (w/v)

YNBGL; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

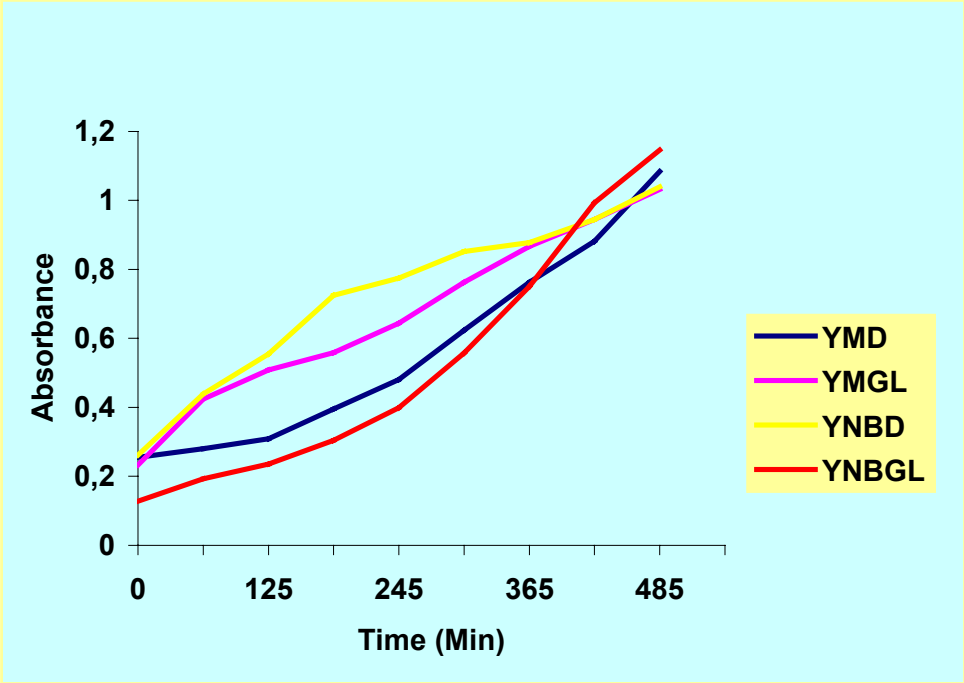


Figure 11: Growth rate of *Pichia jadinii* grown in rich (YM) and minimal (YNB) media with different carbon sources.

Abbreviations:

YMD; YM (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone) supplemented with 2% glucose (w/v)

YMGL; YM (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

YNBD; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glucose (w/v)

YNBGL; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

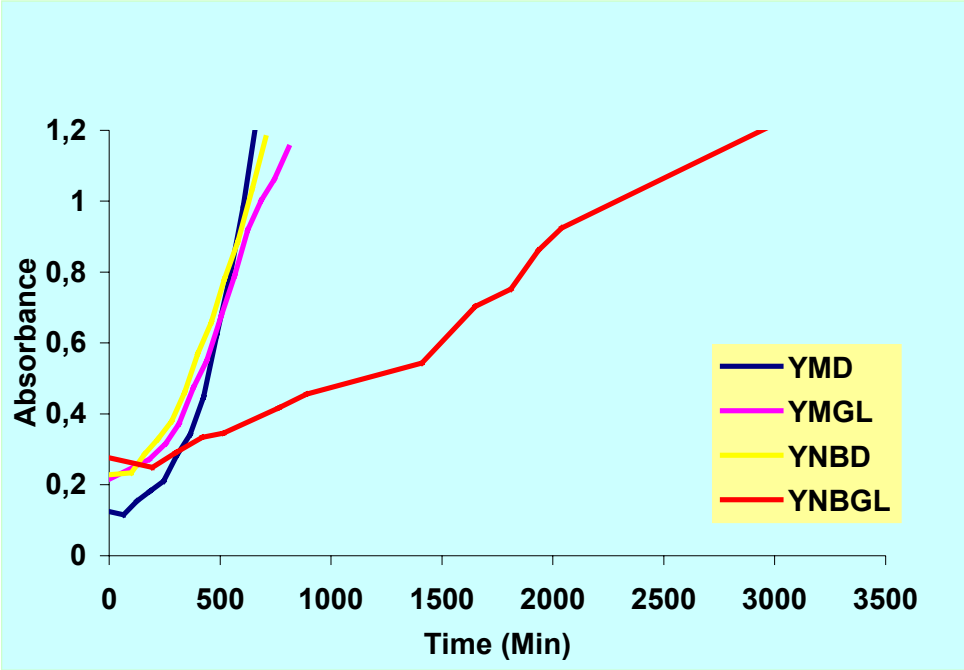


Figure 12: Growth rate of *Candida milleri* grown in rich (YM) and minimal (YNB) media with different carbon sources.

Abbreviations:

YMD; YM (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone) supplemented with 2% glucose (w/v)

YMGL; YM (5g/l Yeast Extract, 3 g/l Malt Extract, 5 g/l Bacto-Peptone) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

YNBD; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glucose (w/v)

YNBGL; YNB (1.6 g/l Yeast Nitrogen Base, 5.0 g/l Ammonium sulfate ($(NH_4)_2SO_4$)) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

Growth pattern of *S. cerevisiae* was given in Figure 13. As shown in figure yeast cells grew better in rich media (YPD and YPGL) then minimal media (YSD and YSGL). Also yeast species grew better in fermentable carbon source (Glucose) than non-fermentable carbon source (Glycerol and Lactate).

All yeast species grew better in rich media than minimal media. *Candida milleri* also grew well in minimal medium supplemented with glucose. *Saccharomyces cerevisiae* grew faster in rich media than minimal media.

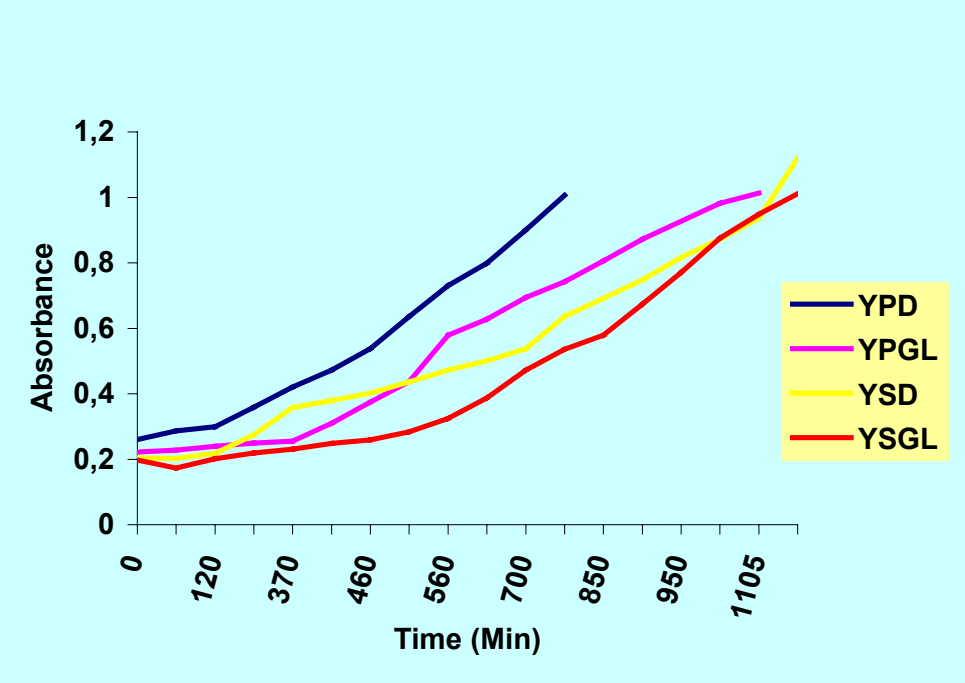


Figure 13: Growth rate of *Saccharomyces cerevisiae* grown in rich (YP) and minimal (YS) media with different carbon sources.

Abbreviations:

YPD; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glucose (w/v)

YPGL; YP (10g/lt Yeast Extract, 20g/lt Bacto-Peptone) supplemented with 2% glycerol (v/v) and 2% lactate (v/v)

YSD; YS (Synthetic Complete Drop out Medium without Uracil) supplemented with 0.5% Uracil (w/v) and 2% glucose (w/v)

YSGL; YS (Synthetic Complete Drop out Medium without Uracil) supplemented with 0.5% uracil (w/v) and 2% glycerol (v/v) and 2% lactate (v/v).

3. 3. Invertase Activities of Yeast Strains Under Repressed and Derepressed Conditions

The addition of glucose to yeast cells growing on a non-fermentable carbon source such as glycerol and lactate causes a global switch in metabolism from gluconeogenesis to glycolysis. Cells performing gluconeogenesis are catabolite derepressed, while fermenting cells are catabolite repressed. The transition from the derepressed to repressed state is achieved by altering the activity and stability of enzymes and switching the expression of large number of genes on or off. For instance, invertase enzyme activity involved in the utilization of sucrose drops dramatically after glucose addition. Subsequently the transcription of *SUC2* gene is shut down by carbon-catabolite repression in *Saccharomyces cerevisiae*. Short-term and long-term repressions of catabolite-repressible genes, such as *SUC2*, are mediated by the sugar kinases or products produced by them, but using different signaling pathways (Hohmann et al. 1999).

Invertase activity of individual yeast cells grown under long-term derepressed and short-term derepressed conditions were determined and given in Table 3.

For short-term derepression yeast species were grown in YP medium (YM medium for NCY16 and NCY17) supplemented with 2% glucose (w/v) until the early-logarithmic stage (OD_{600}: 0.8-0.9). Then yeast cultures were divided into two portions for derepressed growth (YP or YM with

Yeast Strains	Enzyme Activity[a]			
	Short-Term Derepression		Long-Term Derepression	
	R[b]	DR[c]	R	DR
Pichia farinosa	0.0	0.0	0.0	0.0
Pichia anomala	2.159	523.116	12.784	66.111
Pichia jadinii	2590.5	3172.3	6703.0	8094.0
Candida milleri	1050.0	2065.1	3113.9	2951.7
Saccharomyces cerevisiae	0.0	854.4	412.2	2405.9

Table 3: Invertase activities of yeast species under long-term and short-term derepressed conditions.

(a) Invertase activities were given in μmoles of glucose deliberated/min/100mg of dry weight.

(b) R: repressed (2% Glucose (w/v))

(c) DR: derepressed (0.05% Glucose (w/v))

0.05% glucose (w/v)) and for repressed growth (YP or YM with 2% glucose (w/v)). After 3 hours incubation their enzyme activities were determined.

Pichia farinosa (NCY2) did not show any enzyme activity both in repressed and derepressed growth conditions.

Invertase activity of *Pichia anomala* (NCY12) was 2.159 U under repressed and 523.116 U under derepressed growth conditions. Enzyme activity at derepressed growth condition was 260 times greater than repressed activity. Therefore, invertase encoding gene of *P. anomala*, *INV1*, is under the control of glucose repression.

Invertase activity of *Pichia jadinii* (NCY16) was nearly the same both in repressed and derepressed conditions. Among the yeast species used in this research only *P. jadinii* showed the highest enzyme activity in both repressed (2590.5 U) and derepressed (3172.3 U) growth conditions.

Enzyme activity of *Candida milleri* (NCY17) under derepressed condition (2065.1 U) was two times greater than repressed condition (1050 Units).

At repressed condition, *Saccharomyces cerevisiae* (YST124) showed very low enzyme activity, but the activity increased to 854.4 U at derepressed condition. Enzyme activity of YST124 under derepressed condition was 854 fold greater than the repressed condition.

For long-term derepression yeast species were grown in YPGL medium (YMGL medium for NCY17) until the early-logarithmic stage (OD_{600}: 0.8-0.9). Then yeast cultures were divided into two portions for

derepression (0.05% dextrose (w/v)) and for repression (2% dextrose (w/v)) added to YP or YM medium. After 3 hours incubation their enzyme activities were determined.

Pichia farinosa (NCY2) did not show any enzyme activity both in repressed and derepressed growth conditions like in short-term derepression growth conditions.

Pichia anomala (NCY12) showed lower invertase activity than other yeast species. Enzyme activity at derepressed conditions (12.784 Unit) was five times greater than repressed growth condition (66.11 U).

There was no activity difference between repressed and derepressed growth conditions of *P. jadinii* (NCY16) and *C. milleri* (NCY17) under long-term derepression condition. For *P. jadinii*, maximum invertase activity (8094 U) was recorded in all yeast strains and in all conditions. Invertase enzyme activity of *C. milleri* at derepressed condition (2951.7 U) was slightly lower than repressed condition (3113.9 U). Enzyme activity of *P. jadinii* under long-term derepression was slightly greater (2-3 fold) than the enzyme activity in short-term derepression. There was not much more difference in the enzyme activities of *C. milleri* in long-term derepression and short-term derepression conditions.

Enzyme activities of *S. cerevisiae* showed similar pattern, which was increase at derepressed growth in long-term derepressed condition. For *S. cerevisiae,* enzyme activity at derepressed growth (2405.9 U) was six times greater than repressed growth (412.2 U).

P. anomala and *S. cerevisiae* showed higher enzyme activity at derepressed than repressed conditions both in long-term and short-term derepression conditions.

The enzyme activity ratio is the ratio of invertase activity at derepressed condition to repressed condition. This activity ratio of *P. anomala* was 250 for short-term derepression and, it was 5 for long-term derepression. When *P. anomala* grown in long time glucose, enzyme activity was 50 fold greater than the enzyme activity of YPGL grown cells. The enzyme activity of *S. cerevisiae* was 850 and 6 for short-term and long-term derepression, respectively. In the same way, the enzyme activity increased in long-term derepression, which was nearly 141 fold greater than in short-term derepression. After a long time derepressed growth of *S. cerevisiae,* enzyme activity at long-term derepressed-repressed (LTD-R) condition was 412.2 U that is nearly the half of derepression value of short-term derepression.

P. jadinii and *C. milleri* showed nearly the same enzyme activity, which were higher than the enzyme activities of *P. anomala* and *S. cerevisiae* in both repressed and derepressed growth conditions.

Glucose did not affect the enzyme activity of *P. jadinii* and *C. milleri*. Therefore, invertase enzyme synthesis of *P. jadinii* and *C. milleri* may not be controlled by glucose repression. Sometimes the concentration of glucose above 2% must be necessary for the formation of glucose repression signal in the cell. Therefore, for these species 3% to 5% glucose concentration must be used to decide the presence of glucose repression.

The enzyme activity of *P. anomala* and *S. cerevisiae* yeast species became very low under repressed condition than derepressed condition. *SUC2* gene of *S. cerevisiae* and *INV1* gene of *P. anomala* that were coded for invertase enzyme, were controlled by glucose. In the presence of glucose, genes necessary for the utilization of other carbon sources were repressed. But the derepression of *P. anomala INV1* gene was slower than that of *S. cerevisiae*.

3. 4. Analyzing the Time Course Invertase Activities of Yeast Species Under Long-Term and Short-Term Derepressed Conditions

Invertase activity of individual yeast cells grown under repressed and derepressed conditions was determined at different stages of growth. The invertase activities of yeast species were given in μmoles of glucose deliberated/min/100mg of dry weight.

Yeast cells were grown in YP and YM media supplemented with 2% glucose (w/v) for short-term derepression and with 2% glycerol (v/v) and 2% lactate (v/v) for long-term derepression until the logarithmic stage (OD_{600}: 0.8-0.9). Growing yeast cultures were divided into two portions for derepression (0.05% glucose (w/v)) and repression (2% glucose (w/v)). Yeast cells were further incubated and the invertase enzyme activities were determined at 30 minutes time intervals until the stationary stage.

When *Pichia farinosa* (NCY2) were grown in both repressed and derepressed conditions, their invertase activities became zero as shown in Figure 14. The growth of yeast species well in both short-term and long-term dereprresed growth conditions (Figure 15).

Enzyme activities of *Pichia anomala* (NCY12) grown as long-term derepressed (LTD) nearly the same at logarithmic stage under both repressed and derepressed conditions (Figure 16). But the enzyme activity at late logarithmic stage decreased in derepressed cells and remained as it for repressed cells. When the yeast cells were grown as long-term repressed (STD) then transferred to repressed and derepressed growing conditions,

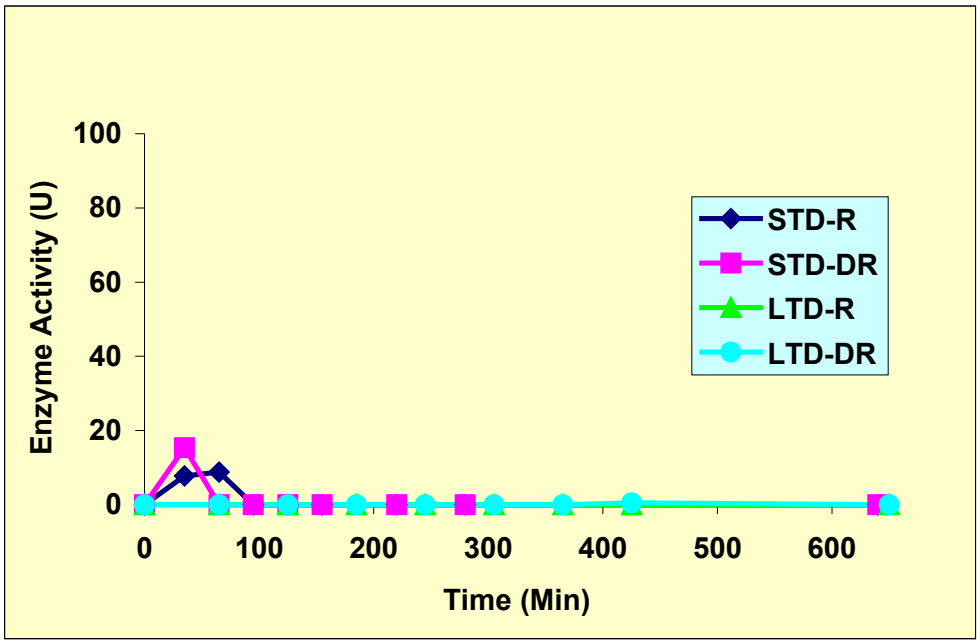


Figure 14: Time-course invertase activity of *Pichia farinosa* under short-term and long-term derepression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v)

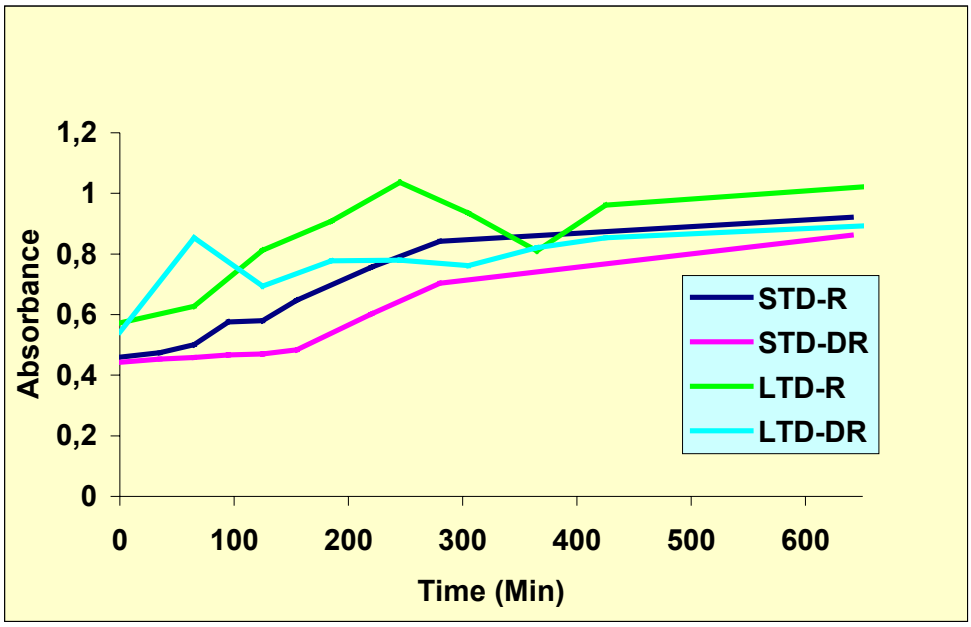


Figure 15: Growth pattern of *Pichia farinosa* at short-term and long-term derepression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v)

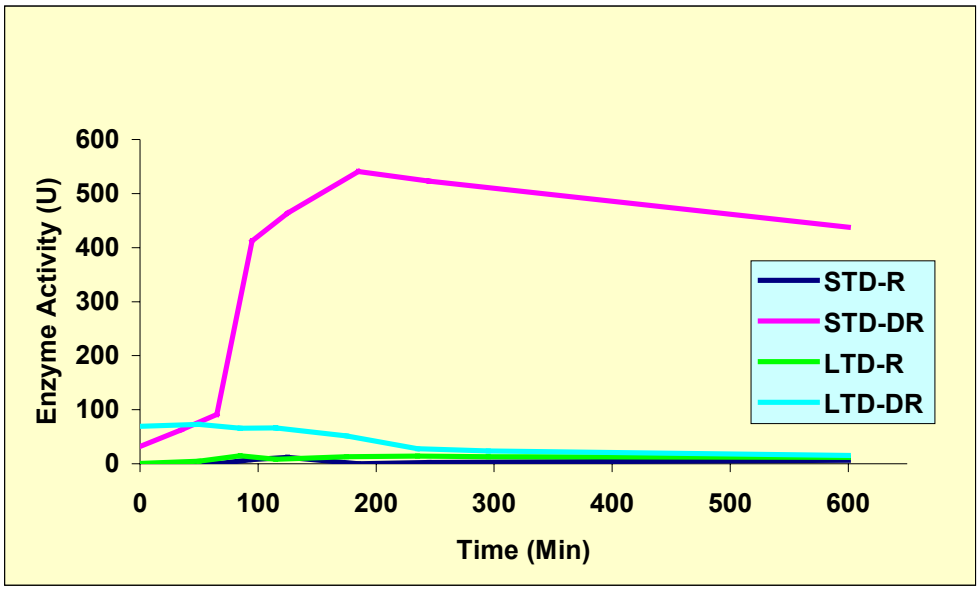


Figure 16: Time-course invertase activity of *Pichia anomala* under short-term and long-term derepression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v)

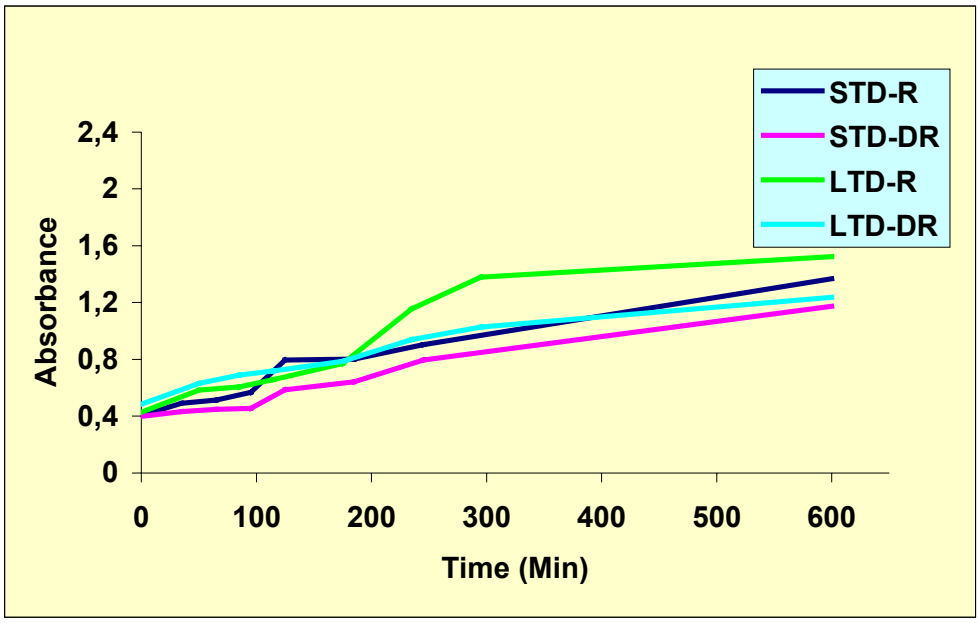


Figure 17: Growth rate of *Pichia anomala* at short-term and long-term derepression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v)

enzyme activities were different. Enzyme activity of short-term derepressed-repressed (STD-R) yeast cells was very low level (nearly zero) during logarithmic and stationary stages. However the activity of short-term derepressed-derepressed (STD-DR) yeast cells was very high at early logarithmic stage and increased till the end of logarithmic phase. Then the activity of enzyme started to decrease at stationary phase. The highest enzyme activity for *P. anomala* was recorded at stationary phase. Growth of yeast cells parallel to sampling time of enzyme activity determination was given in Figure 17.

Time-course invertase activity of *Pichia jadinii* (NCY16) grown both long-term and short-term derepressed was given in Figure 18 and their growth curve at the same time intervals was given in Figure 19. At LTD-Repressed and LTD-Derepressed growth conditions, enzyme activity of *P. jadinii* showed similar pattern. The enzyme activity became high during the logarithmic stage and started to decrease at the beginning of stationary phase then stayed at the same level during stationary phase. The activity of yeast species grown in STD-Repressed and STD-Derepressed conditions became nearly the same during logarithmic and stationary stages.

The enzyme activity of long-term derepressed grown *Candida milleri* (NCY17) yeast species increased during logarithmic stage in repressed and derepressed cells (Figure 20). At the beginning of stationary phase invertase enzyme activity of both repressed and derepressed cells became constant that was around 3000 U for LTD-R and 6000 U for LTD-DR yeast cells. For LTD-DR growing cells, enzyme activity increased up

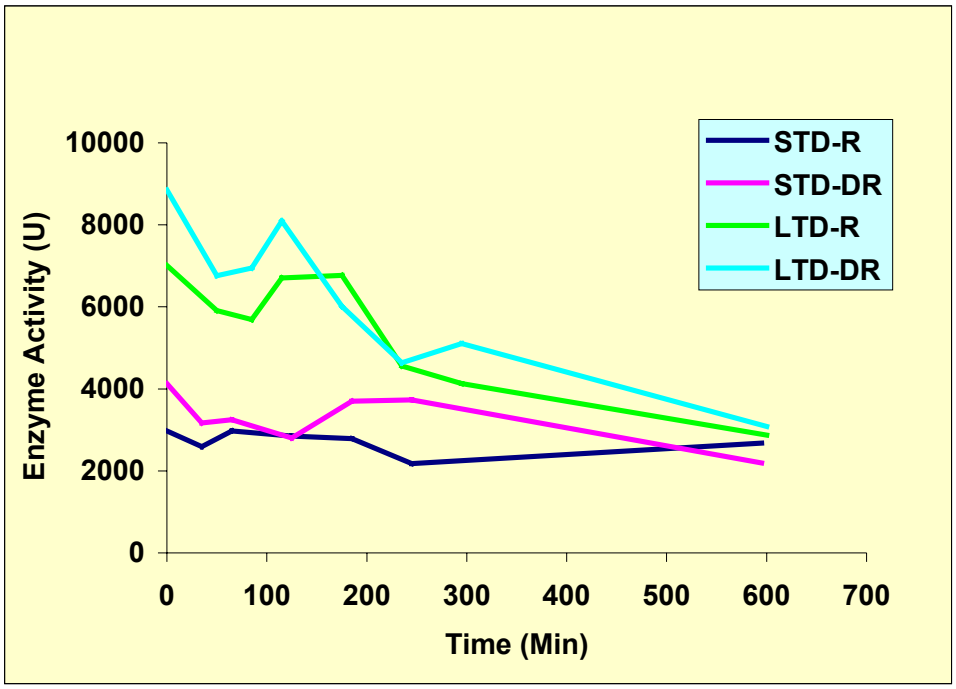


Figure 18: Time-course invertase activity of *Pichia jadinii* under short-term and long-term derepression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v)

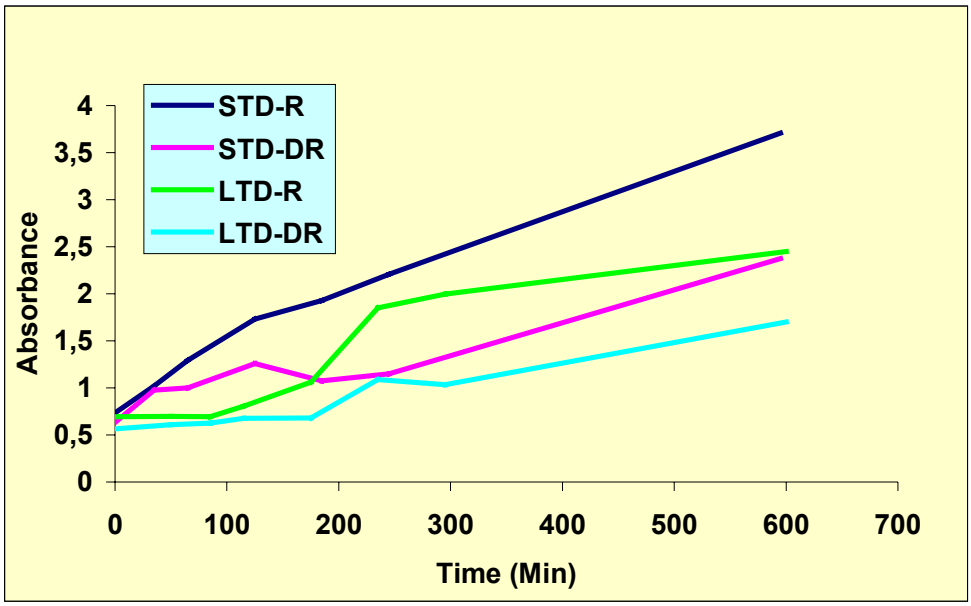


Figure 19: Growth pattern of *Pichia jadinii* at short-term and long-term derepression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v).

to 7000 U at the late logarithmic stage and then decreased to 6000 U at the stationary phase. Growth curve of *C. milleri* parallel to enzyme activity determination times was given in Figure 21.

Invertase activity of *Saccharomyces cerevisiae* (YST124) under short-term derepression (STD) condition was different. When the yeast cells were grown in repressed (STD-R) condition the enzyme activity was very low, nearly zero, during logarithmic and stationary phases. However the enzyme activity at derepressed condition (STD-DR) increased during the logarithmic stage and became constant at the beginning of stationary stage (Figure 22). When the yeast cells were grown in derepressed condition (YPGL) in a long time and shifted to repressed growth (LTD-R), the enzyme activity became very high. Enzyme activity at long-term derepressed--derepressed growth (LTD-DR) increased during logarithmic stage and started to decrease at the end of the logarithmic phase. During the stationary stage of growth invertase activity became constant, approximately 2000 U.

The growth of yeast cells under repressed condition were greater than derepressed growth conditions (Figure 23), even if the invertase activity was an opposite manner.

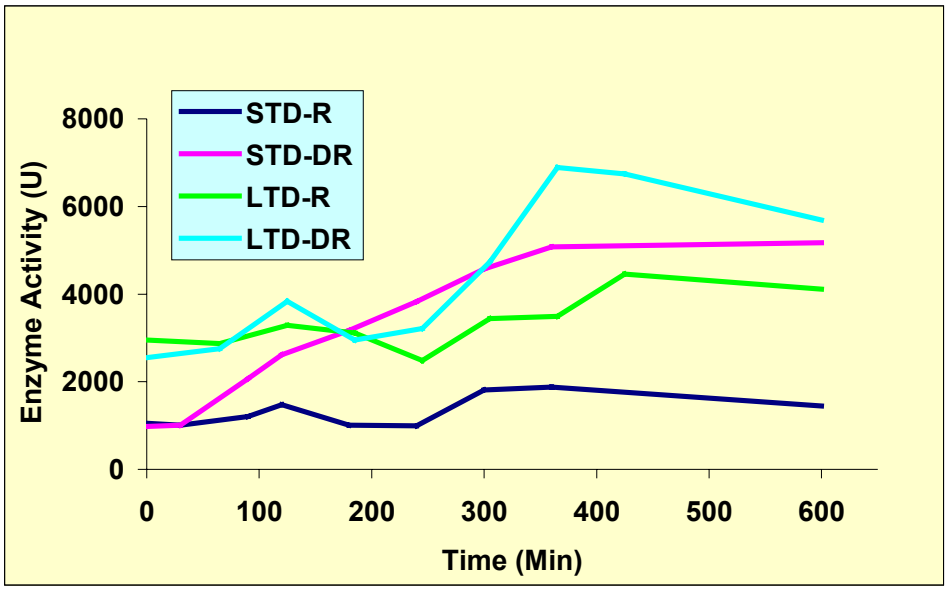


Figure 20: Time-course invertase activity of *Candida milleri* under short-term and long-term dereppression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v)

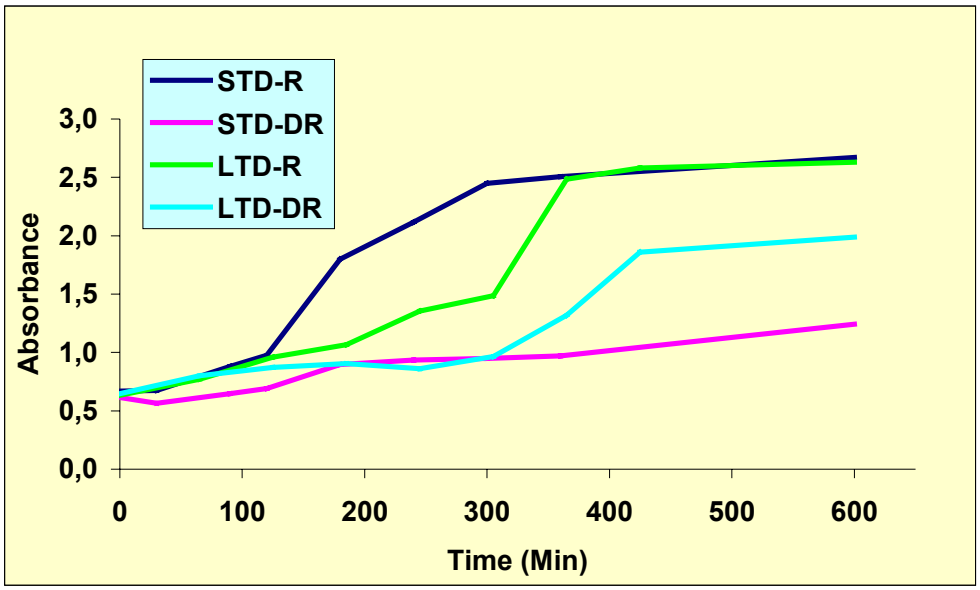


Figure 21: Growth pattern of *Candida milleri* at short-term and long-term dereppression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v).

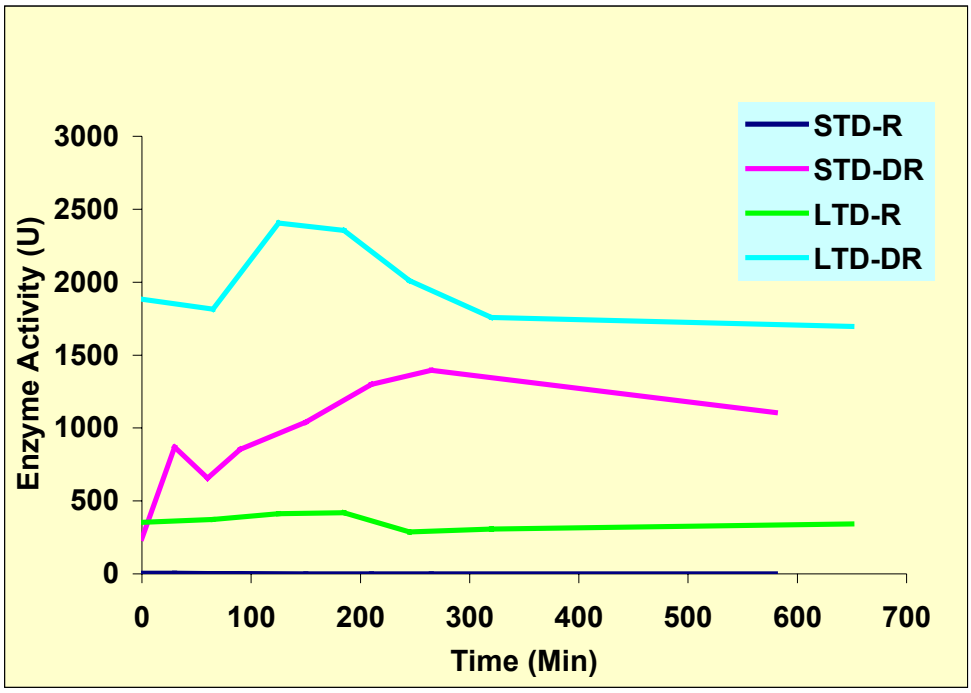


Figure 22: Time-course invertase activity of *Saccharomyces cerevisiae* under short-term and long-term dereppression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v)

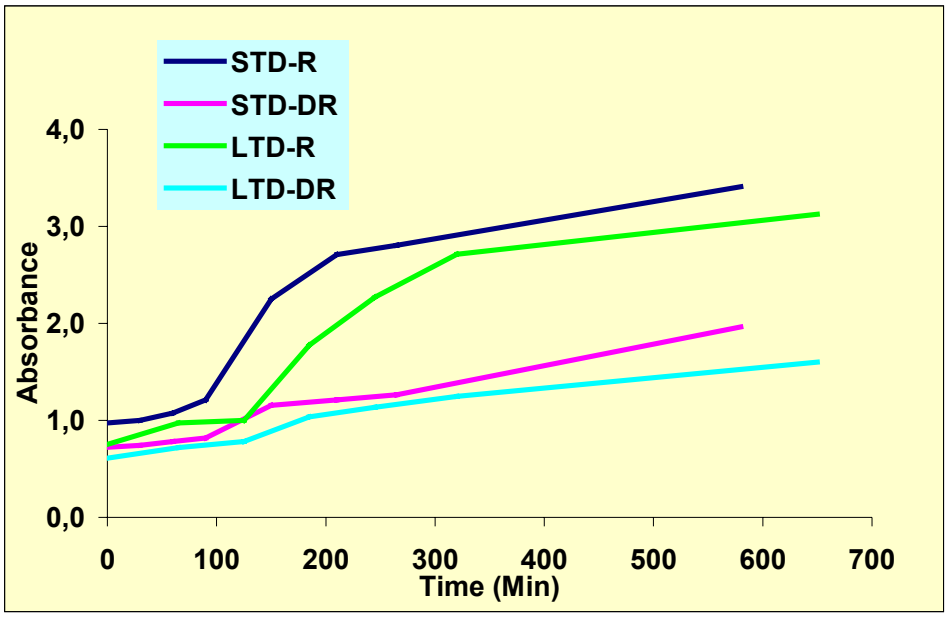


Figure 23: Growth pattern of *Saccharomyces cerevisiae* at short-term and long-term derepression conditions.

Abbreviations:

STD-R (Short-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glucose (w/v)

STD-DR (Short-Term Derepression-Derepression) Yeast cells were grown in YP media with 2% glucose (w/v) then shifted to YP media supplemented with 0.05% Dextrose (w/v)

LTD-R (Long-Term Derepression-Repression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 2% Dextrose (w/v)

LTD-DR (Long-Term Derepression-Derepression) Yeast cells were grown in YP media supplemented with 2% glycerol (v/v) and 2% Lactate (v/v) then shifted to YP media with 0.05% Dextrose (w/v).

3.5. Analyzing the Invertase Activities of Yeast Strains Under Different Temperatures

Yeast cells have evolved to respond rapidly and effectively to fluctuations in temperature, pH, nutrients and other environmental changes. When environmental conditions change abruptly, cells must rapidly adjust their genomic expression program to adapt to new conditions.

Temperature is an important factor for the yeast growth and enzyme activity. Optimum growth temperature for *S. cerevisiae* yeast species is 30°C and optimum temperature for invertase enzyme activity is 37°C. Optimum growth temperature for other yeast species is 25°C, but the optimum temperature for the invertase activity is not known except *Pichia anomala* (NCY12) that is 30°C.

In this part of research, we tried to determine optimum temperature of enzyme activity and effect of temperature on the activity of invertase enzyme. The results were given in Table 4.

Pichia farinosa (NCY2) did not show any invertase activity at derepressed and repressed conditions in all temperatures.

Invertase activity of *Pichia anomala* (NCY12) showed similar enzyme activity pattern at 25°C and 30°C. Under repressed condition, enzyme activity was nearly zero (0.336 U) and 2.159 U at 25°C and 30°C, respectively. Under derepressed condition, the enzyme activity was 98,38 U at 25°C and 523.116 U at 30°C. Enzyme activity did not affected at these

Enzyme Activity [a]								
Yeast Strain	**25 °C**		**30 °C**		**37 °C**		**42 °C**	
	R[b]	**DR[c]**	**R**	**DR**	**R**	**DR**	**R**	**DR**
Pichia farinosa	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pichia anomala	0.336	98.38	2.159	523.116	64.68	48.31	9.349	11.914
Pichia jadinii	2214.2	3126	2590.5	3172.3	2281.4	3233.2	2830.8	2369.4
Candida milleri	1570,1	2462.1	1050.0	2065.1	1722.9	3077.5	2463.3	2546.7
Saccharomyces cerevisiae	22.9	620.9	9.8	739.2	2.2	854.4	19.8	265.3

Table 4: Invertase activities of yeast species under repressed and derepressed growth conditions at different temperatures.

(a) Invertase activities were given in µmoles of glucose deliberated/min/100mg of dry weight.

(b) R: repressed (2% Glucose (w/v))

(c) DR: derepressed (0.05% Glucose (w/v))

temperatures. But at 37°C and 42°C enzyme activity nearly the same in both repressed and derepressed conditions. Enzyme activity at 37°C was 64.68 U for repression and 48.31 U for derepression, and 9.349 U for repression and 11.94 U for derepression at 42 °C. The optimum temperature of enzyme activity for *P. anomala* was between 25°C and 30°C.

Enzyme activity of *Pichia jadinii* (NCY16) and *Candida milleri* (NCY17) under repressed and derepressed conditions approximately the same that was around 2000 U to 3000 U in all temperatures. Therefore invertase activity was not affected by heat in these yeast cells.

In *S. cerevisiae* (YST124), generally the enzyme activity under derepressed conditions was higher than repressed conditions. Temperatures between 25°C and 37°C did not affect enzyme activity. Invertase activity at derepressed condition was around 600 to 800 units and it was 2 to 22 units for repressed condition. At 42°C, invertase activity under derepressed condition was 3 times lower than the derepressed enzyme activity of other temperatures.

Heat stress above 37°C negatively affected the enzyme activity of *S. cerevisiae* yeast species. Temperatures between 25°C and 37°C for *S. cerevisiea* yeast species did not effect glucose repression.

Heat stress affected the enzyme activity of *P. anomala* and *S. cerevisiea*, but *P. jadinii* and *C. milleri* did not affected by the heat.

3. 6. Analyzing the Invertase Activities of Yeast Strains Under Different pHs

Another factor, which affects the enzyme activity, is pH. pH of environment is important for growth and enzyme activity. The optimum pHs of *S. cerevisiae* yeast species for the growth is 6.0-6.5 and for the invertase activity is 5.0-5.2. The optimum pH of enzymatic activity for other yeast species is not known except *Pichia anomala*, which are 5.0.

In order to determine optimum pH for invertase enzyme activity, yeast strains were grown in rich media supplemented with 2% glucose (w/v) until the logarithmic stage (OD_{600}: 0.8-0.9). Then yeast cultures were divided into two portions for repressed growth (2% glucose (w/v)) and for derepressed growth (0.05% glucose (w/v)). Then both repressed and derepressed cultures were separated into five aliquots, and their pHs adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0. Yeast cells were incubated for 3 hours and their invertase enzyme activities were determined and given in Table 5.

Pichia farinosa (NCY2) did not show enzyme activity in all pHs.

Enzyme activity of *Pichia anomala* (NCY12) at pH 4.0 and 5.0 became lower than its activity at pH 6.0, 7.0 and 8.0 under derepressed conditions. There was no activity difference between pH 4.0 and 5.0 at derepressed and repressed conditions. At pH 8.0, enzyme activity was 30 times greater than repressed enzyme activity. The optimum pH for invertase enzyme activity was 6.0, that is the pH of growing medium.

Yeast Strain	Enzyme Activity[a]									
	pH 4.0		pH 5.0		pH 6.0		pH 7.0		pH 8.0	
	R[b]	DR[c]	R	DR	R	DR	R	DR	R	DR
Pichia farinosa	2.9	2.9	1.9	0.5	0.0	0.0	3.6	0.8	3.9	2.6
Pichia anomala	71.8	38.7	15.8	56.1	2.1	523.1	1.8	81.9	10.1	391.8
Pichia jadinii	5506.9	2720.3	4814.6	3896.9	2590.0	3172.3	2769.4	7643.2	1666.0	6720.5
Candida milleri	2047.5	3334.1	1181.8	2317.0	1050.0	2065.1	1728.2	2316.3	1646.7	2431.8
Saccharomyces cerevisiae	9.6	397.8	13.3	593.9	2.2	854.4	12.2	417.4	18.4	184.9

Table 5: Invertase activities of yeast species under repressed and derepressed growth conditions at different pHs.

(a) Invertase activities were given in μmoles of glucose deliberated/min/100mg of dry weight.

(b) R: repressed (2% Glucose (w/v))

(c) DR: derepressed (0.05% Glucose (w/v))

The enzyme activity of *Pichia jadinii* (NCY16) at pH 4.0 and 5.0 under repressed condition became higher than derepressed condition. At pH 6.0, there was no difference between derepressed and repressed enzyme activities. At pH 7.0 and 8.0, derepressed enzyme activity became greater than repressed activity. At higher pHs (pH: 8:0) glucose repression affected on the invertase synthesis, but at lower pHs (pH: 4.0) it was not effective and caused to increase enzyme activity.

The enzyme activity of *Candida milleri* (NCY17) did not change at different pHs. The highest enzyme activity observed at pH 4.0 both in repressed and derepressed conditions. Therefore the optimum pH for enzyme activity for *C. milleri* may be around pH 4.0.

Saccharomyces cerevisiae showed better activity at pH 6.0 and 7.0. At pHs between 4.0 and 7.0 were suitable for enzyme activity, which was repressed by glucose. When the pH of growth medium became 6.0, the derepression of enzyme reached to maximum level (854.4 U). When the pH of the growth medium decreased to 4.0 and 5.0, or increased to 7.0 and 8.0, the derepression of *SUC2* gene became slow. Therefore, pH affected the derepression of enzyme activity negatively.

4. CONCLUSION

The yeast *Saccharomyces cerevisiae* is an industrial microorganism. Different strains of *S. cerevisiae* are commonly used in fermentation and baking industry. It is also favorable eukaryotic organism for the genetic studies and the analysis of metabolic pathways. Its genome has been completely sequenced. The yeast *S. cerevisiae* can utilize various carbohydrates as a carbon and energy source.

A growth patterns and duplication time of yeast cells vary depending on the growth media, and genetic background of related yeast species. Growth rate of yeast cells is always higher in rich medium and fermentable sugars. But the growth rates of the yeast cells decrease in minimal medium and non-fermentable carbon sources.

Addition of rapidly fermentable sugars to cells of the yeast *S. cerevisiae* grown on non-fermentable carbon sources causes a variety of short-term and long-term regulatory effects leading to an adaptation to fermentative metabolism. Ras/cAMP pathway is involved in nutrient sensing and growth regulation (Thevelein, 1992). Activation of this pathway by glucose is dependent on sugar phosphorylation by three hexokinases (*HXK1,*

HXK2 and *GLK1*). Glucose-dependent transcriptional repression exerted through the main glucose repression pathway is primarily dependent on the hexokinase PII (*HXK2*) gene (Entian, 1980). Long-term repression specifically requires Hxk2p (Winde *et al*., 1996).

SUC2 gene of *S cerevisiae* encodes invertase enzyme that hydrolyses the sucrose and raffinose on growth media (Goldstein and Lampen, 1975) Extracellular invertase is highly glycosylated and can form multimeric units. Transcription of *SUC2* gene is regulated by glucose repression and derepression mechanism. In the presence of glucose, transcription of *SUC2* gene is repressed by repressor protein complex, Mig1p-Tup1p-Ssn6p (Lutfiyya and Johnston, 1996). However, the repressor complex by itself is not enough for the long-term repression of *SUC2* gene transcription. Modifications of nucleosomes on the promoter region of *SUC2* gene are essential for the maintenance of repressed chromatin structure during the presence of high levels of glucose (Wu and Winston, 1997; Bu and Schmidt, 1998; Edmondson *et al*., 1996). Gcr1p and Hxk2p that directly interact with the UAS region of *SUC2* are also required for the glucose repression of *SUC2* gene (Türkel *et al*., 2003; Herreno *et al.,* 1998; Hohmann *et al.,* 1999).

In the presence of non-fermentable carbon source in the growth medium, Mig1p complex and Hxk2p dissociate from the *SUC2* promoter. In addition nucleosomes are modified by SAGA (Spt-Ada-Gcn5-acetyltransferase) complex that is recruited to the *SUC2* promoter region with

the Snf/Swi complex. Modified nucleosomes are displaced on the *SUC2* promoter and *SUC2* gene transcription becomes derepressed (Kingston *et al*., 1999; Sundarsanam *et al*., 1999).

After prolonged derepressed incubation and then shifting to repressed growth media, the invertase activity still present but it was lower than the derepressed enzyme activity at short-term derepression condition in *S. cerevisiae*. This activity of invertase may be result from different reasons. Glucose may affect enzyme levels by causing a decrease in their translation rate, or on increase in the degradation rate of the protein. In turn mRNA levels would depend both on the rate of transcription of the corresponding gene and on the stability of the mRNA. Therefore, even if the transcription is repressed by glucose, accumulated mRNA in the cell pool may be translated or mRNA stability may be longer. The half-life of invertase enzyme is not known, so the accumulated enzyme in the periplasmic space may result in the invertase activity under repressed condition. In addition, the slow modifications of nucleosomes on the promoter region of *SUC2* gene may be another reason of this enzyme activity at repressed condition.

Glucose repression is recorded in some *Pichia* and *Candida* yeast species such as *Pichia angusta (Hansenula polymorpha), Candida utilis (Pichia jadini), Candida bordinii* and *Pichia methanolica* that are methylotrophic yeasts. Among proteins of the hexose transporter family of *S.cerevisiae* the amino acid sequence of the *Pichia angusta* Gcr1 protein

encoded by *HpGCR1* gene (*Hansenula polymorpha* glucose catabolite repression) shares the highest similarity with core region of Snf3p, which is a putative high affinity glucose sensor. In *P. angusta* hexose transporter homologue, Gcr1p, controls the glucose repression. Gcr1 protein is functioning in an early stage of repression mechanism in either glucose transport or glucose signaling (Stasyk *et al.,* 2004).

As in *S. cerevisiae*, the synthesis of invertase by *Pichia anomala* is influenced by the carbon source in the culture medium. *P. anomala* invertase enzyme is similar to *S. cerevisiae* invertase enzyme. It is a multimeric enzyme comprised of 3 or 4 subunits of identical size and extracellular form of the enzyme is highly glycosylated. The invertase enzyme is coded by *INV1* gene, which is regulated by glucose repression and derepression mechanism like *S. cerevisiae*. High concentration of glucose in the culture medium completely repressed production of the invertase enzyme. The K_m value of invertase enzyme for sucrose and raffinose is about 40% lower than those *S. cerevisiae* invertase activities on the same substrates under similar assay conditions. Therefore *P. anomala* invertase enzyme has higher affinity to substrate. Because of that the derepressed enzyme activity of *P. anomala* yeast species became lower than *S. cerevisiae* derepressed enzyme activity after prolonged derepressed growth (Rodriguez *et al.,* 1995).

The high synthesis of invertase enzyme in *P.jadinii* and *C. milleri* yeast species after both short-term and long-term derepressed growth

indicated that the sucrose utilization gene or genes might be controlled by a different system or pathway rather than glucose repression. Glucose repression is not so effective on the *KlINV1* gene of *K. lactis* and *INV1* genes of *C. utilis* (Belcarz *et al.,* 2002; Georis *et al.,* 1999). Our results showed that *Candida milleri* invertase enzyme was not affected by glucose repression when the glucose concentration was 2% glucose.

Therefore, it is clear that there are significant differences in the expression patterns and activities of invertase enzymes among the *Pichia* and *Candida* yeast species.

Yeast cells have evolved to respond rapidly and effectively to fluctuations in temperature, pH, nutrients and other environmental changes.

When environmental conditions change, cells must rapidly adjust their genomic expression program to adapt to new conditions. Exposure of yeast cells to physiological stresses such as high temperature, high or low pH and high salinity, activates the stress response pathways in the yeast *S. cerevisiae*. Depending on the nature of stresses, different signal transduction pathways are activated. Heat stress activates the expression of heat shock protein (Hsp). Heat shock proteins are required for the correct folding of misfolded proteins under heat stress. High temperature also activates trehalose synthesis. The accumulation of trehalose in the cell interferes the normal cellular functions. As soon as stressful conditions terminated, accumulated trehalose is degraded to glucose by trehalase enzyme.

Ras/cAMP/PKA pathway regulates the genes encoding heat shock proteins, such as *HSP12* and *HSP104,* which are rapidly repressed on activation of this pathway. Furthermore, PKA activity also causes the repression of *TPS1* and *TPS2* genes encoding trehalose synthase. Trehalose protects the membrane from desiccation and prevents protein denaturation in cellular stress conditions. The transcriptional derepression of HSP and TPS genes is negatively regulated by Ras/cAMP/PKA pathway through one or more AG_4 stress-responsive elements (STREs). Control of STRE-driven *HSP12* and *TPS2* gene expressions by cAPK (cAMP activated protein kinase) is mediated via Msn2 and Msn4 proteins, a pair of partially redundant zinc-finger transcription factors that recognize and bind to STRE sequence (Marchler, 1993; Winderick *et al.,* 1996; Thevelein, 1992; Thevelein, 1994; Estruch, 2000; Thevelein and Winde, 1999; Thevelein *et al.,* 2000; Rep *et al.,* 2000).

For *Pichia anomala, Pichia jadinii* and *Candida milleri* any STRE element or similar functional elements for the invertase gene are not recorded yet.

The yeast *S. cerevisiae* grows better at acidic than at neural or alkaline pH. Maintenance of an acidic environment is based primarily on the active proton extrusion mediated by its plasma membrane H^+-ATPase, and this proton gradient is critical for the uptake of different nutrients. Therefore sudden alkalinization of the environment represents a stress condition for this

yeast. In order to survive, *S. cerevisiae* must detect the change and trigger strong and transient intracellular calcium burst, which results in activation of calcineurin and calcineurin-regulated transcription factor Crz1/Tcn1 (Serrano *et al.,* 2006). Hph1p and Hph2p are components of a novel Ca^{2+} and calcineurin-regulated response required to promote growth under conditions of high Na^{+}, alkaline pH, and cell wall stress (Heath *et al.,* 2004). Activation of calcineurin/Crz1 pathway has relevance in high pH adaptation. The *BCK1* and *SLT2/MPK1* genes that are components of the MAPK cascade are involved in cell wall remodeling and maintenance of cell integrity. Protein kinase C (PKC) phosphorylates and activates the Bck1 kinase in response to cell wall stress. Activated Bck1p can phosphorylate a pair of redundant MAPK Kinases, Mkk1 and Mkk2, which activate the Slt2 MAPK. The membrane sensors, Wsc1-4, Mid2 and Mtl1, transduce the signal through the Rho1 GTPase. Activation of Slt2 results in the phosphorylation of several nuclear and cytosolic targets. Among the nuclear targets are the transcription factors Rlm1, which seems to be responsible for most of the transcriptional effects derived from the activation of Slt2, and SCB-binding factor, Swi4 and Swi6. The alkaline stress rapidly and transiently activates Stl2p. This activation is largely mediated by the Wsc1 cell membrane sensor and this results in an increase in the expression of several specific genes, some of which may be relevant for adaptation to high pH stress (Heath *et al.,* 2004).

The isolation of *Pichia pastoris* gene, *PIM1*, encodes the first MAPK to be identified in this yeast. Pim1p shows the greatest similarity to fungal MAPKs involved in the maintenance of cell integrity (Cosano *et al*., 2001). But the actual mechanism of this maintenance and pH sensing and signaling are not known for *Pichia* yeast cells.

Therefore additional research must be necessary for understanding the effect of pH on the invertase enzyme activity of *Pichia* and *Candida* yeast species.

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