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Doctoral thesis

ASPECTS OF YEAST *SACCHAROMYCES CEREVISIAE* DESICCATION TOLERANCE

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Omnium in mundo lente et perperam eveniant.
(*Benedictus Ierofeievus*)

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Abstract

Doctoral thesis is devoted to particular fundamental and applied aspects of baker's yeast desiccation tolerance. Particulary – nutrient effects on *Saccharomyces cerevisiae* cell survival after desiccation and application of desiccation to increase immunostimulatory properties of carboxylated beer yeast beta glucans.

Cell survival after desiccation was severly affected by media nitrogen and carbon source as well as yeast auxotrophic starvation. Nutrient effects on dynamic derepression of protein kinase A (PKA) and target of rapamycin (TOR) pathways and their subsequent effect on survival after desicction are discussed. Desiccation induced changes in spent brewer's yeast β-D-glucan 3D structure what in turn induced stronger immunoresponse (murine macrophage TNF-α excretion) than commercial immunomodulator (Immunoglikan ©).

Key Words: *Saccharomyces cerevisiae*, nutrient signalization, desiccation tolerance TOR and PKA pathways, β-D-glucan.

Kopsavilkums

Doktora darbs veltīts atsevišķiem maizes rauga *Saccharomyces cerevisiae* sausuma stresa vispārējās fizioloģijas un pielietojamiem aspektiem. Konkrēti – noskaidrota barotnes barības vielu ietekme uz rauga sausumizturību, kā arī pētīta žāvēšanas pielietojums karboksilētu alus raugu beta glukānu imūnostimulējošo īpašību paaugstināšanā.

Šūnu izdzīvotība pēc žāvēšanas bija tieši atkarīga no barotnes slāpekļa un oglekļa avotiem, kā arī no auksotrofiskas badošanās. Darbā diskutēts par proteīnu kināzes A kompleksa (PKA) un *target of rapamycin* (TOR) signālceļu dinamisku derepresiju periodiskās kultivēšanas laikā un to ietekmi uz sausumizturību. Žāvēšana izmaina raugu β-D-glikānu 3D struktūru, kas ietekmē to imūnoloģisko aktivitāti. Žāvētu alus rauga β-D-glikāni uzrādīja augstāku immunoloģisko aktivitāti nekā komerciāls immūnomodulators (Immunoglikan ©).

Atslēgvārdi: *Saccharomyces cerevisiae*, barības vielu atkarīgā signalizācija, sausuma izturība, TOR un PKA signālceļi, β-D-glikāns.

Index

Abbreviations

ADP – adenosine diphosphate

ADPG – adenosine diphosphoglucose

AICAR - 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide

AIR – p-ribosylamyloimidasol

AMP – adenosine monophosphate

ATP – adenosine triphosphate

cAMP – cyclic adenosine monophosphate

CFU – colony forming units

CCR – carbon catabolite repression

DHA – dihydroxyacetone

DHAP – dihydroxyacetone phosphate

DW – dry weight

G6PDH – glucose 6 phosphate dehydrogenase

GLD3P – glyceraldehyde-3 phosphate

GLY – glycerol

GLY3P – glycerol-3 phosphate

GSH – glutathione reduced form

GSSG – glutathione oxidized form

INT – iodonitrotetrazolium chloride

LPS – lipopolysaccharides

MDA – malonildialdehyde

mRNA – messenger RNA

Mya – million years ago

NAD – Nicotinamide adenine dinucleotide

NADH - Nicotinamide adenine dinucleotide reduced form

NADP - Nicotinamide adenine dinucleotide phosphate

NADPH - Nicotinamide adenine dinucleotide phosphate reduced form

NCR – nitrogen catabolite repression

NMR – nuclear magnetic resonance

OD600 – optical density measurement at 600 nm

PDS elements – post diauxic shift specific expression promoter sequences in *S. cerevisiae*, characteristic sequence: T(T/A)AG_3AT

PKA – protein kinase A

PMS – phenazine methosulfate

RNA – ribonucleic acid

ROS – reactive oxygen species

SAICAR - Succino-AICAR

SD – synthetic dextrose broth

STRE elements – stress responsive promoter sequences in *S. cerevisiae*, contains one or several CCCCT sequences

TBA – thiobarbituric acid

TBARS – thiobarbituric acid reactive species

TCA – tricarboxylic acid

TOR - target of rapamycin

UDP – uridil diphosphate

ug – microgram

uM – micromole

UV - ultraviolet

WT – wild type

YPD – yeast extract peptone dextrose broth

Introduction

Baker's yeast *Saccharomyces cerevisiae* natural habitat is a skin of sugar rich fruits. In these habitats, yeasts rely on short periods of "feast" followed by prolonged periods of starvation. Often starvation is followed by gradual desiccation and reversible cease of active metabolism. Therefore changes in the substrate availability could serve as the "early call" for cell signalling: "bad times are approaching". Onset of true starvation could serve as strong "late signal" indicating, that resources are scarce and preparation for starvation of unknown length should be carried out immediately.

S. cerevisiae is famous for its "produce-accumulate-consume" strategy; first fermentable carbon sources are metabolised and ethanol produced; when fermentation ends, ethanol becomes next substrate to be consumed. Diauxic shift is the way how cell reorganises it's metabolism for other substrate consumption (Thomson *et al.*, 2005). Onset of diauxic shift occurs after exhaustion of sugar and could signalize end of "feast". This is accompanied by increase in doubling time and stress resistance. Starvation for different nutrients leads to distinct physiological states but increased stress tolerance, including desiccation tolerance, is typical for cells after natural (phosphorous, nitrogen, sulphur) starvations (Boer *et al.,* 2008). Besides, there is growing body of evidence, that similar, particular stress (oxidative stress) enhancing effects can be related also to starvation for particular auxotrophic agents (tryptophan, methionine).

Since many of intracellular signalling pathways are similar across different eukarytic organisms, this concept foresees possible spots for "desiccation engineering" in yeast cells and beyond. Besides, elucidation of auxotrophy mechanisms in handy eukaryotic model would help to establish strategies for intracellular parasite and/or inherited auxotrophic like metabolic diseases control.

The aim

The aim of the study was to characterise baker's yeast *Saccharomyces cerevisiae* nutritional effects on desiccation tolerance.

Objectives

The objectives of the study were:

- to investigate if prolonged cultivation on oxidative substrates (mimicking diauxic shift and postdiauxie in "natural habitats") affects yeast desiccation tolerance,
- to investigate status of oxidative stress markers of postdiauxic yeasts before and after desiccation,
- to test if redox cofactor engineering can improve desiccation tolerance of *S. cerevisiae* cells in postdiauxie,
- to characterise auxotrophic starvation effects on *S. cerevisiae* desiccation tolerance.
- to characterise desiccation effects on brewer's yeast beta glucans.

The Hypothesis of the study

We hypothesize, that status of intracellular nutrient signalling pathways prior to desiccation, would determine subsequent cell's survivival after desiccation.

1. Literature review

1.1. General information on baker's yeast *S. cerevisiae*

Alcohol fermentation is process known to humanity for thousands of years. Ethanol as natural preservative and gas (CO_2) for bread leavening has been used long before knowledge about their biological agent (yeast). About last two hundred years, people are aware of yeast as a specific group of organisms directly responsible for brewing process. Bakers yeast, *S. cerevisiae* has been classified as separate organism in the 19th century and has been actively investigated since (Chambers and Pretorius, 2010).

S. cerevisiae is unicellular organism belonging to the kingdom of fungi, division *Ascomycotina*. Apart from typical eukaryote, it multiplies not by fission, but budding. At the same time, it contains all structures typical for eukaryotic cell, therefore is used as model organism of higher organisms (including human). *S. cerevisiae* is used extensively in drug screening (Schenone *et al.*, 2013), as disease models (Ocampo and Barientos, 2008), toxicology studies (Kasamets *et al.,* 2009, Smits *et al.*, 2012).

Fermentation of sugar rich substrates to ethanol is typical for *S. cerevisiae.* Main characteristics of this organism are fast glucose consumption and growth together with high (up to 16 -18 %) ethanol tolerance. In contrast to many microorganisms capable to alcoholic fermentation – *S. cerevisiae* not only ferment sugar rapidly, but it also consumes fermentation end products when sugar become depleted. This feature is called "produce-accumulate-consume" strategy: ethanol, toxic fermentation end product, is expelled from the cell and accumulates in environment, thus ensuring that "nutritional resources" are free of other microbes; ethanol is consumed by the same yeast afterwards (Thomson *et al.*, 2005). This "invention" helped ancient *Saccharomycetales* 200 Mya to rapidly ferment sugar rich fruits and ensure their ecological dominance (Rospedowska *et al*., 2011).

S. cerevisiae and number of other yeast species in nature rely on small but lavish periods of fest followed by prolonged periods of starvation. Starvation often is accompanied by cell desiccation.

1.2. Baker's yeast *S. cerevisiae* nutritional physiology

1.2.1. Glucose repression

S. cerevisae and other yeast species may thrive on various carbon sources, but glucose and fructose are the preferred ones (Dynesen *et al.*, 1998). When one of these sugars is present, synthesis of the enzymes required for the utilization of alternative carbon sources (for example, alternative substrate uptake and metabolism) is kept to very low levels or completely blocked. This phenomenon is called carbon catabolite repression, or specifically "glucose repression" (Gancedo, 1998). Effective carbon catabolite repression allows yeast to ferment glucose with high efficiency, even in the presence of oxygen.

Protein kinase A (PKA), the Snf1p[1] protein kinase, and glucose sensors Rgt2p and Snf3p are three partly overlapping carbon-signaling pathways, which are involved in ensuring glucose repression (see fig. 2.2.1). Glucose repression works through transcription inhibition (induction of transcription repressor Mig1p) or transcription derepression (deactivation of transcription repressor Rgt1p). Other sensing effectors are RNA polymerase II mediator by decreasing mRNA stability of unneeded gene transcripts (Schneper *et al.*, 2004, Gancedo *et al.*, 1998).

Glucose is sensed by two membrane proteins Snf3p and Rgt2p. (Özcan *et al.,* 1998, Rolland *et al.*, 2002). Both show approx. 25% sequence similarity to other members of the hexose transporter family. The main difference from hexose transporters is that they have a more than 200 amino acid residues long C terminal cytoplasmic tail. No glucose transport occurs through these proteins, but their deletion results in similar phenotype as if hexose transporters were deleted. From here, it was concluded that both of these proteins act as glucose sensors. Snf3p is characterized as high and Rgt2p - low affinity sensor (Özcan *et al.*, 1998).

Glucose transporter (hexose transporter family genes, *HXT*) expression by Snf3p and Rgt2p is induced through derepression of Rgt1p (Carlson *et al.*, 1998). In the case of high glucose concentration, signal from Rgt2p is transferred to Rgt1p (transcription repressor) via Grr1p, which is part of the SCF^{Grr1} ubiquitin-ligase complex. Skp1p is necessary for Grr1p

[1] In this work, *S. cerevisiae* genes and proteins are named according to the nomenclature of SGDatabase (http://www.yeastgenome.org/): genes are marked by three uppercase letters followed by a number in italic (e.g. *SNF1*), proteins are marked by the relevant gene written as first letter capitalized and letter 'p' attached at the end (e.g. Snf1p).

activity. It binds to Grr1 protein F-box, thus Grr1p is able to perform ubiqutinatination of Rgt1p or it's repressor, see fig. 1.2.1 (Li and Johnston, 1997).

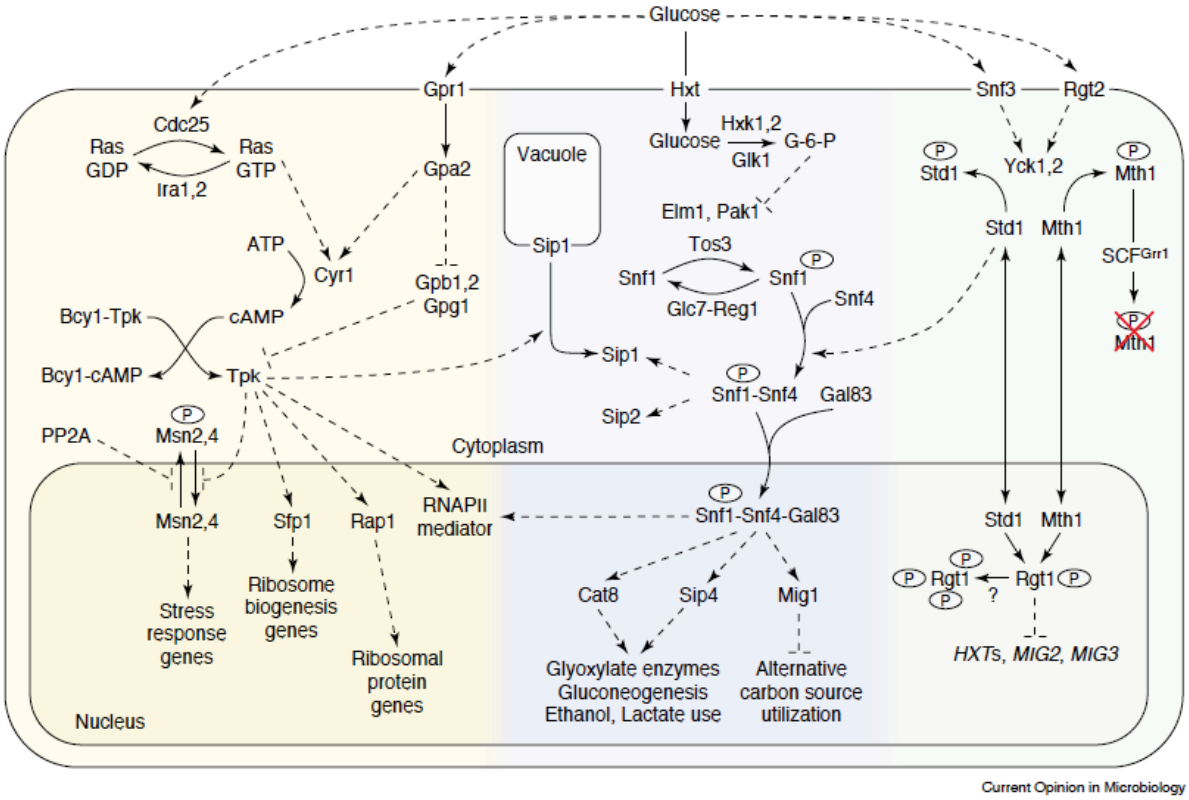


Fig. 1.2.1. *S. cerevisiae* glucose signalling pathways ensuring glucose sensing and repression. Solid arrows represent transformations and/or translocations, dotted lines – regulatory or catalytic influences. Three pathways involved are (from left to right) Protein kinase A, Snf1 protein kinase, glucose sensors Rgt2p and Snf3p. Adopted from Schneper *et al.*, 2004.

The common path for glucose induced transcriptional repression is mediated through Snf1p kinase to the transcription repressor Mig1p, which represses transcription of alternative substrate metabolism. Protein kinase Snf1p (Sucrose non – fermenting) is central element of Mig1p mediated repression. Mig1p displays at least three phosphorylation sites, but only two of them seem to be actually phosphorylated by Snf1p (Ostling and Ronne, 1998). This protein in it's phosphorylated form resides in cytosol, whereas the dephosphorylated form is translocated to the nucleus. In the case of high glucose concentration, Mig1p is dephosphorylated and in the nucleus represses alternative substrate metabolism gene transcription (Ostling *et al.*, 1996). Regulation of Fbp1p (fructose-bi-phosphatase) is example of Mig1p dependent regulation: Snf1p is repressed by high glucose concentrations and therefore Mig1p is dephosphorylated and resides in nucleus repressing transcription of *FBP1*, see fig. 1.2.2 (Zaragoza *et al.,* 2001) .

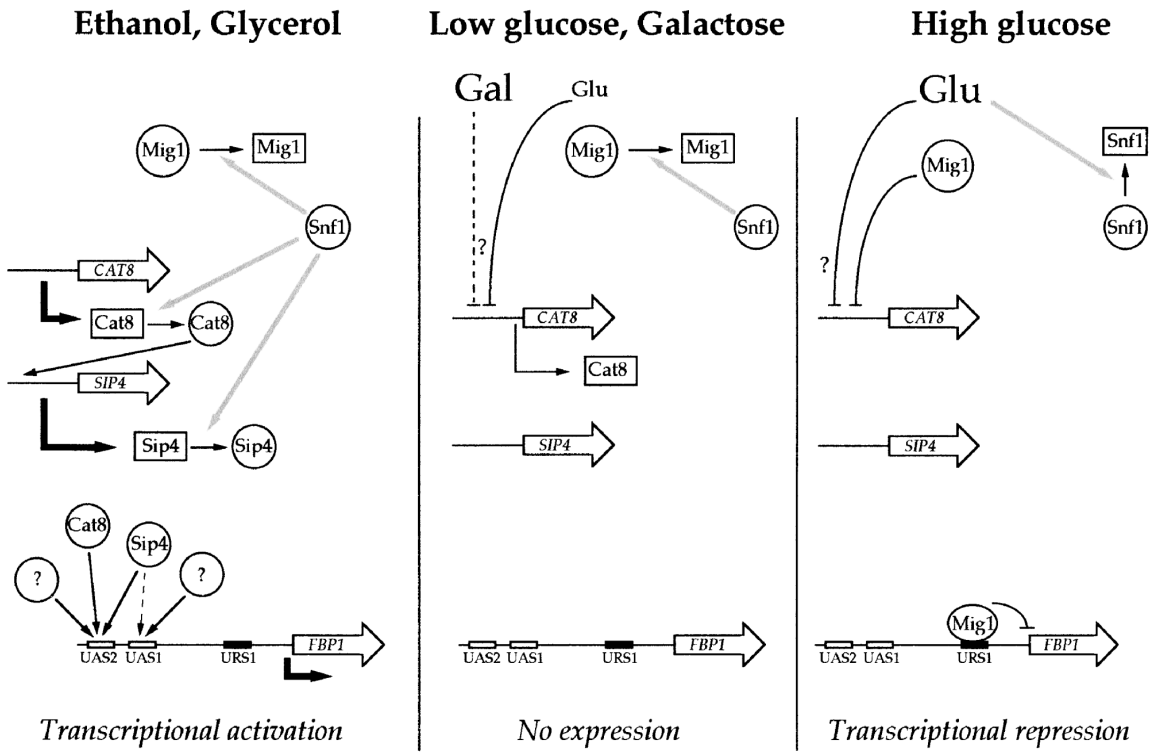


Fig. 1.2.2. Mechanism of Mig1p repression/derepression of glyconeogenic genes depending on glucose concentration.
Cat8, Sip4 are transcription activators, during glyconeogenic growth they bind to certain gene's upstream activating sequences (UAS1/2). When fermentable carbon source becomes abundant, Cat8p and Sip4p are not active, Mig1p is not phosphorylated and can enter nucleus and repress glyconeogenic genes (like *FBP1*) by binding to upstream repressor site (URS1). Adopted from Ostling and Ronne, 1998.

PKA pathway is the third carbon nutrient dependent intracellular signalling pathway. It integrates signals from plasmatic membrane glucose sensor Gpr1p (G-protein-coupled receptor) and glucose intracellular metabolism (glycolysis). Signals from both branches meet at the adenylate cyclase Cyr1p (see fig. 2.2.1.) which produces cAMP, which, in turn, activates protein kinase A (PKA) complex. PKA postpones signal to pathway nuclear effectors: transcription factors Msn2/4p and Gis1p TPK1,2,3 encode the three catalytic subunits of protein kinase A (see fig 2.2.1). (see review Conrad, 2014). Interestingly, PKA complex transmit the signal only if both branches are activated (Gpr1p sensor is saturated and cAMP intracellular level is increased due to active glucose catabolism).

PKA pathway is activated only at rather high extracellular glucose concentration; Gpr1p sensor has low glucose affinity (k_M approx. 50-75 mM). Possibly, sensor low affinity for glucose ensures the switch from respirative/gluconeogenic growth to fully fermentative growth only at glucose concentrations not smaller than 20 mM (Rolland *et al.*, 2000). When activated, PKA pathway depresses stress response (Msn2/4p dependent) gene expression, increases ribosome gene expression and mRNA stability (Yin *et al.*, 2003).

Signalling pathways can have specific and "shared" effectors. mRNA stability is shared target of PKA and Snf1-Mig1 pathways. mRNA stability relates to both – ribosome RNA complex stability as well as to specific gene transcript, including repressor transcript stabilities. For instance, *SDH2* (iron-protein subunit of succinate: quinone reductase complex) gene transcription when growing on glucose has the the same rate as growing on glycerol, however, amount of respective mRNA on glucose is 6 – 12 times less, than glycerol based medium. No differences in *SDH1* transcription activation were observed, therefore an idea of rapid post-transcriptional breakdown in the case of glucose media was considered (Scheffler *et al.,* 1998).

Many factors are involved in mRNA turnover. Possible, glucose dependent mRNA stability "system" involves activity of glucose transporters, glucose sensors (Snf3p), RNA decapping enzymes (like Dcs1p), translation initiation factors (eIF) (Scheffler *et al.*, 1998, Yin *et al*., 2003, Malys *et al.*, 2004, Braun *et al.*, 2014).

1.2.2. Crabtree effect

Crabtree effect is defined as fermentation dominance over respiration under fully aerobic conditions. The effect was first described to better explain reversibility of Warburg effect (extensive lactic fermentation in tumor cells) by Crabtree, 1929. Even though phenomenon was attributed to malignat mammal cells, similar, features has been described in many microorganisms, including baker's yeast (Diaz-Ruiz *et al.*, 2011).

This effect is observed during yeast aerobic fermentation on glucose rich media and is consequence of glucose repression. The main consequences of Crabtree effect during growth on glucose rich media are repression of TCA cycle and oxidative phosphorylation (only anaplerotic reactions of TCA cycle are active). Substrate level phosphorylation is the only way for ATP production when Crabtree effect is on. Ethanol and glycerol are produced to regenerate cytosolic NAD while NADH accumulates due to fast running glycolisis. Besides, NAD^+ and $NADP^+$ can be recovered partly by increased nitrogen uptake (Ronne, 1995).

Ethanol production and it's regulation pathways are of paramount importance in modern biotechnology. Alterations in NAD^+/NADH balance by redirecting flux from glycerol to acetaldehyde production have been proposed to improve yeast ethanol production (Nissen *et al.*, 2001).

1.2.3. Overview of S. cerevisiae nitrogen metabolism

Nitrogen is among the basic elements forming living organisms. Nitrogen pool in the cell is shared by amino acids, purine and pyrimidine bases. Additionally, fungi specific sugars contain nitrogen (glucosamine, like chitin) (Berg *et al.*, 2010).

Nitrogen sources in typical industrially used fermenting substrates differ: grapes are particularly rich in ammonia, arginine and proline (Casalta 2013), whereas barley is rich in ammonia, proline, and glutamic acid (Folkes and Yemm, 1956). Yeast is capable to grow in medium containing various nitrogen sources (ammonia, amino acids, urea, etc.), but, one pathway exist to "unify" all nitrogen forms to glutamate or glutamine (see fig. 1.2.3.).

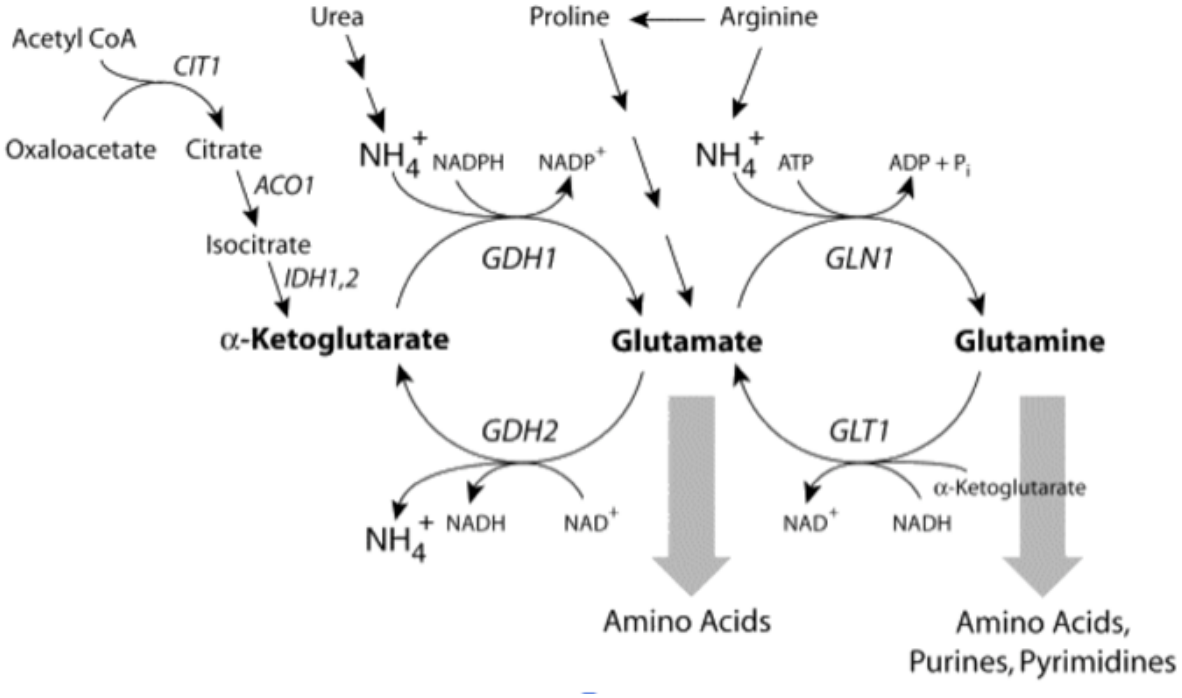


Fig. 1.2.3.Central nitrogen pathway of baker's yeast.
In yeast *Saccharomyces cerevisiae* a set of reactions exist transforming nitrogen containing compounds taken up by the cell into "unified nitrogen forms", like glutamate and glutamine. Gene products involved: *CIT1* (citrate synthase), *ACO1* (aconitase) *IDH1,2* (NAD+-dependent isocitrate dehydrogenase), *GDH1* (NADH linked glutamate dehydrogenase), *GDH2* (NAD^+ linked glutamate dehydrogenase), *GLN1* (gluthamine synthetase), *GLT1* (glutamata synthase). Adopted from Magasanik and Kaiser 2002

Central reactions of yeast nitrogen metabolism are conversion of α-ketoglutarate to glutamate and glutamine to glutamate, catalyzed by glutamate synthase, Glt1p, and glutamate dehydrogenase Gdh1p respectively (see fig. 1.2.3.). In both reactions reduction equivalents in the form of NADH and NADPH are consumed, therefore NAD^+ and $NADP^+$ are recycled.

Nitrogen sources can be taken up by specific ammonia transporters Mep1/3p (Marini *et al.,* 1997), general (Gap1p) and specific (Put4p, Can1p, etc.) amino acid transporters (Regenberg *et al.*, 1999).

1.2.3.1. S. cerevisiae nitrogen catabolite repression

If mixture of different nitrogen sources is available, the most easy convertible nitrogen source is picked up first. Since different nitrogen sources are located nearby or away from nitrogen metabolism core reactions (see fig. 1.2.3) - they are defined as more or less preferred to each other. Easy metabolised (or good, preferred) nitrogen sources are glutamine, asparagine, but proline and urea are poor nitrogen sources (ter Schure *et al.,* 2000). Nitrogen catabolite repression (NCR) is situation when pathways of non-preferred nitrogen source uptake are deactivated due to presence of preferred nitrogen source.

Another criteria (besides to distance from "central nitrogen metabolism reactions") to distinguish good nitrogen source from bad is the level to which systems facilitating alternative nitrogen source usage are de-repressed when grown on particular nitrogen source. As stronger the repression is, as more preferred certain nitrogen source is (ter Shure *et al.,* 2000, Magasanik and Kaiser, 2002). Ammonia is rather good nitrogen source; it needs just one reaction to reach glutamine. Depending on yeast strain, ammonia can fully or partly repress uptake of other nitrogen sources via nitrogen catabolite repression (Magasanik and Kaiser, 2002).

Similarly to carbon catabolite repression (CCR), NCR is realized at various levels of metabolism: transcription, translation and posttranslation. NCR dependent transcriptional control is carried out through Gln3p transcription factor. When good (preferred) nitrogen sources are available, Gln3p is located in cytoplasm, when they become depleted; it is transported to nucleus (Beck and Hall, 1999). Differential localization of Gln3p is achieved by phosphorylation state: phosphorylated Gln3p resides in cytoplasm, dephosphorylated - enters nucleus. Gln3p phosphorylation is regulated by Sit4p phosphatase. The activity of Sit4p in turn, is regulated by it's association-dissociation with protein Tap42p. At last, the complex Tap42p-Sit4p is regulated by TOR (Target Of Rapamaycin) complex proteins, see fig. 1.2.4. (Beck and Hall, 1999, Cooper, 2002).

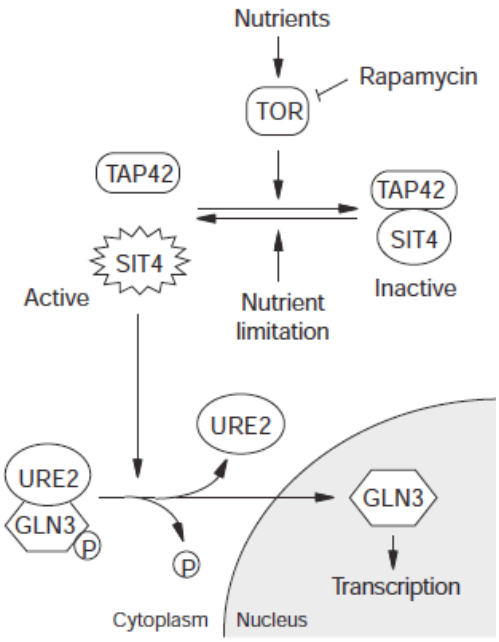


Fig. 1.2.4. Model of Gln3p activation.
In resonse to good nutrient source, TOR stabilzes cytoplasmic GLN3–URE2 complex via Tap42p mediated inhibition of the phosphatase Sit4p, and thereby prevents GLN3 from entering nucleus. Rapamycin works in similar way as nutrient deprivation – inhibiting TOR.
Adopted from Beck and Hall, 1999.

Interestingly enough, Gln3p trasnslocation to nucleus has been attributed not only as a response to changes in nitrogen source, but also to carbon starvation. However, this was resolved to be the result of shared metabolite between carbon and nitrogen metabolism - α-ketoglutarate, which becomes depleted in the case of carbon starvation and subsequently lowers glutamine level and initiates Gln3p nuclear migration even in presence of ammonia. The effect was not present if carbon starved cells were supplemented with glutamine (Cox *et al.*, 2002).

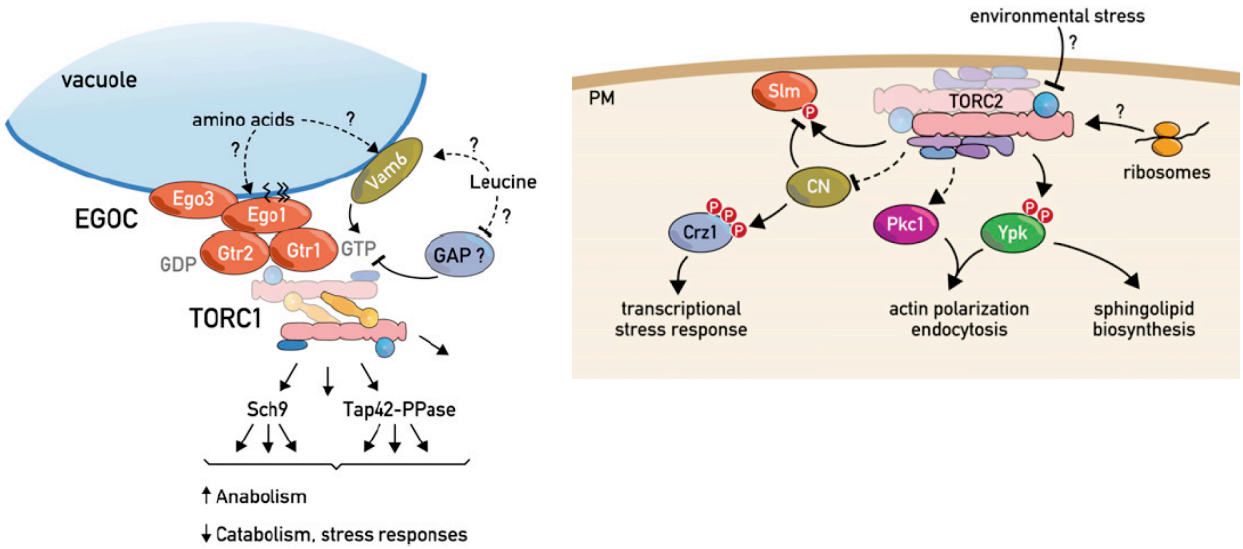
Gln3p targets are genes with one or several upstream GATAA sequences. Also Gat1p, which has high sequence homology to Gln3p is able to induce GATAA dependent transcription (Coffman *et al.*, 1996). Gln3p activated genes are: amino acid permeases, glutamine and glutamate synthesis genes (Daugherty *et al.*, 1993, Cooper, 2002).

1.2.3.2. Target of Rapamycin (TOR) pathway

There is nutrient signal "integration" system in *S. cerevisae* cells to coordinate nutrient supply with cell cycle and growth speed. This large kinase/phosphatase system is called TOR. Historically, this complex has been described as sensitive to antibiotic – rapamycin; it is a secondary metabolite produced by soil bacterium *Streptomyces*

hygroscopicus, isolated from soil samples of Rapa-Nui (Easter Island) in 1965 – hence the name rapamycin. This antibiotics and it's derivatives has gained it's application in oncology as tumor suppressor, immunosuppressant during organ transplantation and it prevents restenosis during angioplasty. Wide application spectrum of rapamycin is due to it's target occurrence in all eukaryotic cells (reviewed by Loewith and Hall, 2011 and De Virgilio and Loewith, 2006).

The core of TOR system in *S. cerevisiae* is formed by two redundant complexes TORC1 and TORC2. These complexes have different protein composition and different functions: TORC1 controls growth in "temporal manner" (integrates N and C source quality signals into growth speed and cell cycle progression), while TORC2 controls "spatial aspects" (for example, actin polarization) of cell growth and proliferation. Only TORC1 is rapamycin sensitive (Loewith *et al.*, 2002).



A **B**

Fig. 1.2.5. Inputs and outputs of TORC1 (A) and TORC2 (B) complexes in *S. cerevisiae.*
EGOC – EGO protein kinase complex, PM – plasma membrane, CN- calcineurin phosphatase.
Adopted from Loewith and Hall, 2011.

In *S. cerevisiae* TORC1 localises in close proximity to vacuole, where it receives signals from EGO complex (EGOC). The latter senses intracellular and vacuolar leucine and other amino acid levels. In the case of high content of free amino acids, phosphorylation signal is passed through EGO complex to TORC1 and then to kinase/ phosphatase cascades. At last, anabolism (ribosomal subunits, etc.) genes are activated. Ussually this would be the case during growth on rich media (huge aboundance of free amino acids); interestingly, this

signal can be simulated by adition of cyclohexamide – protein synthesis inhibitor in tRNA translocation step in eucariotic ribosome (reviewed by Loewith and Hall, 2011).

TORC2 complex is plasma membrane associated and it takes part in organisation of actin cytoskeleton, endocytosis and sphingolipid biosynthesis. Despite high homology to TORC1, which is rather well characterised, TORC2 functioning is not completely understood. Crz1p (stress response transcription factor with consensus binding motif 5'-GNGGC(G/T)CA-3') is calcineurin phosphatase (Cna1p and Cna2p) substrate (see fig. 1.2.5 B), which in turn is regulated by TORC2. In the case of environmental stress, TORC2 derepresses calcineurin phosphatase and it dephosphorylates Crz1p transcription factor, which then enters nucleus and activates target genes. Crz1p dependent genes are involved in protein degradation, vesicle transport, cell wall synthesis and sporulation (Cyert, 2003, Mulet *et al.*, 2006)

1.2.4. S. cerevisiae diauxic growth

Similar to classical bacterial growth experiments on mixed substrates (Monod, 1949), also *S. cerevisiae* growth possesses diauxic characteristics: fermentable, easy metabolised carbon sources are consumed first and then subsequent consumption of other, less favorite carbon sources follows.

S. cerevisiae "produce-accumulate-consume" strategy includes strong diauxic component: when fermentable carbon source depletes, accumulated ethanol becomes next carbon source. To shift from glucose to ethanol consumption, yeast cells perform massive rearrangements in it's transcriptome, translation, metabolome and culture growth dynamics.

1.2.4.1. S. cerevisiae growth phases

When inoculated in rich, glucose containing media - *S. cerevisiae* culture exhibits several distinct growth phases: lag phase – several hours of adaptation after cells are inoculated from saturated preculture into fresh media, exponenential phase – when yeast cells grow with maximum speed consuming glucose, diauxie when shift from fermentation to oxidative growth happens, postdiauxie phase when re-assimilation of fermentation products (ethanol, succinate, glycerol, pyruvate, acetate, etc.) occurs and stationary phase which is defined as set in of starvation for carbon nutrient (Teste *et al.*, 2009, Washburne *et al.*,1993), see fig. 1.2.6. for illustration.

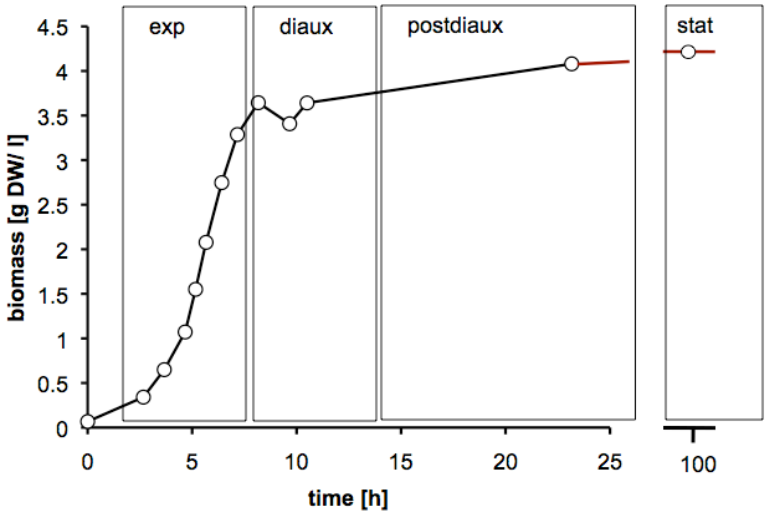


Fig. 1.2.6 Illustration of *S. cerevisiae* growth phases.
Abbreviations: exp – exponential, diaux – diauxic, postdiaux – postdiauxic and stat - stationary phase
The length of each phase is media and strain dependent.

Lag phase

When *S. cerevisiae* cells are transferred from one media to other, for example, from starting inoculum containing ethanol to fresh media with glucose as main carbon source, several hours' putative absence of growth is observed. This is called by lag phase. Indeed, no cell proliferation is observed during very first hours of cultivation even though nutrients are available at ample amount (Brejning and Jespersen, 2002).

During this culture growth phase, cells adopt to new media. To rearrange metabolism from slow growing, glucose derepressed to fast growing under strong glucose repression, rapid nitrogen, carbon source uptake and protein synthesis starts. Glucose transporters (like Hxk2p) and core amino acid metabolism proteins (like glutamate dehydrogense, Gdh1p) are strongly induced. In transcriptional level, induction of genes facilitating glucose catabolism and protein synthesis are upregulated, while genes of gluconeogenic and stress response pathways are repressed (Brejning *et al.*, 2003). G1 transition, bud formation and cell proliferation follows (Brejning and Jespersen, 2002). It has been suggested, that resuming activity of TOR signalling complex promotes cell transition from G1 arrest to rapid proliferation (Barbet *et al.*, 1996).

Exponential phase

S. cerevisiae is Pasteur effect negative yeast: it is capable to ferment sugars in the presence of oxygen. When growing on glucose, fermentation is the main process to gain ATP and it happens in so called "exponential" growth phase. Ethanol, acetate and glycerol together with CO_2 and biomass are the *S. cerevisiae* fermentation products (Walker, 1998).

When lag or adoption phase ends, rapid yeast cell proliferation starts. Cells fully exploit all the preferred nutrients and thrive by maximizing growth speed. At exponential phase, especially at the very beginning of it (when population haven't reached 1/100 to 1/10 of it's maximum density) no limits exist for cell growth: there are enough of nutrients, small number of concurrents and no toxic effects of fermentation products have set in. This growth phase is used for determination of various strain specific physiological characters, like specific growth rate (μ), product yields (Y_x) (Dalgaard *et al.*, 1994). While prolifirating at maximum speed, PKA, TOR and glucose sensing signaling pathways are active ensuring optimal transcriptional mode to sustain fast growth: maximal ribosome production, induction and stabilization of mRNA transcripts coding for glucose catabolism, blocking stress responsive genes (mainly through Msn2/4p phosphorylation) and alterantive carbon source utilization, and reserve carbohydrate accumulation (Smets *et al.*, 2010).

Diauxic shift and diauxie

When broth glucose reserves are going to end, a drastic shift from fermentation to oxidative growth happens. This growth phase is called diauxic shift followed by postdiauxie.

Sequential shift from fermentative (glucose) to oxidative (ethanol, organic acids) substrate consumption is achieved by fundamental reorganization of *S. cerevisiae* metabolism. This reorganization takes place at all levels of metabolism: transcriptional, translational, protein, and metabolite level (De Risi *et al.*, 1997). In principle, *S. cerevisiae* glycolisis pathway can work in both directions – as glucose catabolic and gluconeogenesis pathway from ethanol, see fig. 1.2.7. However, during growth only one direction is preferred and the choice is made by nutrient signaling pathways. When glucose is exhausted, glucose repression ends and different growth phase starts. Ethanol becomes major substrate; mitochondrial oxidative phosphorylation becomes the main pathway for ATP production, cell doubling time increases and specific growth rate μ decreases. PKA pathway is inactivated, since it Cyr1p does not receive signals from both of it's branches (high glucose sensor Gpr1p and hexose catabolism). As a result, transcription of ribosome coding genes decreases, transcription of stress response genes increases in Msn2/4p dependent manner, respiratory metabolism genes become derepressed (both PKA and Mig1p dependent) (Smets *et al.*, 2010).

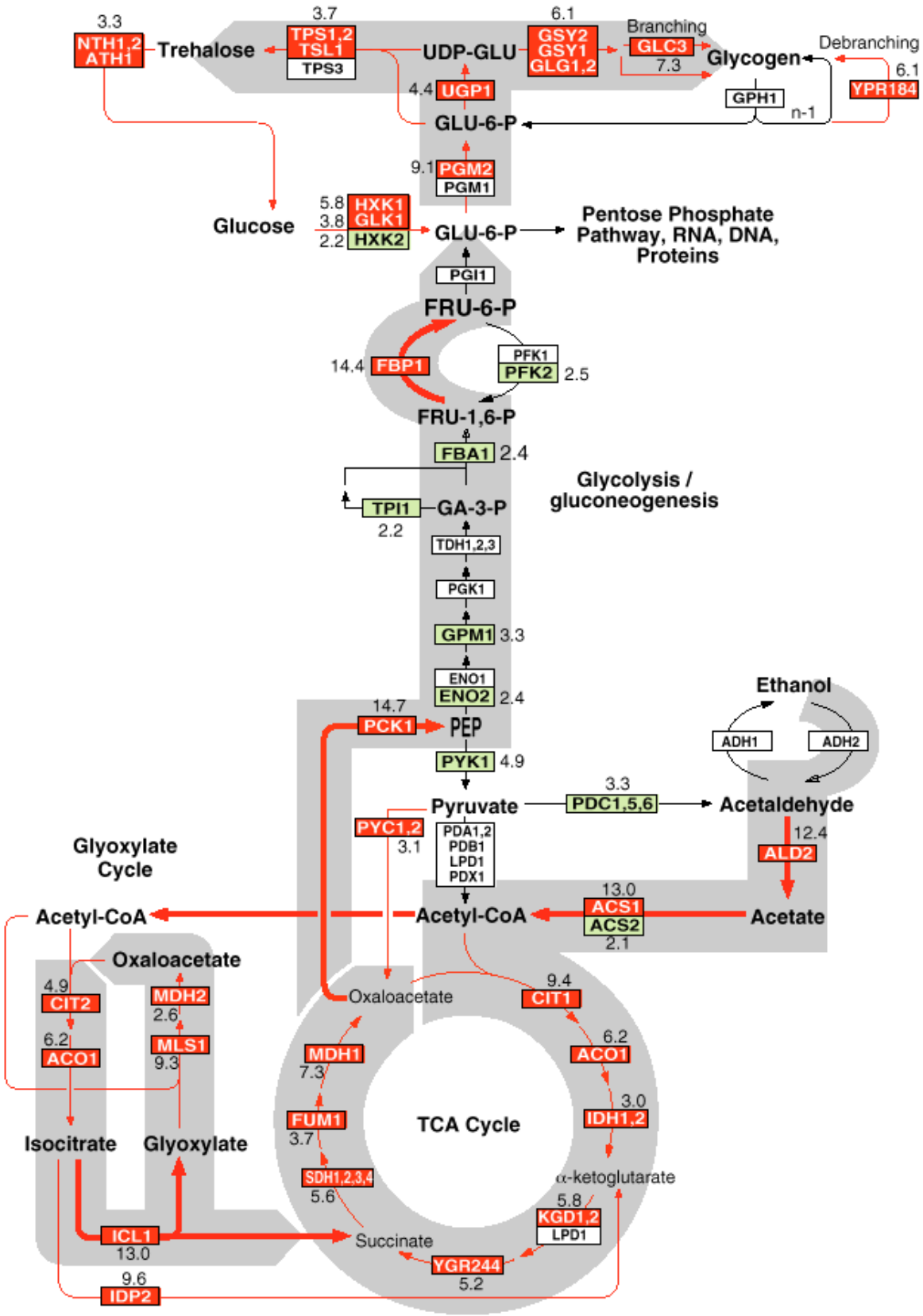


Fig. 1.2.7. Glycolitic flux rearrangement after diauxic shift in *S. cerevisiae.*
Arrows depict carbon flow. Genes in red boxes are overexpressed, in green – expression is decreased. Note, that transcriptional regulation only partly explains carbon flow (for example, Adh2p is not upregulated while ethanol flux is increased). Adopted from De Risi *et al.*, 1997.

Postdiauxie

After diauxic shift, when glucose is depleted, yeast cells transiently arrest growth. Because there is still some carbon available in the medium: ethanol and acetate, cells reorganize their metabolism to obtain energy from these sources. It is said, that cultures enter the post-diauxic phase, once cell division is reinitiated. Postdiauxic phase is a period of slow growth and it can last for several days in a batch culture till all carbon sources are over (Gasch and Werner-Washburne, 2002).

Specific, postdiauxic (PDS) genetic element T(T/A)AG_3AT has been identified. Similar to STRE (AG_4) sequences, it is recognized by specific transcription factor. Gis1p is downstream effector of Rim15p kinase, which in turn is repressed by PKA pathway In the case of decreased PKA signal (glucose depletion), Rim15p gets derepressed and activates Gis1p, which in turn initiates a set of postdiauxie typical gene transcription in PDS element dependent manner. In parallel, the same time Rim15p activates Msn2/4p which in turn enchances various stress responsive gene transcripts in STRE dependent manner (Pedruzzi et al., 2000). Genes activated in posdiauxie in Gis1p dependent manner are *GDH3, GND2, GRE1, SSA3, TKL2 ALD2* (stress response). Genes co-activated by or/ and Gis1p and Msn2/4p are*: PHO8, PH084, PHO89* (phosphate permeases), *MET2, MET6* (methionine synthesis and sulphur metabolism), *PLC1, SRY1, STE11, ZWF1 ALD3, ARG80, BMH2, GIS3, GPA2, GRE2, GUT1* (stress responsive genes) (Westholm *et al.*, 2012).

Stationary phase

When yeasts are cultivated in batch mode for prolonged time - stationary phase sets in. This term in literature is used for prolonged cultivation episodes (5-7 days). “Starvation” is the typical term used to describe yeast cell nutritional status in stationary phase (Werner-Washburne *et al*., 1993 Gasch and Werner-Washburne, 2002). In laboratory model fermentations – starvation for carbon source induces stationary phase phenotype. However, starvations for other nutrients, like nitrogen or phosphate, might be induced or set in leading to similar phenotypic traits (Klosinska *et al.*, 2011, Gasch and Washburne, 2002).

During prolonged starvation, part of yeast culture can enter into a “quiescent state”. Theese are comperatively young, nonbudding cells with characteristic higher density. At the same time, part of culture consists of large, senescent cells with many bud scars and increased ROS content. Only small, nonbudding cells are stress resistant (Allen *et al.*, 2006). During stationary phase cells acquire specific, nutrient dependent phenotype, what helps to survive starvation and to resume proliferation if refeeded (see review De Virgilio, 2012).

During yeast cultivation gradual changes in nutrient signalisation occur (due to depletion of particular nutrient). Nutrient signalling pathways converge to similar effectors which are repressed or derepressed depending on nutrient availability, see fig. 1.2.8. for illustration.

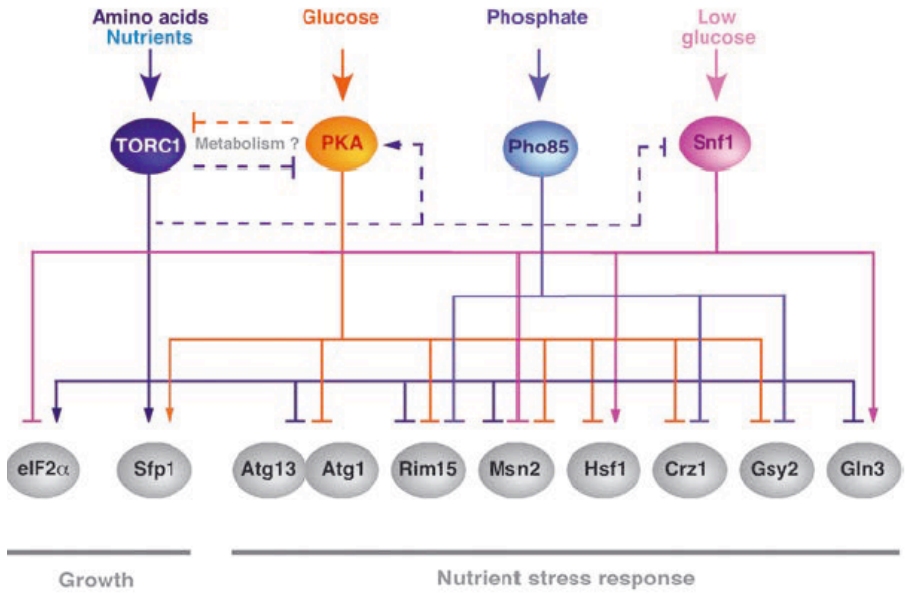


Fig. 1.2.8. Nutrient responsive pathways (TORC1, PKA, Pho85, and Snf1) converge on common transcription factors.
Here arrows and bars denote positive and negative interactions, respectively, which can either be direct or indirect. Dashed arrows and bars refer to cross-talk mechanisms between nutrient sensing pathways. Adopted from De Virgilio, 2012.

As a result, similar set of genes induced by Rim15p, Msn2p and Crz1p. However, due to different overlap of nutrient signalling pathways, different "transcriptional signature" can appear as the result of particular starvation. Cells in stationary phase have increased tolerance to heat, prolonged starvation, zymolase treatment, hydrogen peroxide and desiccation (Klosinska *et al.*, 2011, De Virgilio 2012).

Trehalose accumulation is typical reaction/ response observed in cells in stationary phase. It is generally assumed that yeast cells establish a core quiescence program regardless of which nutrient is limiting.

1.3. On biology of desiccation tolerance

Among many abiotic stresses, air drying is one of the most common. Organism desiccation is the extreme decrease of organism's water content due to air-drying. It means complete loss of organism's free water, including significant portion of bound water. Broadly speaking, water loss through evaporation is key problem for terrestrial life (Alpert, 2006). Organism exploit several strategies how to cope with water loss:

- try to accumulate and save water inside the organism, or
- let the water evaporate and enter the metabolically quiescent state and wait for "better times to come" (Wharton, 2002).

The latter strategy is typical for group of organisms capable to survive desiccation, stop their metabolism and resume it when re-hydrated again thus these organisms are called "anhydrobiotes" (Keilin, 1959). Typical examples of anhydrobiotic organisms are single cell prokaryotes (for example spore forming Gram+ and heterocyst forming cyanobacteria), and eukaryotes (for example, yeasts), some plants and animals (reviewed by Potts *et al*., 2005 and Alpert, 2006). There are many physical and physiological restrictions why desiccation tolerance is typical for fungi, small worms (nematode, rotifers), plants (moss, spores, most of seeds of seeded plants and some adult angiosperms). Organisms capable of anhydrobiosis in their vegetative state generally are of small size, their cells have rigid cell walls or organism posess exoskeleton, they are capable to accumulate large amounts of sugar or other osmolytes (reviewed in Alpert, 2006, Wharton, 2002). Anhydrobiosis is widespread among fungi. Yeasts, including *S. cerevisiae*, are typical anhydrobiotes (Calahan *et al.*, 2011).

Habits with low water potential (hot and cold deserts, arctic territories, surfaces of plant leaves) posess other life threatening features. For example, increased degree of UV radiation. Anhydriobiotic organisms posess "stress cross tolerance": they are capable to whitstand other environmental stresses while desiccated, for example, elevated temperature, irradiation, UV, etc.. Thus, organisms in desiccated state can whitstand many different otherwise detrimental environmental stresses (oxidative stress, heat, UV, etc.) (Warton, 2002, Cornette and Kikawada, 2011).

1.3.1. S. cerevisiae desiccation tolerance physiology

The baker's yeast *S. cerevisiae* population is capable to survive prolonged periods of water activity close to 0. As response to desiccation, this organism does not form specific water reserves, nor endospores. Despite the lack of water reserves or endospore formation, *S. cerevisiae* cells are capable to survive almost complete desiccation. In *S. cerevisiae* evaporation leads to water loss, meaning not only loss of free water, but also great part of molecular hydratation layers or bound water (Beker *et al.*, 1984). Water loss changes not only volume (shape of the cell), but also intracellular "organization" of the cell. Desiccation induced alterations of *S. cerevisiae* cell shape, membranes and organoid organization are well studied. Collapse of cell due to volume loss is typical change after desiccation observed in *S. cerevisiae*, while restoring the shape and wrinkling of cell surface is typical for

rehydrated cells (Ventina *et al.*, 1984). Organoid (for example nucleus) membrane disruptions, plasma membrane invaginations are often observed in dried *S. cerevisieae* cells (reviewed in Beker and Rapoport, 1987).

There is a debate on particular biochemical mechanisms and factors ensuring *S. cerevisiae* desiccation tolerance. Here we will review effects of: trehalose content, oxidative stress response system and nutritional signalling, on *S. cerevisiae* deisccation tolerance.

1.3.2. Trehalose metabolism in S. cerevisiae

Trehalose is among the most common disaccharides in nature. It consists of two glucose moieties linked together by α(1,1)-glycosidic bond, see fig. 2.3.1.

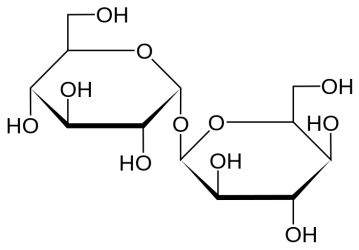


Fig. 1.3.1 Trehalose structure.
Source: public domain.

Trehalose is met in all kingdoms of life: fungi, plants, animals, protists and monera. Goddijn *et al.*, 1997 reported on trehalose in plants, Elbein, 1974 - in insects and Ishihara *et al*., 1997 reported on trehalose presence in mammal kidney tissues.

Trehalose content in fungi cells can be as high as 20% of cell's dry weight. In yeast *S. cerevisiae* main functions of trehalose are: osmoprotection, carbon reserve formation, and termotolerance. Schematic presentation of trehalose synthesis and breakdown in *S. cerevisiae* is shown in fig. 1.3.2.

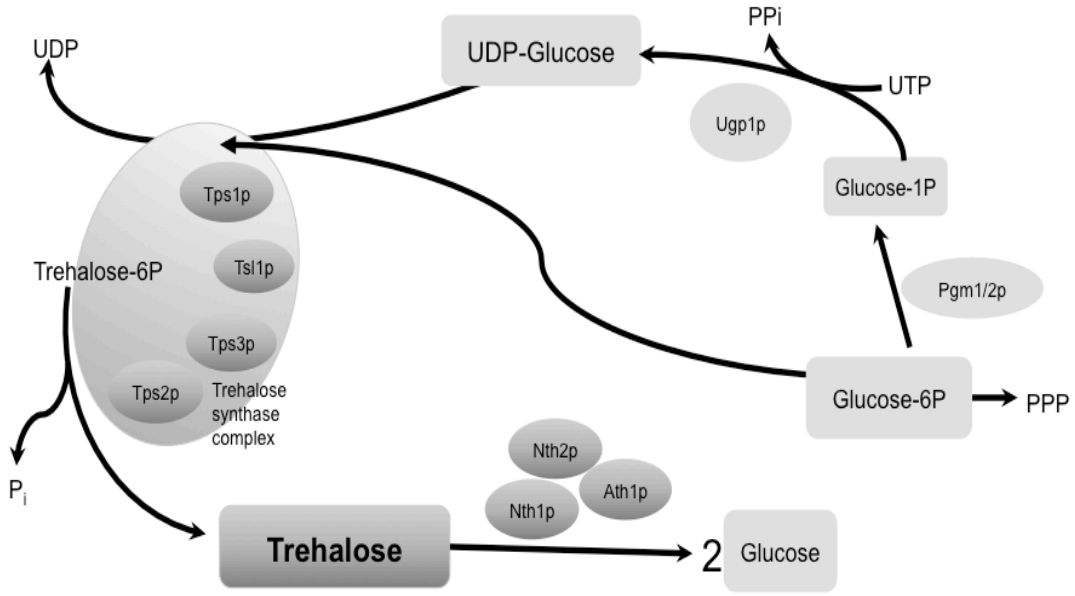


Fig. 1.3.2. Trehalose synthesis and breakdown in *Saccharomyces cerevisiae.*
Proteins involved: Acid trehalase (Ath1p), neutral trehalase (Nth1/2p), trehalose-6 phosphate synthase (Tps1p), trehalose-6 phosphate phosphatase (Tps2p), Trehalose phosphate synthase subunit 3 (Tps3p), Trehalose phosphate synthase complex (Tsl1p) Phosphoglucomutase, isoforms 1 and 2 (Pgm1/2p), Uridine – 5´ - diphospho – glucosepyrophosphorylase (Ugp1p).
Adopted from Francois and Parrou, 2001.

Trehalose is synthesized from glucose – 6–phosphate and UDP - glucose molecules (Panek, 1962). This is done by trehalose synthase complex consisting of four subunits, Tps1p, Tps2p, Tps3p and Tsl1p (see fig. 1.3.2.). Tps1p catalyses synthesis reaction of trehalose-6P from glucose-6P and UDP – glucose. Tps2p dephosphorylates trehalose-6P thus forming trehalose. Tps3p and Tsl1p are believed to be stabilizing units of the trehalose synthase complex. (Reinders *et al.*, 1997; Bell *et al.*, 1998).

TPS1, TPS2, TPS3 and *TSL1* all have several (6, 5, 2 and 4 respectively) upstream STRE sequence elements. Evidence for Msn2/4p dependent induction of those genes has been demonstrated in protein and mRNA level. In both cases their expression correlated with Msn2/4 induction pattern (Boy-Marcotte *et al.*, 1998, Winderickx *et al.*, 1996).

ADPG (adenosine diphosphoglucose) dependent pathway has been suggested as alternative pathway of trehalose synthesis observed in presence of maltose in *tps1* mutants (Ferreira *et al.*, 1997). However trehalose accumulation in tps1 mutants revealed to be trehalose transport occurring when cells are grown in rich broth (Plourde-Owobi *et al.*, 1999).

Yeasts have three enzymes responsible for trehalose breakdown: acid trehalase (Ath1p) and neutral trehalases (Nth1/2p), as illustrated in fig. 1.3.2.

Acid trehalase has maximal activity at a pH 4.5 and it is located in vacuoles. (Destruelle *et al.*, 1995). Main function of Ath1p is extracellular trehalose breakdown, *ath1*

strain is unable to grow on trehalose as sole carbon source (Nwaka *et al.*, 1996). *ath1* deletion does not affect intracellular trehalose concentration (Nwaka *et al.,* 1995a).

Neutral trehalase 1 (Nth1p) is the enzyme that degrades intracellular trehalose (Nwaka et al., 1995b). *NTH1* has three upstream STRE sequences therefore it is a target of the stress response transcription factors Msn2p and Msn4p (Martinez-Pastor *et al.*, 1996, Zahringer *et al.,* 2000). Active form of Nth1p enzyme is phosphorylated, (App and Holzer, 1989), PKA phosphorylates Nth1p and thus activates trehalose breakdown as a response to nutrient availability, besides, Nth1p associated proteins Bmh1p and Bmh2p are necessary to gain maximum trehalase activity. Phosphatase, mRNA decapping enzyme, Dcs1p (YLR270W) dephosphorylates Nth1p and thus deactivates it – as a result, trehalose accumulates (Schepers *et al.*, 2012, Malys *et al.*, 2004).

The neutral trehalase 2 (Nth2p) does not show any significant trehalase activity, however its amino acid sequence is 77 % similar to Nth1p (Wolfe and Lohan 1994). The main role of Nth2p is still unclear; it has been suggested to play a role in stress response (Nwaka *et. al* 1995).

Various models have been proposed for trehalose accumulation in the cells, which include trehalose synthesis and breakdown. Since trehalose is universal stress metabolite, various external signals can induce its breakdown through stress - nutrient response pathways: cAMP dependent PKA pathway, (Thevelein and Winde 1999), cAMP independent PKA activation (Crauwels *et al.*, 1997), TOR dependent transcription regulation by Msn2/4p (Martinez – Pastor *et al.*, 1996, Boy – Marcotte *et al*., 1998, Zahringer *et al.*, 2000).

1.3.3. Trehalose supports desiccation stress tolerance in S. cerevisiae cells

Trehalose is abundant in all branches of life: bacteria, fungi, plants and animals. It has been extracted in comparatively large amounts from organisms or their parts capable of anhydrobiosis (spores, conidia, cysts, larvae, pupae, lichens), reviewed in Elbein, 1974. Presence of large amount of dissacharides (trehalose or sucrose) in anhydrobiotic rotifers, nematodes, arthropods, plants in dry state have led to assumption on dissahcarides as direct cause of organism desiccation tolerance (Wiemken, 1990, Elbein, 1974, Eleutherio *et al.*, 2014).

Extensive research has been done to elucidate trehalose role in desiccation tolerance. First, the enhancement of desiccation tolerance by trehalose is believed to be related with its

ability to form hydrogen bounds with lipid hydrophilic parts thus lowering their phase transition temperature, see fig. 1.3.3. for details (Crowe et al., 1984).

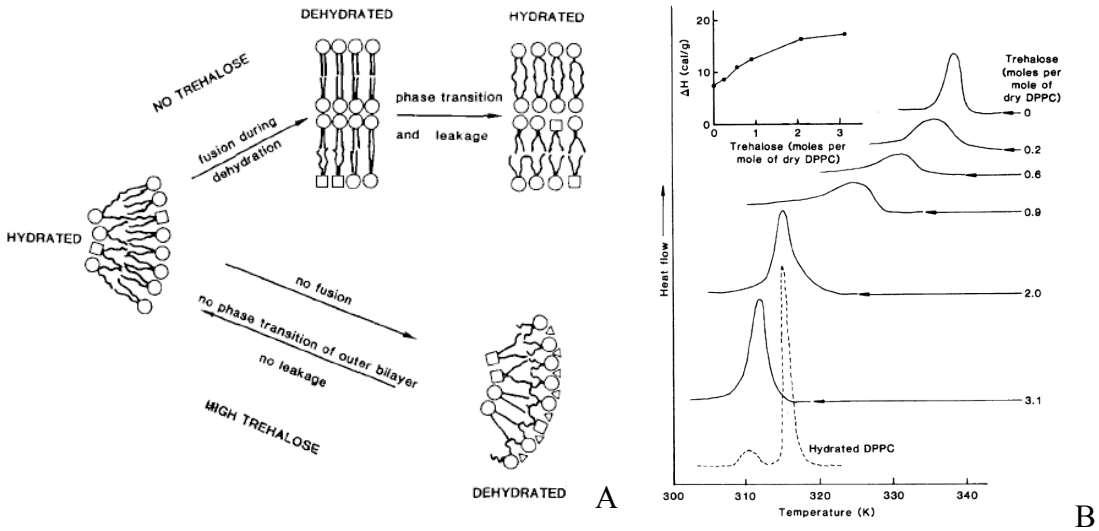


Fig. 1.3.3. A. **Trehalose – lipid liquid crystalline to gel formation model in the dry state**. Adopted from Crowe *et al.*, 1997.
B. **Differential scanning calorimetric traces of dry, hydrated dipalmotoylphosphatidylcholine (DPPC) and in the presence of trehalose**. Adopted from Crowe, 1984.

Phospholipid- trehalose interaction via hydrogen bonding has been confirmed by NMR (Carolyn *et al.*, 1986) and molecular modeling studies (Golovina *et al.*, 2010) and *in vivo* yeast experiments (Leslie, 1994). The hydratation water replacement hypothesis (demonstrated in fig. 1.3.3.A) is dominant model how trehalose acts in preserving molecular structure of membranes intact in the absence of water (reviewed by Crowe *et al.,* 1997). To guarantee full membrane protection in dehydrated state, trehalose has to be present at both sides of membrane (Eleutherio *et al.*, 1993). Additionally, trehalose has exhibited protein antiaggregation effect (Singer and Lindquist, 1998). Besides, some authors have attributed antioxidative activity to trehalose and this has been demonstrated *in vitro* and *in vivo* (Herdeiro *et al.*, 2006, Oku *et al.*, 2003). In practice, trehalose accumulation has coincided with many instances of yeast desiccation and subsequent enhancement of survival (Gadd *et al.*, 1987, reviewed also in Crowe *et al.*, 1997). However, there are many anhydrobiotes, including trehalose synthesis deficient strains of *S. cerevisiae*, capable of comparatively high desiccation tolerance in absence of trehalose (Ratnakumar and Tunnacliffe, 2006). Recently, trehalose is perceived as heat shock and nutrition dependent metabolite rather than desiccation specific protectant (Eleutherio *et al.*, 2014). Trehalose seems to enhance desiccation tolerance of the yeast, however, it is not necessary prerequisite of yeast survival

in desiccated state (Rapoport *et al.*, 1988, Krallish *et al.*, 1997, Ratnakumar and Tunnacliffe 2006, Rozenfelde and Rapoport, 2014).

2.3.4. Oxidative stress during S. cerevisiae desiccated state

Reactive oxygen species (ROS) are produced in every cell in the presence of oxygen. It is estimated, that 1-5 % of oxygen in mitochondria is reduced incompletely and can appear in the cell in one or other form of ROS. Hydrogen peroxide (H_2O_2), the hydroxyl radical (OH•), and the superoxide anion ($O_2•^-$) are active oxygen forms capable to damage yeast cell. ROS attack and change almost all groups of biological macromolecules: lipids, proteins and nucleic acids. ROS leads to lipid peroxidation, protein carbonylatian, degradation and nucleic acid strand brakes (Kohen and Nyska, 2002). Since ROS can seriously harm cells activity (proteins), integrity (plasma membranes) and inheritance (nucleic acids), there is antioxidant system in place. Yeast cell contains enzymatic and non-enzymatic antioxidation systems capable to neutralize ROS.

Enzymatic antioxidant system consists of peroxidases, catalases and superoxide dismutases. Interestingly enough, many elements of antioxidant enzyme systems are conserved among prokaryotes and eukaryotes, including yeasts and human.

Glutathione and thioredoxin peroxidases reduce alkyl hydroperoxides:
ROOR' + electron donor (2 e^-) + 2H^+ → ROH + R'OH (Culotta, 2001, Hirt *et al.*, 2002).
Metal (Mn and Cu-Zn) containing superoxide dismutases (MnSOD and CuZnSOD) scavenge O_2^-. *SOD2* encodes yeast mitochondrial matrix superoxide dismutase containing manganese. *SOD1* encodes yeast cytosolic superoxide dismutase containing Cu-Zn (O'Brien, 2004). Heme- and manganese-containing catalases scavenge H_2O_2 via following reaction:
2 H_2O_2 → 2 H_2O + O_2.

S. cerevisiae contain two catalases: peroxisomal Cta1p and cytosolic, Ctt1p. Places of ROS generation and "enzymatic actors" of their neutralization are depicted in fig. 1.3.4.

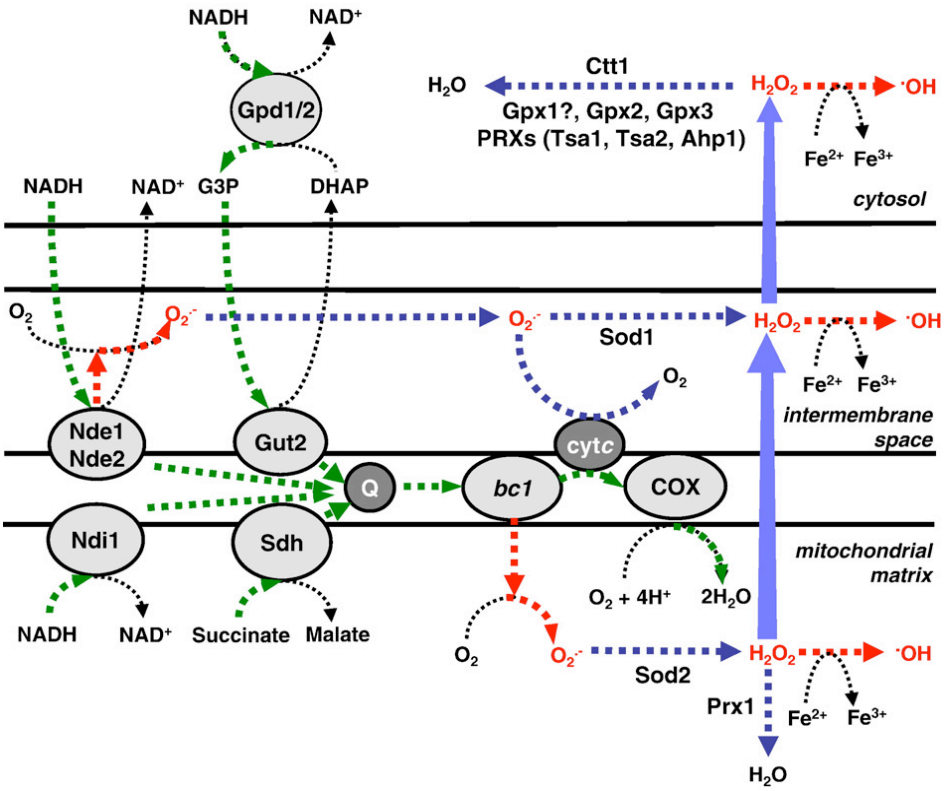


Fig. 1.3.4. ROS production and neutralisation in *S. cerevisiae*.
ROS forms predominantely in respiratory chain. The main antioxidant enzymes involved are: bc1, cytochrome bc1 complex; cyt c, cytochrome c; cox, cytochrome c oxidase; Ctt1, cytosolic catalase T; Gpd1/2, cytosolic NADH-linked glycerol-3-phosphate dehydrogenase; Gpx, glutathione peroxidase; Gut2, membrane-bound glycerol-3-phosphate:ubiquinone oxidoreductase; Nde1/2, external NADH dehydrogenase; Ndi1,internal NADH dehydrogenase; Prx, peroxiredoxin; Q, ubiquinone; Sdh, $FADH_2$-linked succinate dehydrogenase complex; and Sod1/2, superoxide dismutase. Adopted from Herrero *et al*., 2008.

Antioxidant molecules – reduced glutathione (tripeptide γ-glutamylcysteinylglycine) and thioredoxins (small, sulphydril rich, 12 kD proteins) work as cytoplasmic ROS scavengers in *S. cerevisiae* cells. Each of these peptides contains thiol moiety, capable to reduce ROS. Peptide, in turn, gets oxidised. Specific, NADPH dependent glutathione and glutaredoxin peroxidases regenerate oxidized glutathione or thioredoxin. Although, not typically considered an antioxidant, this pyridine dinucleotide can have a major impact on oxidative stress resistance, as it provides the reducing equivalents needed to regenerate GSH and reduced thioredoxin (Herrero *et al.*, 2008).

Glutathione metabolism including synthesis, nonenzymatic ROS scavenging and GLR1 glutathione reductase dependent regeneration of the reduced glutathione supply is depicted in fig. 1.3.5.

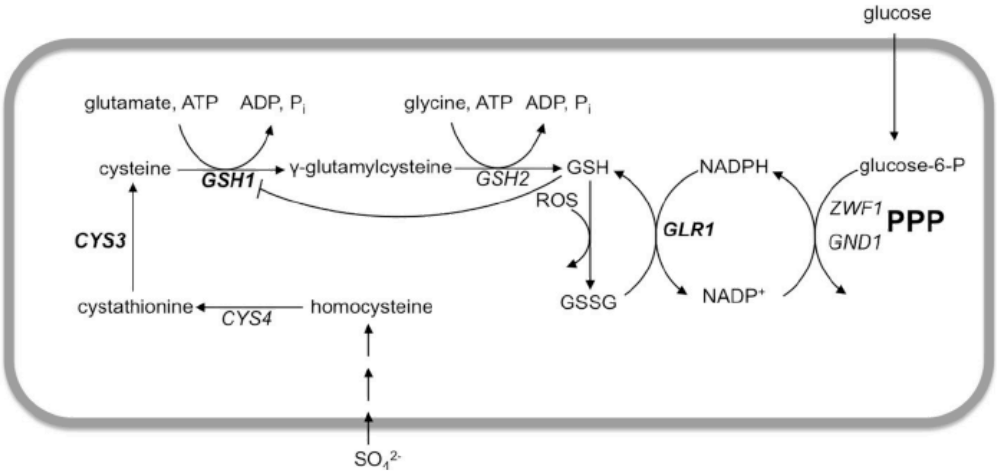


Fig. 1.3.5. Simplified illustration on glutathione metabolism in *S. cerevisiae*.
GSH1- gamma glutamylcysteine synthetase, GLR1- glutathione reductase, ZWF1 – glucose-6 phosphate dehydrogenase, GND1 6-phosphogluconate dehydrogenase, CYS4 cystathionine beta-synthase, CYS3 cystathionine gamma-lyase, GSH – glutathione reduced form, GSSG – glutathione oxidized form. ROS – reactive oxidized species. Adopted from Ask *et al.*, 2013.

Oxidative stress can be defined as overflow of ROS production what exceeds antioxidative capacity of the cell. Oxidative stress happens due to weakening of cell's antioxidative system or notable increase of ROS production (Culotta, 2001).

When cells are desiccated under fully aerobic conditions, their antioxidative capacity is attenuated, meanwhile ROS production increases (endogenous and air born ROS during storage). Since no solute is available, no enzyme dependent ROS scavenging can occur in dry state (reviewed in Franca *et al.*, 2007).

Alterations of desiccation tolerance if cell's antioxidative system is challenged have been observed. Superoxide dismutases Sod1/2p are necessary to sustain cell viability in *S. cerevisiae* cells after freezeing-thawing cycle (Park et al., 1998). GSH1/2 deletions have also been identified to lower desiccation tolerance, albeit this effect can be alleviated by external glutathione or its chemical analog supplement (Espindola *et al.*, 2003). Yeast cell's antioxidant system is perceived as target to improve its desiccation tolerance.

1.3.5. Specific proteins as chaperons during S. cerevisiae desiccated state (Hsp and LEA)

Term "LEA like proteins" comes after discovery of specific group of proteins in angiosperm plant seeds becoming abundant during seed ripening. At that point, these proteins were termed Late Embryogenesis Abundant (LEA) proteins. Two important features are characteristic to these proteins: they accumulate at very late stage of seed development and they contain domains particularly rich in hydrophilic amino acids (Dure *et al.*, 1989).

Later, it turned out, that expression of these proteins is not restricted only to seeds, but can occur also in other plant tissues during water stress. Also similar proteins (at least with respect to their amino acid sequence) are found in other organisms (bacteria, fungi, animals). Therefore, to broaden the term "LEA" which initially was applied to seeds of higher plants, a term "LEA-like proteins" was invented. Typical features of LEA-like proteins are: high hydrophilic nature (20 times more bound water than for other proteins of the same size), lack of strict secondary or tertiary structure ("natively unfolded") in water solutions (Tunnacliffe, Wise, 2007).

In *S. cerevisiae*, Hsp12p is characterized to belong to LEA like proteins. It is expressed in stationary phase or during growth on ethanol. It does not have strict 3D structure when purified alone in water solution, albeit it changes rapidly to alpha helical structures when incubated together with membranes. These structural features are linked with its functions: strengthen membrane integrity during heat, desiccation or ethanol stress (Sales, 2000, Welker et al., 2010), as well as it is required in dietary induced life span extension (Herbert, 2012). Hsp12p prevents protein aggregation during desiccation; this effect is enchanced by trehalose (Goyal, 2005). Absence of Hsp12 can be functionally complemented by trehalose (Shamrock and Lindsey, 2008).

Attempts to apply LEA-like proteins to improve otherwise desiccation non-stable animal cell tolerance were made: p26 gene transfection from anhydrobiote brine shrimp, *Artemia franciscana*, significantly improved human embryonic kidney (293H) cell line survival during air-drying (Ma *et al*., 2005).

1.3.6. Genome wide investigations of S. cereivisiae desiccation tolerance

Recent advancments in eukaryotic (including *S. cerevisiae*) and prokaryotic genetics have created great tools for physiology research in genomic or transcriptomic scale. These tools include: rapid genome sequencing, automated annotation, genome wide transcription analyses and genome wide *knock-out* strain collections (Boyle and Gill, 2012).

To decipher important genetic factors affecting desiccation tolerance, two approaches are used: to look for specific, desiccation dependent transcription response, other – to search for genes specifically affecting desiccation tolerance.

With regards to desiccation induced transcriptome response – several attempts have been made to identify desiccation associated gene transcription. Depending to experimental setup – desiccation induced transcriptome patterns are different. Nakamura *et al.*, 2008, identified groups of genes upregulated of commercially pressed yeast during gradual

desiccation: protein folding (chaperones were upregulated within first hours of desiccation, then downregulated) and fatty acid metabolism genes (activated throughout desiccation). Singh *et al.*, 2005, investigated global gene transcription pattern during *S. cerevisiae* S288c strain suspension gradual desiccation and found it similar to stationary growth phase cells. Other groups have identified, that genes of nitrogen derepression, amino acid synthesis and transport are induced during yeast cell desiccation (Ratnakumar *et al.*, 2010). Stress related transcription factors Msn2/4 appeared to be involved, but not necessary for desiccation tolerance. On the other hand, deletions of elements of PKA and TOR kinase signaling pathways (*rim15*, *bcy1*) revealed to dramatically lower desiccation survival (Ratnakumar *et al.*, 2010).

S. cerevisiae strain S288c non-essential gene deletion library (Brachmann *et al*., 1998) have helped to find out important genetic elements of desiccation tolerance. Rodriguez-Porrata with colleagues tried to identify critical gene necesarry to rescue "desiccation vulnerable phenotype". They identified, that overexpression of SIP18 (apoptosis repressor), increases desiccation tolerance of parent strain two folds (Rodriguez-Porrata, 2011).

Calahan *et al*., 2011 published wide survey where each *S. cerevisiae* nonessential gene deletion strain's desiccation tolerance was assessed. Team set a treshold for their ambitious aim – a strain was assessed as desiccation intolerant, if it's survival droped by more than 10 times in comparison to parent strain. By this method authors focused just on small portion of gene deletions having most prominent effects on desiccation tolerance. Group of genes related to mitochondria metabolism revealed to be significant for cell desiccation tolerance. Strangely, no *hog1*, *msn2/4*, trehalose synthesis deletions *tps1,2,3*, no *hsp12* and *sip18* decreased survival of yeast 10 or more times after desiccation. Instead, mitochondrial metabolism revealed to be critical for desiccation tolerance. Petite mutants revealed to be extremely desiccation vulnerable, similarly to wild type when cultivated with respiratory inhibitor myxothiasol. Mitochondrial dependent desicction intolerance could be complemented by several random mutations in: mediator repressor and thioredoxin metabolism (Calahan *et al.*, 2011).

1.3.7. Nutrient signaling effects on **S. cereivisiae** *desiccation tolerance*

Many publications on yeast desiccation tolerance have noted, that stationary phase cells are far more desiccation tolerant, than exponentially growing cells. Besides, sudden media shift from N-rich to N-poor media is used for dry baker's yeast production already for

some time (reviewed in Beker and Rapoport, 1987). These observations has been linked to decreased growth speed, accumulation of "specific tolerance factors" or specific nutrient starvation impact on survval after desiccation. When resolving these aspects one by one – it turned out, that *S. cerevisiae* does not produce "specific desiccation tolerance factors", neither growth speed is critical for desiccation tolerance. Starvation for particular nutrient turned out to be the case. This observation linked desiccation tolerance to TOR signaling and cell's nutritional status (Welsh *et al*., 2013).

Recent research on yeast desiccation tolerance mechanisms has revealed nitrogen signaling pathway TOR as important player to ensure desiccation tolerance. Additionally experiments on specific nutrient signaling pathways (TOR and PKA) revealed negative effector Sch9 protein kinase element to ensure desiccation tolerance. When deleting Sch9 – Msn2/4 gets derepressed and "full scale stress resistance is induced", see fig. 1.3.6. for illustration (Welch *et al.*, 2013).

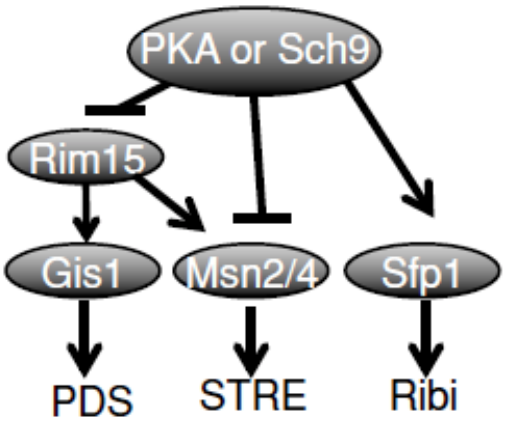


Fig 1.3.6. Common effectors of PKA and Sch9p having effect on desiccation tolerance.
PKA or TOR (through Sch9) negatively affects STRE responsive transcription factors Msn2/4 and protein kinase Rim15. Finally, in the case of halt of PKA or TOR activity – ribosome synthesis decreses (Ribi), STRE responsive gene transcription (STRE) and postdiauxic shift element (PDS) genes get activated. Adopted from Welch *et al*., 2013.

1.3.8. Desiccation – applied aspects in food technology and medicine

Historically desiccation is one of the simplest, yet most effective methods for food preservation. It has been and still is widely exploited for fruit, meat and seed/grain preservation. Interestingly, approximately 50 % of food in NASA SpaceShuttle program is transported in dried form. Predried food preparations are suspected to form large part of food supply also for future space (like Mars) missions (reviewed in Perchonok *et al.*, 2012).

Raw food might contain more than 90% of water thus making it vulnerable to temperature, oxidation and bacterial spoilage. Due to low water potential, dry food has several advantages: it is stable over long period of time and temperature, it does not spoil (rot

or ferment), it is comparatively easy to transport (since large portion of water is evaporated, products are light and takes less space). Thus dried food products are popular up till now. Bed drying, spray drying, air drying, vacuum and freeze-drying are the most often used food drying technologies (see review in Sachin and Jangam, 2011).

Besides traditional raw food drying, production of microbial starting cultures is industry where desiccation is being extensively applied. Baking and brewing industries are two examples exploiting dry microbial (yeast) starting cultures: active dry yeast, wine and brewer's yeast respectively (Beker and Rapoport 1987, Bauer and Pretorius 2000).

Besides dry yeast, probiotics industry is growing segment of modern food market. Microbial starters are essential elements of probiotic food production. Nowdays freeze-drying is standard method for production of industrial starting cultures and medical probiotic drug production (Santivarangkna *et al*., 2011).

Probably due to successive history in area of food processing, applications of desiccation is of growing interest also in medicine and pharmacy. Researchers are optimistic in their prognosis on desiccated protein, cell and tissue usage in daily medicine practice; however, safe and widely accepted desiccation protocols are just emerging. There are some examples of effective knowledge transfer from fundamental microbial desiccation physiology research to medicine applications. Patent on blood platelet preservation is one of them. Trehalose addition is found to be effective method to preserve freeze-dried blood platelets and not loosing their blood clotting activity (Wolkers *et al.*, 2001a and 2001b). Other attempts for trehalose applications as cryopreservation or of other human cell types have been published: embryonic stem cells (He*,* 2011), mesenhymal stem cells (Jamil *et al.,* 2005). In general, "anhydrobiotic engineering" of human cells have proven to be of limited success, yet potential field of application of principles discovered in model organisms.

Vaccine preparation and storage is another area for desiccated material usage. "Cold chain" is term describing transportation of termo-sensitive material from producer to consumer. Transportation of pharmaceutical materials are often subject of "cold chain". Biopharmaceutical drugs (proteins, vaccines) are typical examples which are transported by applying cold chan. However, there are many occasions where "cold chain" can broke (hot climate, lack of nearby electricity, long transportation episodes in hot climate areas). Desiccated vaccines are perceived as alternative form of traditional pharmaceutical, which can not only withstand high temperatures, but also change immunization practices: away from spike and towards to inhale (Lin *et al*., 2011, Savage, 2014).

As demonstrated by literature data - there are many findings and interpretations on mechanisms of *S. cerevisiae* desiccation tolerance. This work was done to clarify if *S. cerevisiae* culture nutritional status before desiccation determines desiccation outcome. Since *S. cerevisiae* lacks specific "desiccation sensors", it should relay on other intracellular or extracellular signals to "prepare" itself for harsh environmental conditions, like desiccation. We choose to characterise *S. cerevisiae* desiccation tolerance during postdiauxic growth thus mimicking "natural habitats" after sugar depletion. We explored desiccation tolerance of yeasts in early and late stage postdiauxie.

Also we aimed to engineer desiccation tolerance via improving glutathione metabolism. We also investigated how desiccation affects brewer's yeast β-D-glucans in terms of their immunological activity.

2. Materials and methods

2.1. Yeast strains

All strains used in current work are listed in table 3.1.

Table 2.1. Yeast strains used in this work

Name in this study	Name in literature	genotype	source
SC14	*Saccharomyces cerevisiae 14*	Putative diploid, nonsporulating	Institute of Microbiology and biotechnology, University of Latvia
BY4741	BY4741	*MAT**a**; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	EUROSCARF
tps1	Y00775	*BY4741 tps1::kanMX4*	EUROSCARF
CEN.PK prototroph	CEN.PK2 prototroph	*CEN.PK2-1C MAT**a***	Dr. Peter Richard, VTT Biotechnology, Espoo
CEN.PK ADE8	CEN.PK2	*CEN.PK2-1C MAT**a**; ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8C; SUC2*	
CEN.PK ade8		*CEN.PK2 ade8Δ0*	This study*
R0		*CEN.PK fcy1Δ0 ade8Δ0*	This study*
R1		*CEN.PK fcy1::DAK1*	This study*
R2		*CEN.PK2 lys2::GLD2*	This study*
R3		*CEN.PK2 lys2::GLD2 fcy1::DAK1*	This study*
R4		*CEN.PK2 ade8::GLD2 fcy1::DAK1*	This study*
R5		*CEN.PK2 ade8::GLD2*	This study*
W303 ade2	W303A	*MAT**a**; ura3-1; trp1Δ 2; leu2-3,112; his3-11,15; ade2-1; can1-100*	Dr. Peter Richard, VTT Biotechnology, Espoo
W303 prototroph	W303A 2832-1B	*W303A MAT**a*** prototroph	Dr. Fred R. Cross (The Rockefeller University, New York)
W303 ADE2	AKY 489	*W303A ade2-1::ADE2*	Dr. Arnold Kristjuhan, (Institute of Molecular and Cell biology, Tartu)
Brewer's yeast		Bottom fermenting brewer's yeast, Stain a.s, Bratislava brewery	Dr. Smogrovicova (Slovak University of Technology, Bratislava)

* gene disruptions and integration was done by homologous recombination using a *URA* cassette and screening for ura- mutants on 5-FOA plates as described in Sadowski *et al*., 2007.

2.2. Cultivation media and cultivation

YPD, or yeast extract peptone dextrose (per litre 10 g of Yeast extract (Biolife), 20 g of peptone (Biolife), 20 g of dextrose (Sigma-Aldrich))

SD or synthetic dextrose media (per litre 1.7 g of Yeast Nitrogen Base w/o amino acids and ammonium sulphate (Becton, Dickinson and Company), 5 g, of $(NH_4)_2SO_4$, 20 g L-1 of dextrose) supplemented with leucine (260 mg/L), of tryptophan (80 mg/L), of uracil (100 mg/ l), histidine (100 mg/l) and adenine (100 mg /l), (Sigma-Aldrich) (concentrations chosen after Pronk, 2002).

For auxotrophic starvation experiments SD media with either adenine, leucine, tryptophan or histidine omitted was used.

Yeasts were cultivated in shake flasks or test tubes at 180 rpm in 30°C with broth volume not exceeding 1/5 of the flask or 1/10 of test tube volume.

2.3. Yeast desiccation tolerance (viability) tests

Yeast biomass was harvested by centrifugation, uniformly pressed through 1 mm sieve and left to desiccate in +30°C, for approx. 18 h till the moisture content reached 8-12%.

Alternative desiccation microassay was developed similar to Calahan et al., 2011: 1 ml of OD600=1 yeast biomass was harvested by centrifugation, washed, pelleted and left to desiccate in +30°C in desiccator for 6-10 h. Moisture content reached approx 10 %.
Yeast viability after dehydration was determined either by the primuline staining or serial dilution spot test.

Primuline staining

Yeast cell suspension after rehydration was stained with primuline (1:30 000) and evaluated microscopically. Cell viability was expressed as proportion in percents of live versus total cell count (Rapoport and Meisel, 1985).

Spot test

Alternative assessment of desiccation tolerance was done by estimating colony forming units (CFU) per ml, before and after dehydration. 1 ml of culture at OD_{600} = 1 was washed with distilled water twice, diluted serially, and spotted on YPD plate. Desccated pellet was rehydrated with distilled water for 10 min in room temperature. The suspension of rehydrated cells was serially diluted and spotted on YPD plates. The viability was calculated

by dividing the number of CFU per ml before and after desiccation, as described in Calahan *et al*., 2011.

2.4. Cell extract preparation

For metabolite measurements

For tiobarbituric acid, glutathione and trehalose measurements, cells were resuspended in 600 ml of the 20 mM Tris HCl buffer, containing 10% trichloroacetic acid and 1.5 g of glass beads (400 - 600 um diameter). The yeast samples were disrupted by three cycles of 1 min disintegration in Retsch MM 301 device. Samples were left on ice for 1 min in between disintegration cycles. Cell lysates were centrifuged for 10 minutes 20 000 rpm on table top centrifuge Eppendorf 5417R.

For enzymatic reactions

For enzymatic reactions (spectrophotometric or zymograms), cells were resuspended in sodium phosphate buffer (0.05 M, pH 7) supplemented with protease inhibitor cocktail (EDTA free, Roche), containing 1.5 g of glass beads (400 - 600 um diameter). The yeast samples were disrupted by vortex for 20 min at +4°C.

Cell lysates were centrifuged for 25 minutes 20 000 rpm on table top centrifuge Eppendorf 5417R, at 0°C.

2.5. Reduced glutathione (GSH) quantification

o-phtalaldehyde (OPA) fluorescence method was used for GSH quantification as described by Senft *et al.,* 2000.

To determine GSH unspecific (-CN, $-SO_3$ groups also can form complexes with OPA). Complex formation with OPA – a set of reactions with N-ethylenmaleide (NEM) capable of blocking GSH-OPA complex formation, were included into assay.

Pipetting scheme is given in table 3.2. To prepare reaction mix, pipetting was done according to table 3.2 from left to right (first sample was introduced into test tube, then TCA-RQB etc.).

Table 2.2. Pipetting scheme for GSH quantification

Cuvette	Sample	TCA-RQB*	NEM 150 nm	1M KP	0,1M KP	OPA 0.7 mg/ml
A	0-10 ul	Up till 200 ul	7 ul	325 ul	1.35 ml	200 ul
B	0-10 ul	Up till 200 ul	-	325 ul	1.35 ml	200 ul

* Redox quenching buffer with TCA (RQB-TCA, 20 mM, HCl, 10 mM ascorbic acid, 5% TCA).

All ingredients are mixed and incubated in dark at room temperature for 30 min. OPA-GSH complexes were measured by excitation at 365 and emission at 430 nm by Yvon-Horriba FluoroMax3 device. Results of A sample (GSH unspecific OPA complexes) were subtracted from B sample. Results are expressed as uM/ g DW.

2.6. Lipid peroxidation assay

For lipid peroxidation, thiobarbituric acid reactive species (TBARS) spectrofluorometric detection similar to Yagi *et al.,* 1998 was used. The cell extracts were mixed with 0.1 ml of 0.1 M EDTA and 0.6 ml 1 % (w/v) thiobarbituric acid in 0.05M NaOH. The reaction mixture was incubated in a boiling water bath for 15 min, after cooling, the excitation was set to 520 and emission to 553 nm. Fluorescence was measured by Yvon-Horriba FluoroMax3 device. Malonildialdehyde dimer (Sigma Aldrich) was used as standard.

2.7. Trehalose and cell polysaccharide quantification

Cell polysaccharide (including trehalose) content was determined by anthrone assay (Trevelyan and Harrison, 1956). TCA cell extract (diluted with water when necessary) was mixed with anthrone (2 g/ l in 75% H_2SO_4) in a 1:6 ratio. The mixture was heated at 100 °C for 10 min, and absorbance at 625 nm was measured.

Fractional cell polysaccharide purification for quantitative assays was done as described earlier (Stewart, 1975). Each fraction's glucose content was determined by anthrone assay as described above.

2.8. Yeast transformation

Yeast transformation was done by lithium acetate - PEG method as described by Gietz and Woods, 2002.

2.9. Zymogram staining

Native PAGE (10 % acrylamide) in glycine buffer (pH 6.8, 0.1 M) was run. The gel was dyed in a zymogram staining solution containing 200 mM Tris-HCl, pH 9.0, 0.1 M substrate, 0.25 mM iodnitrotetrasolim (INT) chloride (Sigma Aldrich), 0.06 mM phenazine methosulfate (PMS), and 1.5 mM oxidised cofactors (NAD or NADP). 50 ug of protein were applied for each lane.

2.10. HPLC analyses

Glucose, ethanol, acetate, pyruvate, succinate and glycerol content were measured simultaneously by Agilent 1100 HPLC system. Shodex Asahipak SH1011 column was used to fraction all measured metabolites (glucose, glycerol, ethanol, pyruvate acetate and succinate). Glucose, glycerol, acetate and ethanol were analysed by refraction index detector (RI detector RID G1362A), but pyruvate and succinate were detected by diode matric detector set to 210 nm. The flow of the mobile phase (0.01 N H_2SO_4) was 0.6 ml min^{-1}, the sample injection volume was 5 μL.

2.11. Purification of yeast β-glucans and qualitative analyses

Water-insoluble (1-3)-β-d-glucan was extracted from spent brewer's yeast cells by autolysis- alkaline extraction. Yeast biomass was suspended in distilled water and subjected to autolysis at 50° C during 24 h. Sediment was washed twice with water; resuspended in 3 % NaOH 1:5 (w/vol, initial biomass g: vol NaOH ml ratio) and incubated for 4 h at 55°C. Then additional volume of distilled water was added and suspension was incubated at room temperature overnight. Sediment was washed twice with distilled water, resuspended in 1:5 (initial biomass : vol NaOH ml ratio) in 3 % NaOH and heated during 2 h at 100°C, additional volume of distilled water was added and mixture was incubated at room temperature overnight. Sediment was washed several times; extraction with 1N HCl at room temperature for 2 h was performed 3 times. Residual sediment (pure β-glucans) was washed until neutral pH reaction was reached. Purified β-glucans were freeze-dried and stored in room temperature.

2.12. NMR spectroscopy

The ^{13}C-NMR spectra were recorded at 298 K in D_2O solutions by Bruker AM-300 instrument, as described previously by Kogan and Aldolfi, 1988.

2.13. Congo red assay

Measurements of the absorption maximum shift by complex formation between purified β-glucans and Congo red were performed as following: Congo red dye was used at a final concentration of 60 uM, NaOH concentration was varied from 0.025 M to 0.5 M. Absorption spectra between 460 and 530 nm in 1 nm increments were recorded on Tecan Infinite 200 M Pro microplate reader.

2.14. β-glucan O-carboxymethylation

Derivatization of the (1-3)-β-d-glucan was performed using the procedure described previously (Williams *et al*., 1991). Briefly, 3 g of the glucan was suspended in a 40 ml of 3 % NaOH in isopropanol. The suspension was vigorously stirred at 4°C for 1 h. 4.7 g Na-monochloroacetic acid was added immediately and the mixture was stirred at 70°C for 2 h. The excess of NaOH was neutralized, remaining cream-like substance was dialysed against water to remove salts. The dialised carboximethylated β-glucan suspensions were lyophilized. The substitution degree of resulting β-glucan preparations were 0.8 as confirmed by potentiometric titration with 0.05 M KOH.

2.15. Preparation of murine macrophages and TNF- α induction assay

ICR mice aged 8 to 12 weeks were obtained from the animal farm of the Institute of Experimental Pharmacology, Slovak Academy of Sciences (Dobrá Voda, Slovakia).
All animal experiments were conducted according to the ethical guidelines issued by the Institute of Virology, Slovak Academy of Sciences.

Peritoneal ICR mice macrophages were elicited by intraperitoneal injection of thioglycolate broth (Difco). After 5 days, the mice were sacrificed and peritoneal macrophages were collected by peritoneal lavage using Hanks balanced salt solution.

TNF-α induction assay

Cells were washed by centrifugation and 1×10^6 cells were resuspended in 0.5 ml RPMI-1640 with L-glutamine (PAA Laboratories GmbH, Austria), supplemented with 10 % heat-inactivated fetal bovine serum (Gibco) and placed to each well of 24 wells culture microplates (Sarstedt) for 2 h at 37°C in a humidified atmosphere of 5% CO_2. Non-adherent cells were washed away. 0.5 ml of complete RPMI-1640 medium, containing appropriate

stimulant (pleuran or spent brewers yeast carboxymethylated beta glucans), was added to each well. After cultivation, the supernatants were collected and stored at –40°C.

The level of TNF-α was determined in cell culture supernatants collected after 3 an 6 h of cultivation by ELISA kit (TNF-α Instant ELISA, Bender MedSystems) according to the manufacturer's instructions. Mouse TNF- α was used as a standard.

2.16. Statistical treatment of data

All the represented values are means from biological triplicates. Error bars and variation depict standard errors. Two tailed, two-sample unequal variance Student's t-test was used to compare means of physiological parameters. P-values less than 0.05 were considered statistically significant.

Calculations of means, standard error and t-test were performed with MS Excel 2008 for Mac. vers. 12.2.9 .

3. Results

3.1. *SC14, BY4741* and *tps1* strain growth dynamics and extracellular metabolite analyses

To characterise yeast desiccation tolerance depending on nutritional status – we choose SC14, BY4741 and *tps1* strains. SC14 is prototroph, diploid strain, specifically selectioned for dry baker's yeast production (Beker and Rapoport, 1987). BY4741 and its trehalose synthesis mutant *tps1* are auxotrophic (*his, met, ura, leu*), haploid strains of S288c origin (Brachmann *et al*., 1998). To characterise these strain desiccation tolerance during postdiauxie – exact timing of diauxic shift and postdiauxie growth phase needed to be determined. We recorded strain growth dynamics and analysed extracellular metabolites at corresponding time points.

SC14, *BY4741* and *tps1* (*BY4741* derived trehalose synthase mutant, incapable to accumulate trehalose) strains were grown in YPD (yeast extract, peptone, glucose 2%) media; fig. 4.1.1 depict strain biomass growth dynamics over 54 h. Yeast growth was monitored spectrophotometrically as absorbance at 600 nm (Smits *et al.*, 2012). Yeast biomass concentration as gram of dry weight per liter was calculated using experimentally determined transformation coefficients, which are specific for each strain g/L/ OD600: 0.2798 (*CEN.PK2*), 0.2282 (*BY4741*), 0.346 (*SC14*). Theese transformation coefficients were determined gravimetrically from growing yeast cultures.

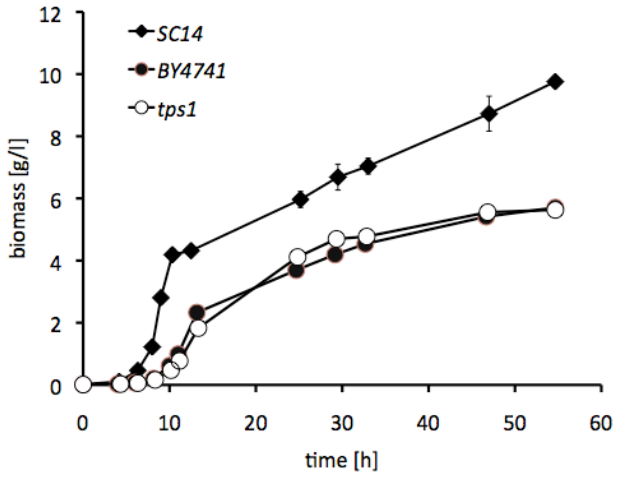


Fig 3.1.1. Growth dynamics of *SC14, BY4741* and *tps1* strains.
Biomass is expressed as grams of dry weight per litre (g DW / l). Growth curves are constructed as average of biological triplicates. Error bars represent standard error. In most cases error bars are smaller than graph markers.

In parallel to determination of yeast biomass dynamics over time, samples for extracellular metabolite analyses were collected. Acetate, succinate, pyruvate, glucose, glycerol and ethanol levels are plotted for each strain, refer to fig. 3.1.2 A-F.

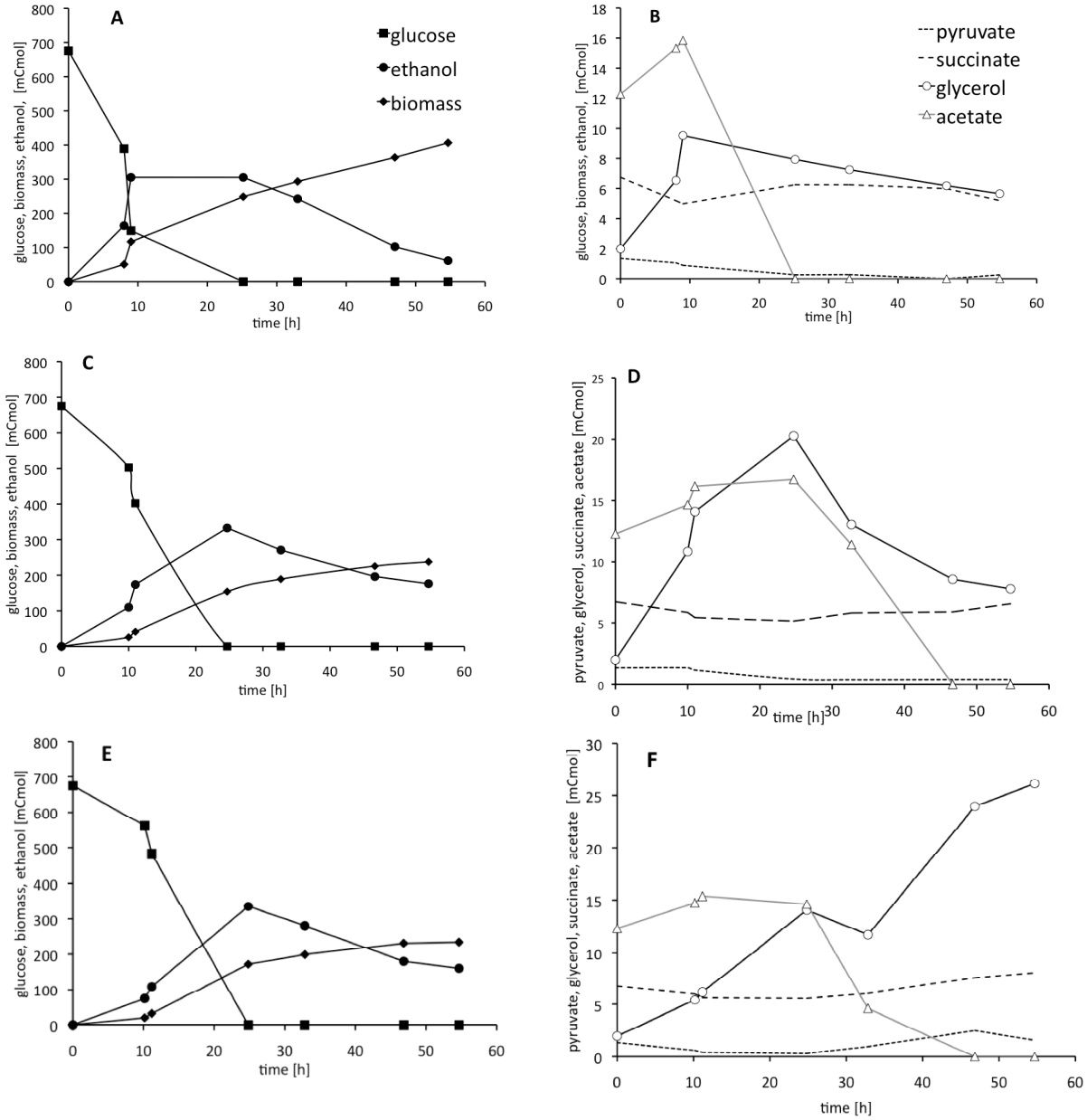


Fig 3.1.2. Extracellular metabolite dynamics during *SC14* (A, B), *BY4741* (C, D) and *tps1* (E, F) cultivation. Metabolite concetration were determined by HPLC analyses.
All metabolites are depicted as mili C-mols. Legend is similar for A, C and E graphs and F, D, B. Each data point is average of biological triplicates. Standard deviations did not exceeded 10 %. They are not included in figures for the sake of clarity. Detailed metabolite data are available at Annex I.

First rapid yeast biomass growth happens on expense of fermentable carbon source (glucose). This is exponential phase and it lasts till 10 h for *SC14* and approx. 12 h for *BY4741* and *tps1* (fig. 3.1.1). Then growth on fermentation products continues (ethanol acetate, glycerol), this is diauxic growth and it continues till the very end of experiment (54

h). Both biomass dynamics and extracellular metabolite results testifies this as diauxic rather than stationary phase. When glucose is depleted, ethanol and other extracellular metabolites are sequently consumed. No carbon substrate got depleted over 54 h of cultivation for none of strains (fig. 3.1.2 A-E); in the same time biomass growth does not ceases till the very end of cultivation. Stationary phase for these strains does not set in when cultivated in YPD media for 54 or more hours, instead, postdiauxic phase lasts from 10 or 12 till 54 h of cultivation or more.

3.2. Desiccation tolerance depending on cultivation time

Traditionally, *S. cerevisiae* desiccation tolerance has been explored in "stationary phase cells" (Borovikova *et al.,* 2014, Espindola *et al.,* 2003, Guzhova *et al,*, 2008). We aimed to test *S. cerevisiae* desiccation tolerance during postdiauxie. We determined, that for *SC14*, *BY4741* and *tps1* strains postdiauxic growth lasts from 12 till at least 48 h. This was testified by biomass dynamics and extracellular metabolite analyses: oxidative growth on ethanol lasted all over from 10 or 12 h till the 54 or more hours of cultivation (see fig. 3.1.2). We choose 24 and 48 h cultivation time points as representatives for "early" and "late" postdiauxic growth phases respectively.

We analyzed cells survival rate, trehalose, reduced glutathione and malonildialdehyde content. First set of experiments was done with *S. cerevisiae* strain *SC14,* traditional model for desiccation stress physiology investigations in LU MBI (Zikmanis *et al.,* 1982, Khroustalyova *et al.,* 2001, Trofimova *et al.,* 2010).

We checked if there are any differences in *SC14* strain desiccation tolerance in cells harvested at 24 h and 48 h of cultivation. Yeast biomass was air-dried for 16-18 h to reach residual moisture content of 8 – 12 %.

Desiccation tolerance was estimated by primuline fluorescence test (Rapoport and Meisel, 1985). Results are depicted in fig. 3.2.1.

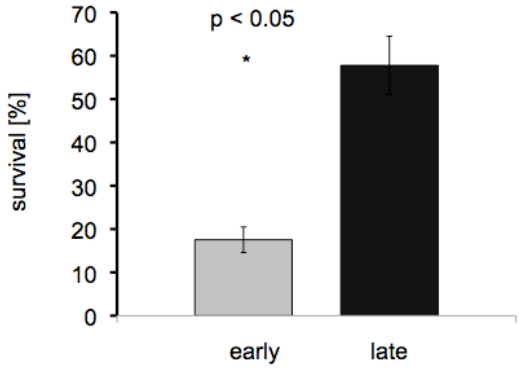


Fig. 3.2.1. *SC14* strain desiccation tolerance when harvested in early (early) and late (late) postdiauxie phase.
Mean values are calculated from biological triplicates, error bars depict standard deviations.
Star represent statistically significant difference between survival of different growth phases (t test, $p<0.05$)

Desiccation tolerance (survival %) of *SC14* strain when harvested in early or late postdiauxie phases were 18.5 ± 2 % and 55 ± 8 % respectively.

Trehalose has been often described as typical metabolite ensuring *S. cerevisiae* stress (including desiccation) tolerance (Gadd *et al.,* 1987, Francois and Parrou 2001, Herdeiro *et al.,* 2006). Besides, there are data on trehalose increase during desiccation process (Krallish *et al.*, 1997). So, we decided not only measure trehalose content in early and late postdiauxic yeast cells, but also before (fresh) and after (dry) desiccation. Results are depicted in figure 4.2.2.

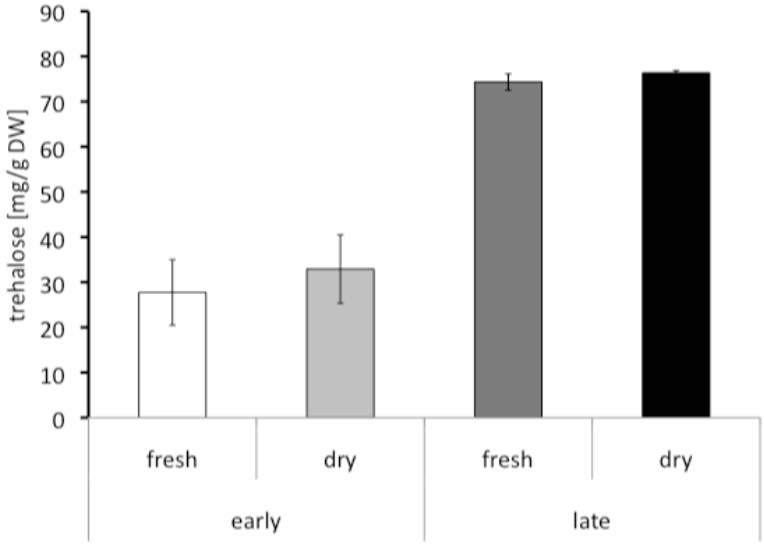


Fig. 3.2.2. *SC14* strain trehalose content [mg/ g DW] before (fresh) and after (dry) desiccation when harvested in early postdiauxie (early) and late postdiauxie (late) phases.
Mean values are calculated from biological triplicates, error bars represent standard deviations.

Early postdiauxie cells had trehalose content of 28 +/- 5 mg/ g DW before and 30 +/- 4 mg/g DW after desicication; whereas late postdiauxie phase cells contained 70 +/- 1 mg/g DW before and 72 +/- 1 mg/g DW after desiccation. These results revealed no significant differences between trehalose content before and after desiccation (t test, $p>0.05$), but showed statistically significant difference between trehalose content in early and late diauxic phase.

Primuline method used to estimate desiccation tolerance in a way demonstrates cell membrane integrity (fluorophore enters cells with compromised cytoplasmatic membranes). Cell membrane lipid peroxidation is one of cellular damages taking place during desiccation (Espindola *et al.,* 2003, Franca *et al.,* 2005). Malonyldialdehyde accumulation is s biochemical marker of lipid peroxidation. Malonyldialdehyde colour reaction with thiobarbituric (TBA) acid is common method how to evaluate these damages. We choose TBARS assay to evaluate desiccation effect on lipid peroxidation. Results are presented in figure 3.2.3.

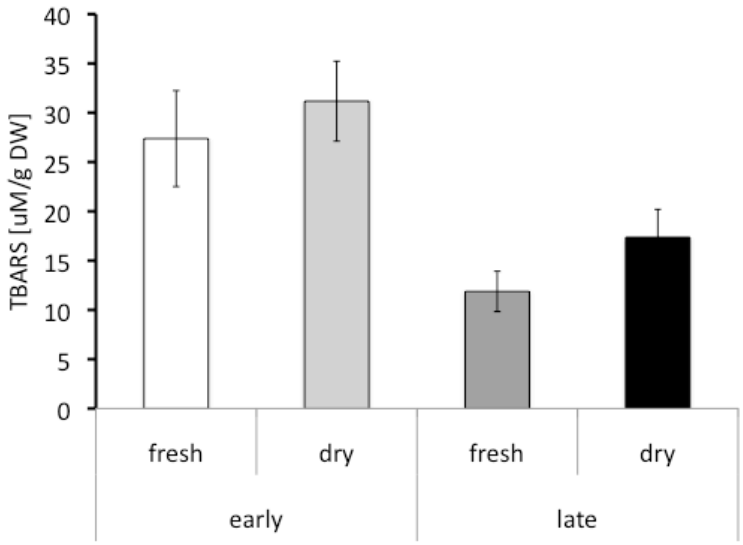


Fig. 3.2.3. TBARS content [uM/ g DW] of *SC14* strain before and after desiccation when harvested in early and late postdiauxie phases.
Mean values are calculated from biological triplicates, error bars represent standard deviations.

Higher lipid peroxidation levels correlate with lower survival rates (compare 3.2.1. with fig. 3.2.3.). Besides, increase in average TBARS levels after desiccation is observed in both cases – cells from early and late postdiauxie; however, no significantly different with respect to desiccation (t test, $p>0.05$).

Glutathione is simple, non-enzymatic system for ROS scavenging. Concentration of glutathione reduced form characterizes cells ability to scavenge ROS. Glutathione dependent ROS scavenging system is reported to be actively involved in lowering cytoplasmic ROS during desiccation and subsequently – enchancing cell's survival (Espindola *et al.,* 2003).

We tested content of glutathione reduced form (GSH) before and after desiccation of early and late postdiauxic phase cells (see fig. 3.2.4.).

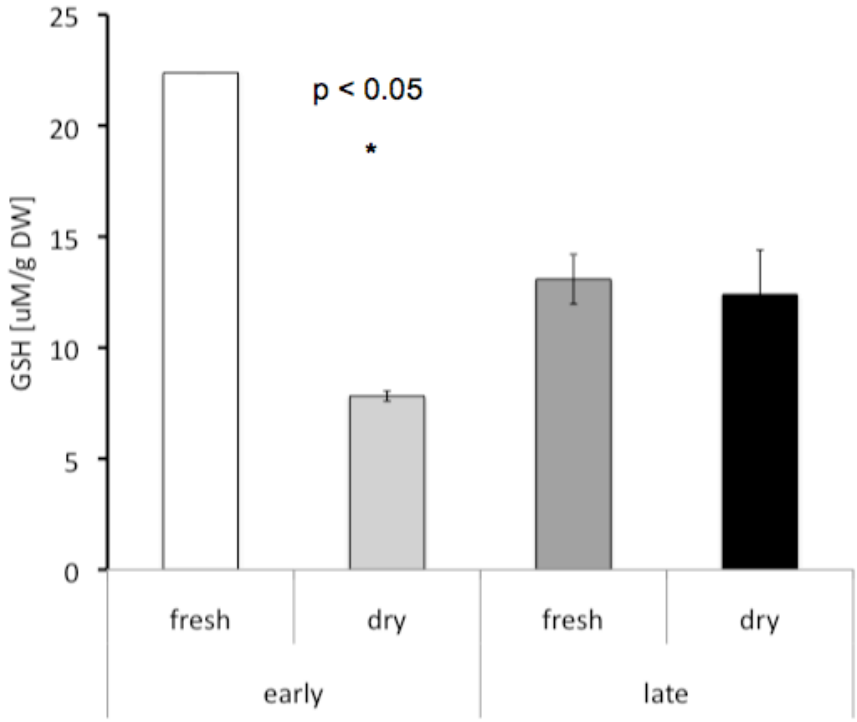


Fig. 3.2.4. GSH content [uM/ g DW] of *SC14* strain before and after desiccation when harvested in early and late postdiauxie.
Mean values are calculated from biological triplicates, error bars represent standard deviations.
Star represents statistically significant difference between GSH content of postdiauxic cells before and after desiccation. (t test, $p<0.05$).

Interestingly enough, fresh cells from early postdiauxie phase contain more GSH than fresh late postdiauxie phase cells (22+/- 0.2 and 14 +/-1 uM/ g DW respectively). Cells from early postdiauxic growth phase lose GSH after desiccation most probably in reaction to accumulated ROS. After desiccation, early postdiauxie phase cells contained 7+/- 0.35 and late - 13+/- 2 uM/ g DW of GSH. Thus early postdiauxie phase cells loose significant amount of GSH while no statistically significant changes in GSH content in late postdiauxie phase cells before and after desiccation are observed.

Here, it seems, that increased trehalose content might be sufficient to ensure high desiccation tolerance. *SC14* cultivation for prolonged period after glucose exhaustion ensures

cells to accumulate large amount of trehalose, what subsequently leads to elevated desiccation tolerance. Additionally, membrane peroxidation of early postdiauxic cells is high (what correlates to low survival); GSH level is high in fresh early postdiauxic cells and low in dry cells – displaying putative ROS scavenging activity.

To further elucidate role of trehalose during desiccation tolerance in cells of early and late postdiauxic phase cells, we did set of experiments on haploid reference strain *BY4741* and respective *tps1* deletion strain (incapable to accumulate trehalose).

Similar cultivation time points (early postdiauxic, 24 h and late postdiauxic, 48 h) were chosen similarly to experiments with *SC14*. Similarly to *SC14*, desiccation tolerance, trehalose content, GSH and TBARS content were measured.

Similarly to *SC14* strain, survival after desiccation of *BY4741* in early and late postdiauxic growth phases differed significantly, albeit to different degree ($p<0.025$ for *tps1* strain and $p<0.0015$ for *BY4741*), see fig. 3.2.5.

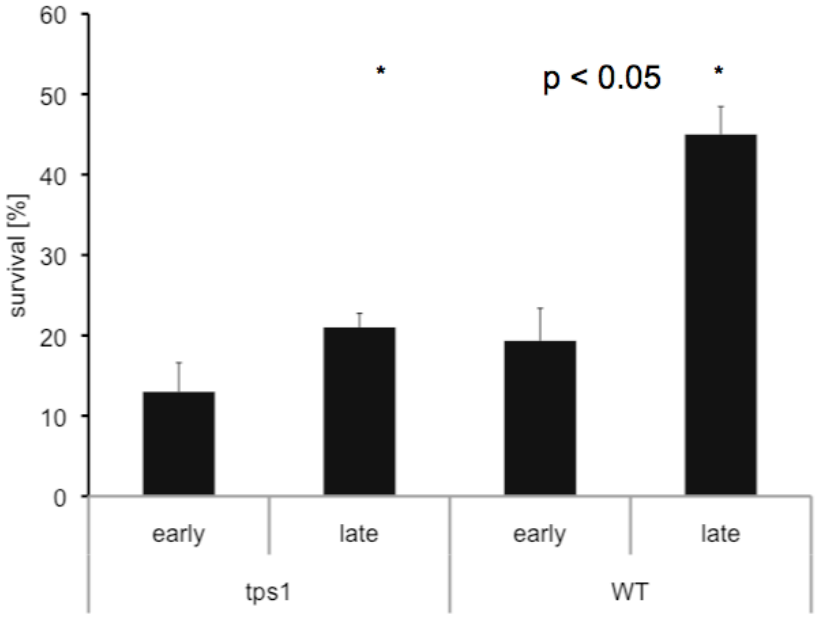


Fig. 3.2.5. *BY4741* (WT) and respective *tps1* strain survival after desiccation when harvested in early and late postdiauxie phase.
Mean values are calculated from biological triplicates, error bars represent standard deviations. Stars represent statistically significant differences between survival of different growth phases (t test, $p<0.05$).

Trehalose content of *BY4741* and respective *tps1* mutants before and after desiccation were estimated. No significant, desiccation dependent, differences in trehalose content (mg/ g DW) were observed (see fig. 3.2.6).

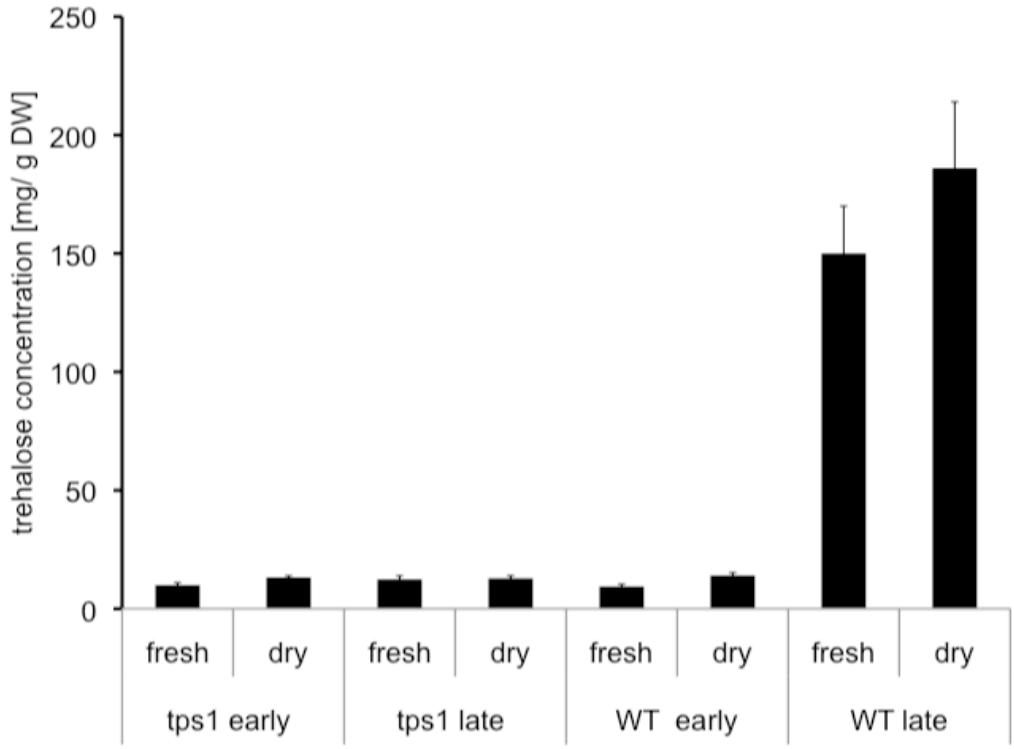


Fig. 3.2.6. *BY4741* and *tps1* deletion strain trehalose content [mg/ g DW] before and after desiccation when harvested in early and late postdiauxie phases.

Mean values are calculated from biological triplicates, error bars represent standard deviations.

Since analytical method (anthrone assay) used to measure trehalose content is sensitive to glucose moiety rather than disaccharide trehalose itself, nonspecific analytical signal might come from cytoplasmic, water-soluble sugar phosphates (Mokrasch, 1954). This might explain some trehalose presence seen in fig. 3.2.6. even in trehalose synthesis deficient mutant

Similar to previous results from strain *SC14*, we did not observe statistically significant changes in trehalose content before and after desiccation. Thus we concluded, that desiccation *per se* has no effect on trehalose content in *S. cerevisiae* cells under our experimental conditions. Therefore, we decided to measure trehalose concentration just before desiccation in all following experiments.

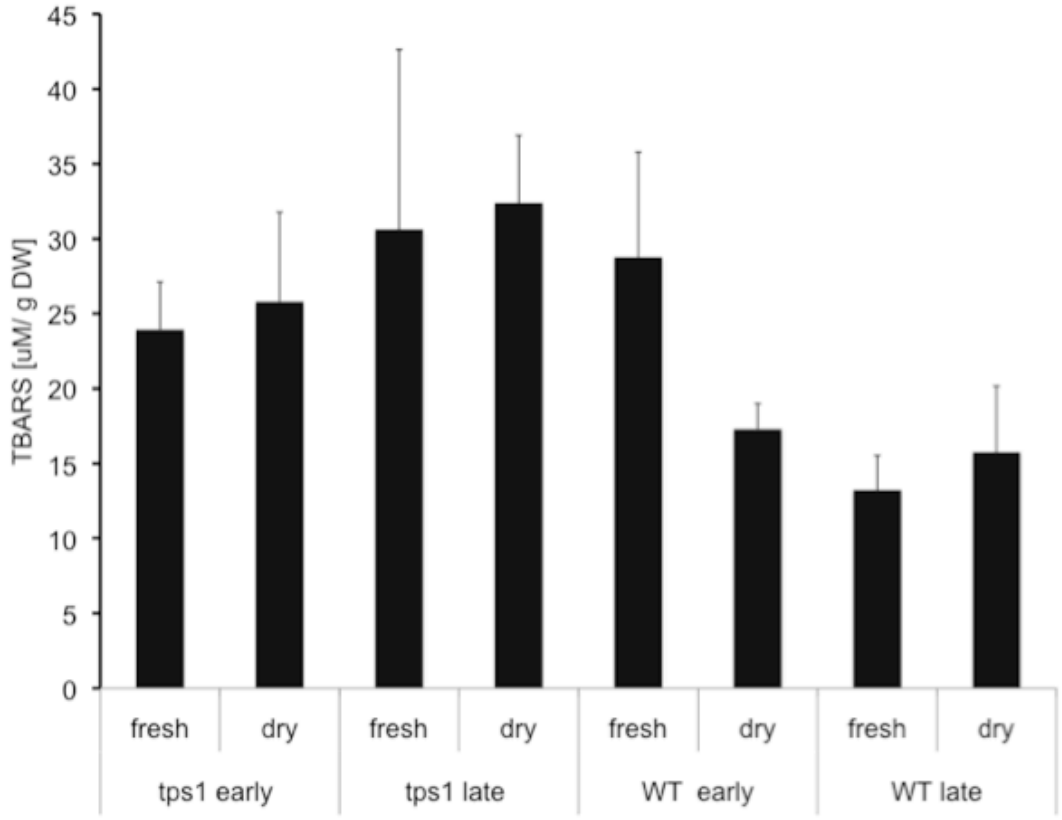


Fig. 3.2.7. TBARS content [uM/ g DW] of BY4741 (WT) and corresponding tps1 deletion strain before and after desiccation when harvested in postdiauxie and stationary (stat) phases. Mean values are calculated from biological triplicates, error bars represent standard deviations.

Results of TBARS analyses of *BY4741* and it's *tps1* deletion strain before and after desiccation in culture early and late postdiauxic growth phases are similar to those obtained from *SC14* strain, see fig. 3.2.7. Interestingly, highest desiccation tolerance corresponds to lowest concentration of TBARS (in the case of *SC14* and *BY4741* stationary phase cells). Although mean values of TBARS before and after desiccation are different, they are not statistically significant (t test, $p>0.05$).

At last we checked changes in reduced glutathione concentrations before and after desiccation of *BY4741* and respective *tps1* deletion strain when sampled in early and late postdiauxic phase (fig. 3.2.8.).

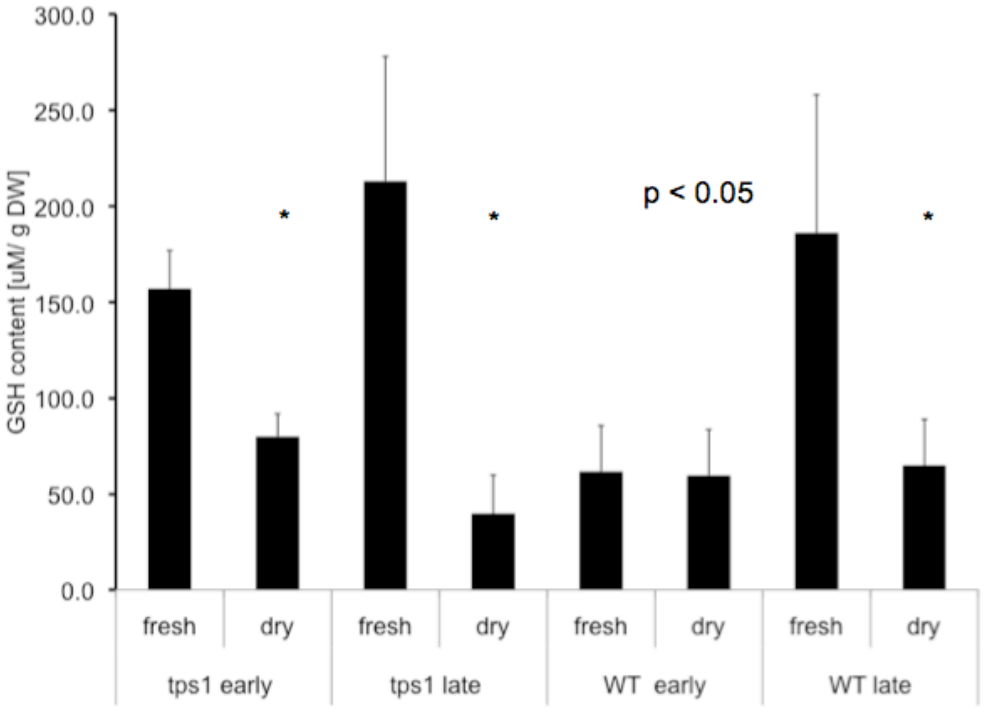


Fig. 3.2.8. BY4741 (WT) and corresponding tps1 deletion strain reduced glutathione (GSH) content [uM/ g DW] before (fresh) and after (dry) desiccation when harvested in early and late postdiauxie phases.
Mean values are calculated from biological triplicates, error bars represent standard deviations
Stars represent statistically significant differences between GSH content in fresh and dried yeast (t test, a<0.05).

It is hard to interprete oxidative marker results (like cell GSH and TBARS content) for cell population after desiccation, since part of the cells have damaged, leaking membranes and oxidation processes proceed differently as in the case of cells with fully integral plasma membrane. To fully elucidate oxidative stress development during desiccation stress – cell population enrichment into highly tolerant and intolerant fractions should be done and their "oxidative fate" be estimated separately.

3.3. *S. cerevisiae* redox cofactor engineering to enhance glutathione turnover and desiccation tolerance

3.3.1. Glycerol cycle construction in S. cerevisiae

As demonstrated in previous results, GSH is depleted after desiccation or during desiccated state (fig. 3.2.4. and 3.2.8.). One possible mechanisms of desiccation in-tolerance can be related to ineffective oxidative stress response – cell's inability to neutralise accumulated ROS after desiccation (Franca *et al*., 2007). Our intention was to try to increase GSH content of the cells, thus increasing oxidation stress tolerance while in desiccated state.

Reduced – oxidized glutathione pair forms the main non-enzymatic oxidative stress defense system in *S. cerevisiae*. Here, the reduced glutathione (GSH) is the active component, capable to sequester reactive oxygen species (Penninckx, 2002).

There are two strategies how to increase GSH concentration in *S. cerevisiae* cell:

- increase in *de novo* synthesis,
- increase the rate of GSH recycling (NADPH dependent GSSG reduction).

De novo synthesis is tightly regulated by GSH negative feedback, thus making it nonattractive spot for engineering (illustrated in fig. 1.3.5.). So, we chose to increase cytoplasmic NADPH supply, thus enchancing GSH turnover.

Several enzymes produce NADPH in *S. cerevisiae* cytoplasm:
glucose-6-phosphate dehydrogenase (Zwf1p), aldehyde dehydrogenase *(*Ald6p), isocytrate dehydrogenase (Idp2p) (Minard and McAlister-Henn, 2005). Additionally there is a putative enzyme glycerol dehydrogenase (EC 1.1.1.72), catalyses NADP dependent reaction from glycerol to glyceraldehyde, putatively encoded by YPR1 and GCY1 in *S. cerevisiae*.

We decided to construct "NADP dependent glycerol cycle" – similar to transhydrogenase cycle active in moulds see fig. 3.3.1. Glycerol cycle presence in native *S. cerevisiae* has not been proven despite numerous efforts (Costenoble *et al,* 2000, Norbeck and Blomberger 1997).

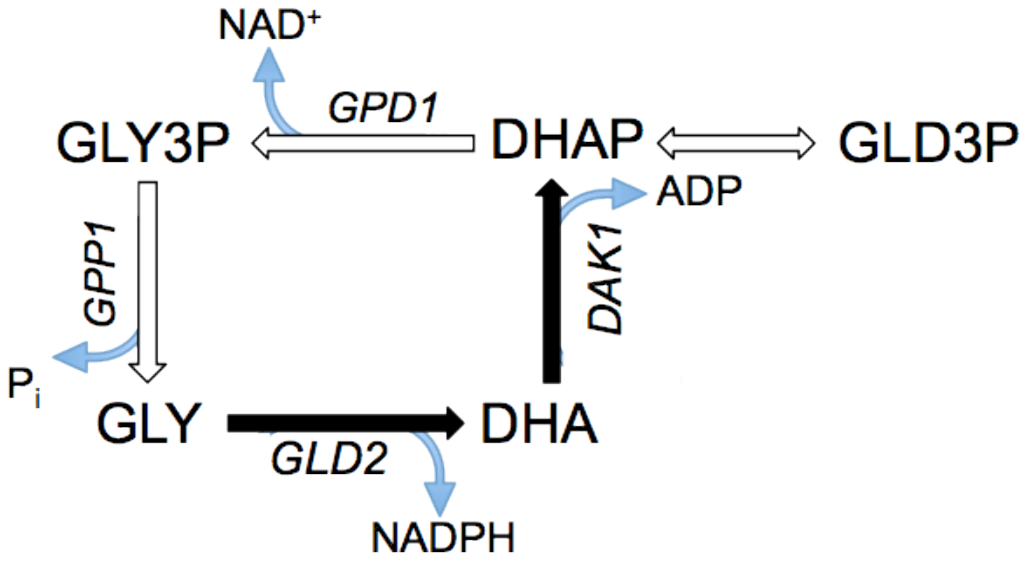


Fig. 3.3.1. Redox cofactor engineering in S. cerevisiae via introducing glycerol cycle.
Dark arrows depict engineered reactions (*GLD2* NADP dependent glycerol dehydrogenase from *H. jecorina* and overexpression of *S. cerevisiae* DHA kinase *DAK1*) .
GPD1- glycerol-3phosphate dehydrogenase
GPP1 – glycerol-3phosphate phosphatase

In order to find out candidates for glycerol cycle engineering, we did a ClustalW alignment of known NADP dependent fungi and mould glycerol oxidoreductases, see fig. 3.3.2.

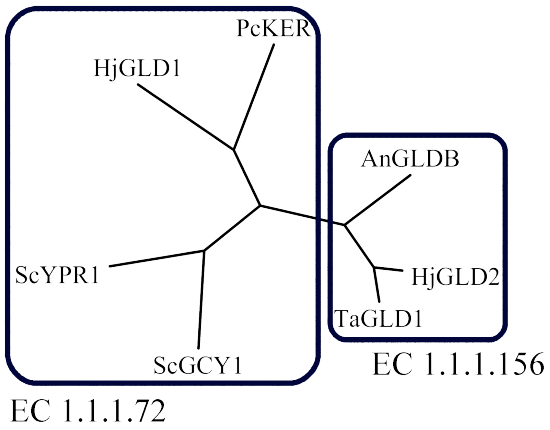


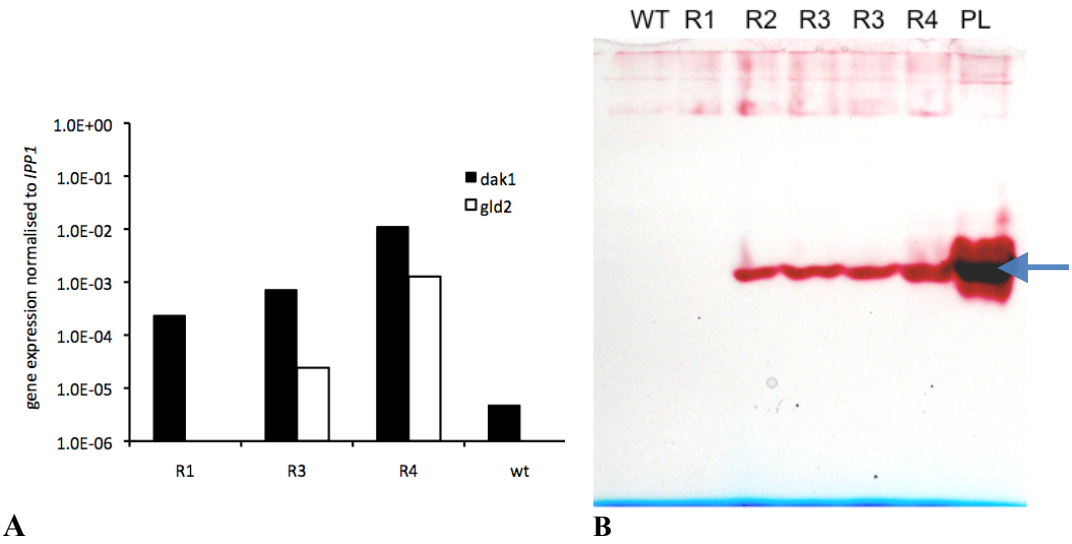
Fig. 3.3.2. Clustalw analyses of fungal NADP dependent glycerol dehydrogenases protein sequences.
EC. 1.1.1.156 – a glycerol: NADP 2-oxidoreductase (facilitates the reaction of glycerol and NADP to form DHA and NADPH).
EC. 1.1.1.72 – NADP+ oxidoreductase dependent (facilitates the reaction of glycerol and NADP to form d-glyceraldehyde and NADPH). Enzymes are grouped based on their gene sequences. Names depict organim and respective gene.
Hj- *Hypocrea jecorina*, Pc – *Penicillium citrinum*, Sc – *Saccharomyces cerevisiae*, An – *Aspergillus nidulans*, Ta – *Trichoderma atroviridiae*

Theese proteins group into two domains – what are described by two enzymatically different activities: EC.1.1.1.72 ($NADP^+$ dependent glycerol dehydrogenase) and EC.1.1.1.156 (NADP dependent glycerol 2-dehydrogenase). *H. jecorina GLD2*, the *A. nidulans GLD*B and the *T. atroviride GLD1* form one NADP dependent glycerol dehydrogenase cluster (Liepins *et al.,* 2006). *S. cerevisiae YPR1* and *GCY1* were also included into analyses, however they grouped together with H. jecorina *GLD1* and *Penicillium citrinum* oxidoreductases. Literature data shows, that theese enzymes also posess NADP dependent glyceorl oxidative activity. However, this activity was about 4000 times lower than in the reducing direction with dl-glyceraldehyde and NADPH (Ford and Elis, 2002).

We chose to link glycerol with dihydroxyacetone (DHA) via introducing *H. jecorina* NADP dependent glycerol dehydrogenase gene (*GLD2*) (Brusbardis and Liepins, 2010, Liepins *et al*., 2006). Additionally we overexpressed *S. cerevisiae* endogeneous DHA kinase *DAK1* (Molin *et al.*, 2003) thus completing the cycle. Both *GLD2* and *DAK1* were integrated into CEN.PK2 strain genome in various positions: *DAK1* in *fcy1*, *GLD2* in *ade8* or *lys1*.

Integration was done by two-step gene disintegration system as described by Sadowski *et al.*, 2007.

Successful transformants were checked for *DAK1* and *GLD2* expression by qPCR (see fig. 3.3.3. A). All transcripts were normalized to housekeeping gene *IPP2* (inorganic polyphosphatase) transcription level.



A **B**

Fig. 3.3.3. A *GLD2* and *DAK1* specific qPCR normalized to *IPP2* transcripts.
B Glycerol dehydrogenase specific zymogram of Gld2p activity in CEN.
PK2 background (WT) strains.
Arrow points to specific NADP dependent glycerol dehydrogenase fraction. Staining was done with INT and PMS mix in the presence of 0.1 M glycerol and 0.2 mM NADP.
All strains tested here are of *CEN.PK2* background:
WT *CEN.PK2 trp2 ura3 his2 leu2,*
R1 WT *fcy1:: DAK1,*
R2 WT *lys2:: GLD2*
R3 WT l*ys2:: GLD2, fcy1:: DAK1*,
R4 WT *ade8:: GLD2, fcy1:: DAK1*
R5 WT *ade8:: GLD2*
PL – WT + *GLD2* expression plasmid

Strain *R4* (CEN.PK2 *GLD2::ade8 DAK1::fcy1*) revealed the highest *GLD2* and *DAK1* expression. This was partly confirmed by GLD2 zymograms – R4 had the second highest *GLD2* activity after strain expressing *GLD2* in plasmid (see fig. 3.3.3., B lane 6).

To estimate if all elements of glycerol cycle are present, we did additional zymograms, specific for glucose-6P dehydrogenase (to estimate status of cells main NADPH production site), glycerol dehydrogenase (Gld2p) and glycerol-3phosphate dehydrogenase (Gpd1p). Yeast were grown in full SD media on two substrates – glucose and glycerol to see if parts of cycle are glucose repressed. Results are depicted in fig. 3.3.4.

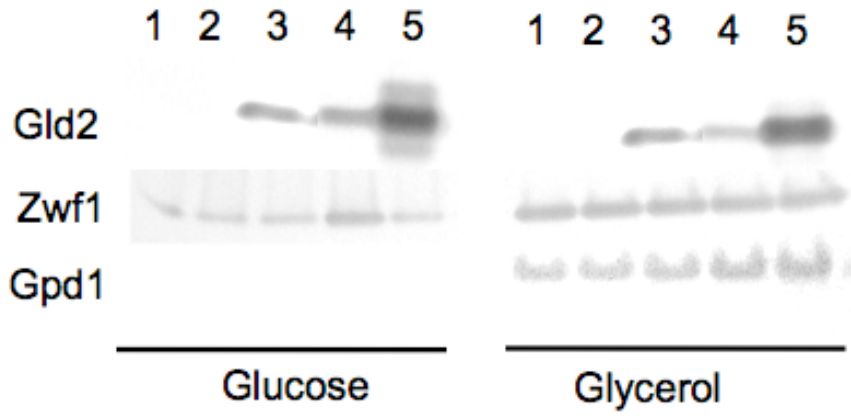


Fig. 3.3.4. Expression of glycerol (Gld2), glycerol-3phosphate (Gpd1) and glucose-6phosphate (Zwf1) dehydrogenases as analysed by zymograms in CEN.
PK2 strain and derivative strains when grown on glucose and glycerol. 50 ug of protein was applied to each lane.
1 *CEN.PK2* parent strain (WT),
2 R3 WT *fcy1::DAK1*,
3 R5 WT *ade8::GLD2,*
4 R4 WT *fcy1::DAK1 ade8::GLD2,*
5 WT plasmid 3324 expressing *GLD2*

All zymograms were stained by iodonitrotetrasolium chloride (INT) in the presence of electron acceptor/donor phenazine methosulphate (PMS). 0.1 M of glucose-6 phosphate and 0.2 mM of NADP were used for Zwf1p zymograms; 0.1 M of glycerol and 0.2 mM of NADP were used for Gld2p zymogram, 0.1 M of glycerol-3 phosphate and 0.2 mM of NAD were as used as Gpd1p zymogram substrates. As seen from staining of different protein samples – Gpd1p activity is absent when cells are grown on glucose, therefore eventual glycerol cycle might work as "real" cycle only when cells are grown on non-repressive carbon sources, otherwise cycle might work as "half cycle" (Gld2p and Dak1p) converting glycerol to DHA and DHAP. Interestingly, Zwf1p activity is higher in derepressed (glycerol grown) cells.

3.3.2. Effects of glycerol cycle on yeast physiology

To check if *GLD2* insertion and/or *DAK1* overexpression has effect on engineered strain growth kinetics, we tested these strain growth on glucose in SD media, see fig. 3.3.5. Not *GLD2*, nor *DAK1* overexpression had any affect on yeast growth on glucose. All of engineered strains grew similarly to *CEN.PK2* series parent strains H1346.

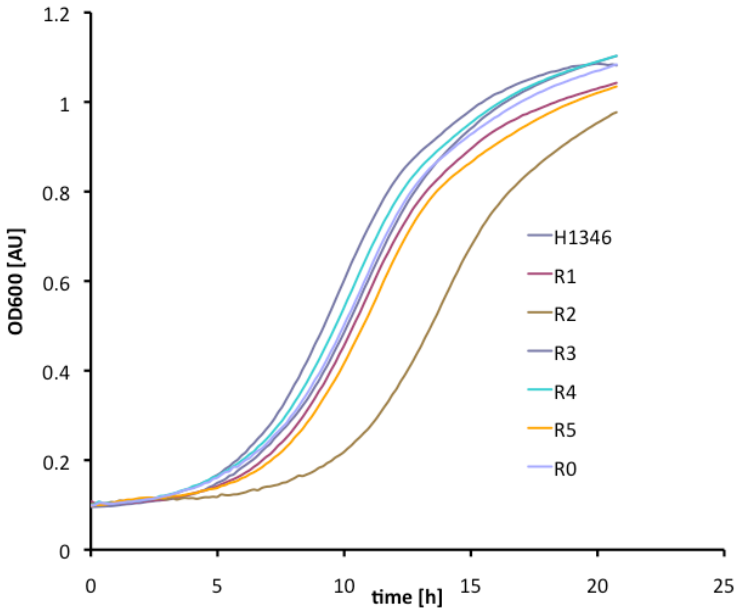
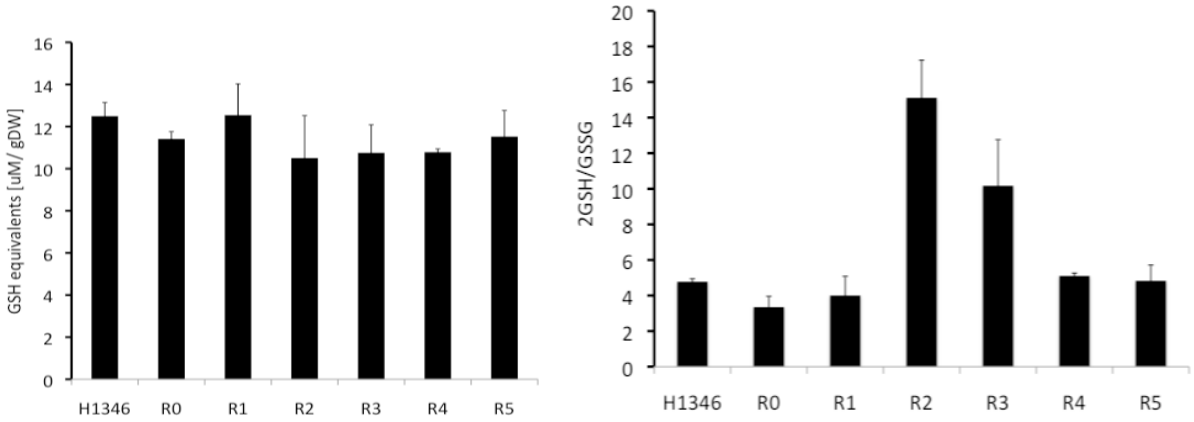


Fig. 3.3.5. *CEN.PK2* series strain growth in synthetic dextrose (SD) medium.
Strain genetics are as follows:
H1346 CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1 MAL2-8c SUC2*
R0 H1346 *ade8Δ0, fcy1Δ0*
R1 H1346 *fcy1::DAK1*
R2 H1346 *lys2::GLD2*
R3 H1346 *lys2::GLD2, fcy1::DAK1*
R4 H1346 *ade8::GLD2, fcy1::DAK1*
R5 H1346 *ade8::GLD2*

Samples to estimate glutathione content were harvested after 29 h of growth in YPD media supplemented with additional adenine in surplus (50 mg/l). Total glutathione content was expressed in reduced glutathione (GSH) equivalents: GSH concentration + 2x GSSG concentration. Glutathione content of developed strains did not differ from parent strain (see fig. 3.3.6. A). In the same time 2GSH/GSSG proportion differed (fig. 3.3.6. B). Strains R2 and R3 revealed significant increase in 2GSH/GSSG ratio - from 4.5 in parent strain to 15 and 10 in engineered R2 and R3 respectively.

The increased proportion of GSH versus GSSG is indicator of more reduced media inside the cell, what could increase oxidative tolerance and possibly desiccation tolerance.



A B

Fig. 3.3.6. CEN.PK2 series strain glutathione content (A) in reduced glutathione (GSH) equivalents (uM/ g DW) and GSH/GSSG ratio (B).
Strain genetics are as follows:
H1346 CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1 MAL2-8c SUC2*
R0 H1346 *ade8*Δ0, *fcy1*Δ0
R1 H1346 *fcy1::DAK1*
R2 H1346 *lys2::GLD2*
R3 H1346 *lys2::GLD2, fcy1::DAK1*
R4 H1346 *ade8::GLD2, fcy1::DAK1*
R5 H1346 *ade8::GLD2*

Desiccation stress tolerance of newly developed strains was assessed. Results are depicted in fig. 3.3.7. In fact, no correlation to desiccation tolerance with increased 2GSH/GSSG ratio was observed. Strangly only R4, where *GLD2* was integrated in *ade8* gene, showed the strongest increase in desiccation tolerance albeit it did not had significant improvement in GSH/GSSG ratio (fig. 3.3.6. B).

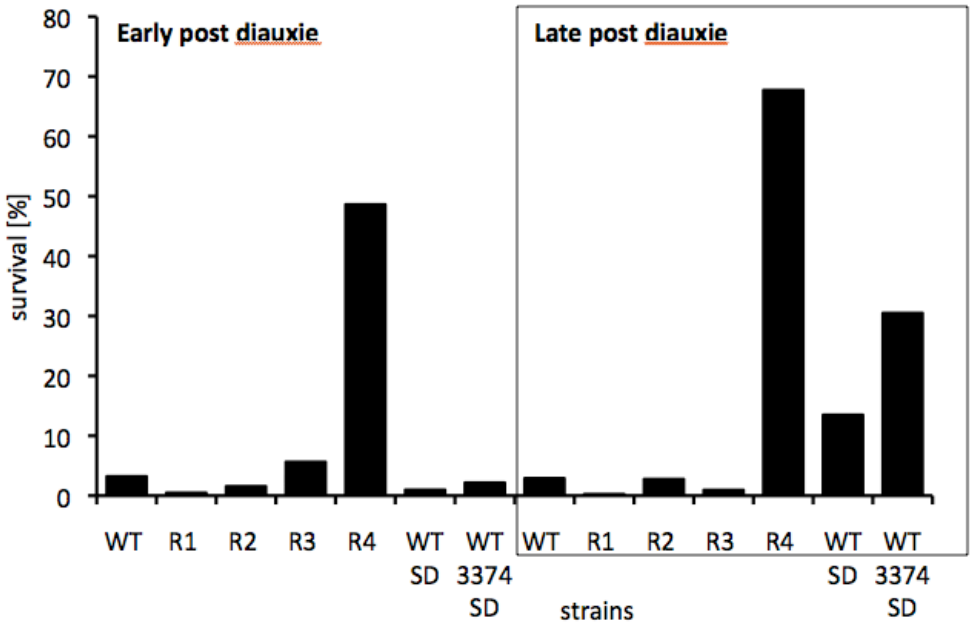


Fig. 3.3.7. H1346 (WT) and derived recombinant strain (R1-4) desiccation tolerance when grown in YPD media and harvested in early or late postdiauxic growth phase.
Additionally two strains (WT and WT with *GLD2* expression plasmid 3374) were grown in SD medium till early (24 h) and late postdiauxie (48 h) phase.
Viability after desiccation was assayed with primuline method.

From here (fig. 3.3.6. B) we concluded, that cofactor engineering has potential to improve intracellular glutathione species levels, however, it does not improve yeast desiccation tolerance (fig. 3.3.7.).

3.4. Nutritional effects on yeast desiccation stress tolerance

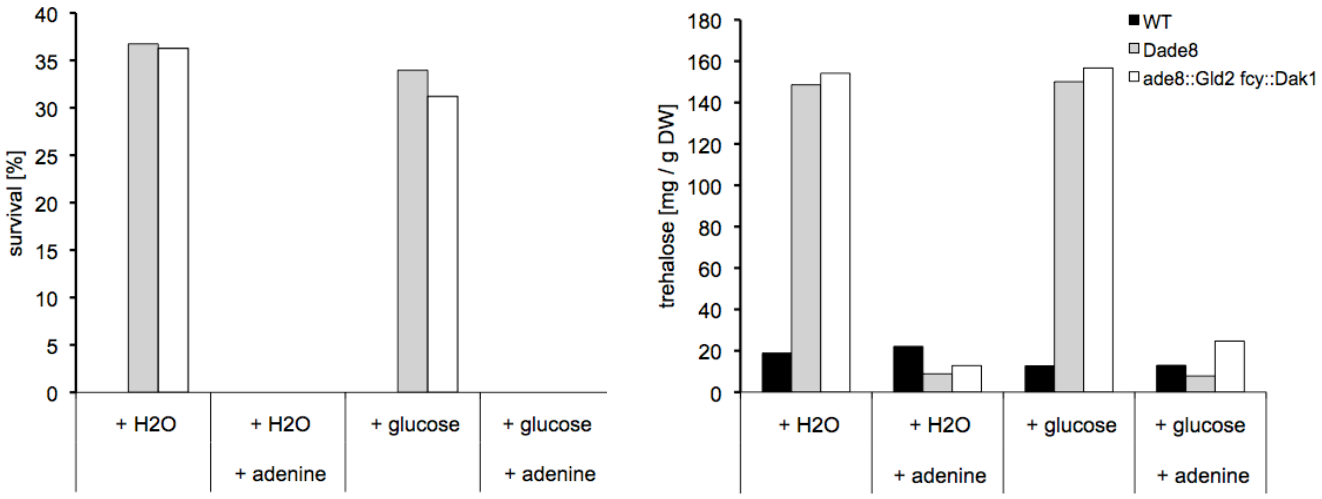
3.4.1. Adenine auxotrophy effect on yeast desiccation stress tolerance

When studying glycerol cycle effects in *CEN.PK2* derived strains, we observed bizarre side effect: only strain with *ade8* disruption displayed high desiccation tolerance in early and late postdiauxie phase when cultivated in YPD medium. Additionally, WT and WT containing *GLD2* expression plasmid displayed enhanced desiccation tolerance when harvested in late postdiauxie phase. See fig. 3.3.6.

Since *ade8* disruption leads to stop of *de novo* adenine synthesis (Rebora *et al.*, 2001), then engineered strains could behave differently when media adenine is exhausted. To check if this is the case, we tested if desiccation tolerance of *ade8* disrupted strains is adenine dependent.

We cultivated CEN.PK2 *ade8::0* and *ade8::GLD2 fcy1::DAK1* co-expressing strain in YPD medium and monitored increase in OD600. When exponential increase of OD600 ended (glucose depleted), we added adenine (40 mg/l final concentration) and/ or glucose

(2% final concentration), and sterile water was used as negative control. After 2 h of incubation, cultivation was interrupted, and cells were harvested and dried. Based on previous results, trehalose content of the cell was determined once - before desiccation. Survival around 30 – 35 % was observed only for strains with *ade8* deletion (either deletion or integration vector with *GLD2*) in combination with media w/o additional adenine supply, fig. 3.4.1 A. In parallel, trehalose concentration for the same strain- media combination increased remarkably (around 140 mg/ g DW), fig. 3.4.1 B



A B

Fig. 3.4.1. *CEN.PK* and derived recombinant R5.0 (*CEN.PK ade8*) and R4 (*CEN.PK ade8::Gld2 fcy::Dak1*) strain desiccation tolerance (A) and trehalose content (B).
Cultures were grown in YPD media till the end of exponential growth phase when broth were supplemented with water, glucose and adenine in the combinations depicted above. Cultures were cultivated for another 2 h and then harvested and dried for 16 h. Survival after desiccation was estimated by primuline fluorescence method. Trehalose content was estimated by anthrone assay.

Yeast extract is the only component of YPD media containing adenine. Depending on vendor, adenine content can be highly variable there – it can vary from 0.34 up till 1.91 mg / g yeast extract) (Van Dusen *et al.,* 1997). We checked adenine content of yeast extract used in our experiments by hypoxanthine oxidase – horseradish peroxidase coupled reaction and it was 1.3 mg / g yeast extract or 13 mg / l of broth.

Increased stress tolerance induced by auxotrophy *per se* is novelty for the desiccation tolerance studies in baker's yeast model, therefore we decided to explore it in detail.
Previous results point to the fact that prolonged postdiauxic cultivation always leads to increased desiccation resistance. These results point to additional nutrition dependent effects which might play a role in auxotrophic strains in poorly defined medias. Additionally, adenine depletion in media *per se* might induce stress response reactions also in adenine prototrophs, therefore different genetic screens should be used to reveal genetic aspects of

auxotrophy induced desiccation tolerance. We decided to explore adenine dependent desiccation tolerance and trehalose accumulation throughout exponential, diauxie and postdiauxie growth phases by *W303* prototroph, *W303 ade2* and *W303 ADE2* strains (see for genotype details in material section, table 2.1.).

W303 ade2 strain has nonfunctional *ade2* and in the case of adenine deficiency it accumulates red pigment – oxidized AIR (ribosylamyloimidasol). When available in medium, adenine is actively transported in the cell and stored there. If media adenine is depleted, intracellular resources are used and at last – adenine *de novo* synthesis sets in. In the case of *W303 ade2* strain, when *de novo* synthesis starts, oxidised intermediate AIR (red pigment) accumulates (VanDusen *et al.,* 1997). In this way, accumulation of red pigment is reliable marker of adenine starvation in yeast cell.

To determine cultures survival of low desiccation tolerance (1 % or less) primuline method gives erroneous results, since impact of each putatively alive cell is large (if 1000 cells per sample are counted). Classical method for viability estimation is serial dilution followed by plating and CFU count. We compared primuline and plating results of desiccated *CEN.PK* yeasts in parallel, results are depicted in fig. 3.4.2.

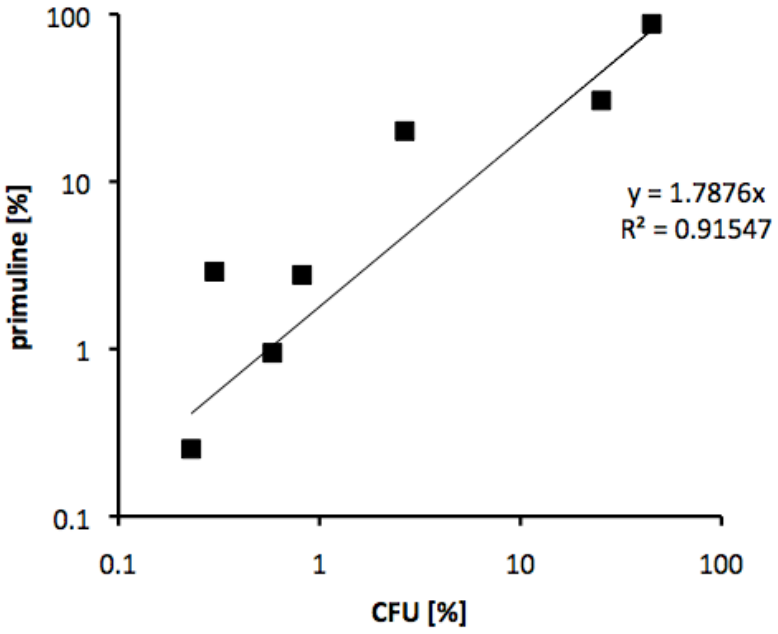


Fig. 3.4.2. Primuline fluorescence and plating method (CFU) comparison.
CEN.PK2 WT and developed R1-4 cultures were grown till early and late postdiauxie, desiccated and their survival were determined in parallel by plating and primuline method.
1000 cells were counted for each microsope slide, plate counts were done between 20 – 200 colonies/ plate.
Logarithmical scale was chosen to display all results.

As demonstrated in fig. 3.4.2, there is poor consensus between primuline and plating method if culture viability is 1% or less. Also systematic overestimation of viability by primuline method persists, slope coefficient 1.78.

Calahan *et al*., 2011 and Welsh *et al*., 2013 have reported on serial dilution spot test as semi- quantitative tool to estimate desiccation tolerance in large viability interval (0.0001 – 100 %). From our previous results on adenine dependent desiccation tolerance (fig. 3.4.1), we learned that 10 fold or more difference in desiccation tolerance among strains are suspected (similar metrics to Calahan *et al.*, 2011), thus we chose to apply the same viability estimation method.

To estimate dynamics of desiccation tolerance over culture growth in YPD medium, we did shake flask cultivations of *W303 prototroph*, *W303 ade2* and *W303 ADE2* strains and sampled it starting from OD600=1. 1 ml of culture at OD600=1 was washed with distilled water, spotted on YPD media for initial cell count, centrifuged, supernatant was removed and left to desiccate at desiccator for 10 h. After desiccation cells were rehydrated with water, serially diluted and spotted on YPD solid agar media. Survival rates were calculated as proportion of CFU before and after desiccation. Experiment was done in triplicates, spot test was done in technical duplicate. Yeast growth and desiccation tolerance dynamics are depicted in figure 3.4.3.

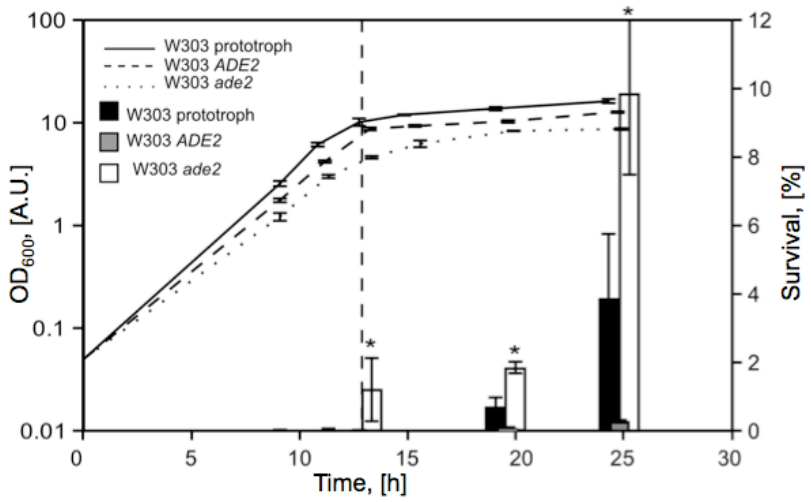


Fig. 3.4.3. Desiccation tolerance during *W303 prototroph, W303 ADE2* and *W303 ade2* cultivation in YPD media.
Cells were desiccated in +30°C for 10 h. Desiccation tolerance was quantified by serial dilution spot test (Calahan *et al*., 2011).
Lines denote strain growth curves in logarithmic scale and bars survival rates. Error bars depict standard deviation from biological triplicates. Viability in first two time points (8 and 12 h) is nearly zero for all strains. Vertical interrupted line depicts time of adenine depletion.
Asterisks depict difference between *W303 ade2* and both *W303* prototroph and *W303 ADE2* strains desiccation tolerance as significant ($p<0.05$). Adopted from Kokina e*t al.,* 2014.

Desiccation tolerance of *W303 ade2* strain peaked immediately after adenine depletion. Additionally, adenine depletion happened at the same time as glucose depletion and subsequent slowing of cell growth speed took place (fig. 3.4.4.). However, desiccation tolerance of other strains (adenine prototrophs) lagged - portion of those strain population became desiccation tolerant just after 20 and more hours of cultivation. Based on these results, we concluded, that increase in desiccation tolerance for *W303 ade2* strain is caused by adenine depletion purely.

In parallel we monitored trehalose content of the *W303* series strain cells. Results are depicted in fig. 3.4.4.

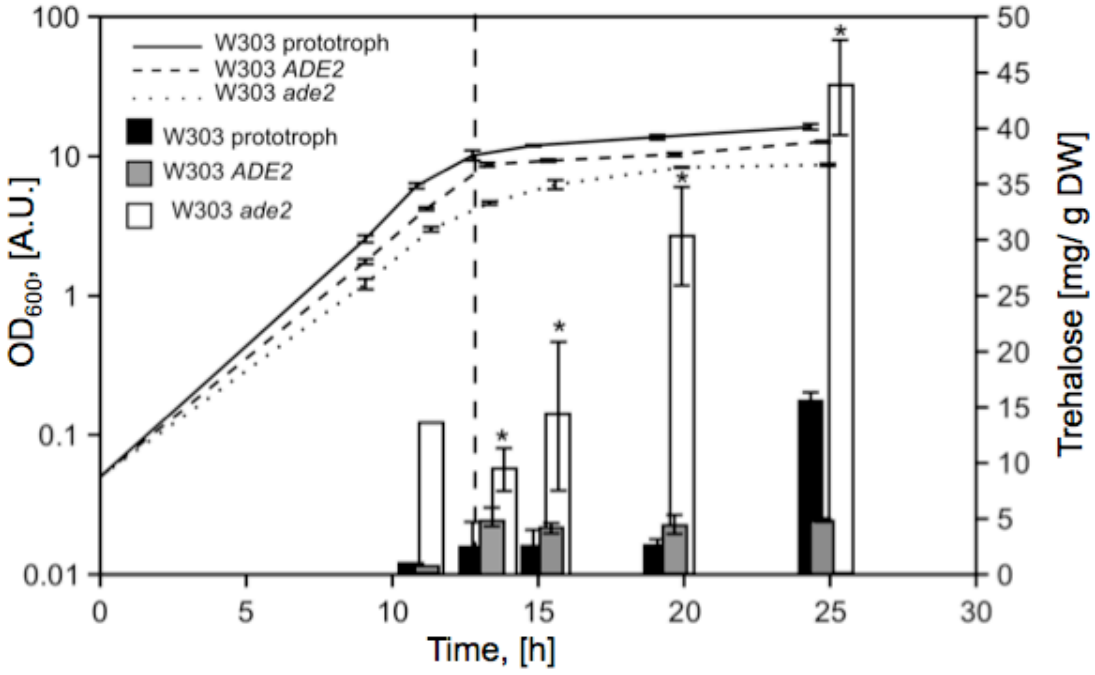


Fig. 3.4.4. Trehalose accumulation during *W303 prototroph, W303 ADE2* and *W303 ade2* cultivation in YPD media.
Trehalose was measured by anthrone method. Lines denote strain growth curves in logarithmic scale and bars trehalose content. Error bars depict standard deviation from biological triplicates. Vertical interrupted line depict time of adenine depletion.
Asterisks depict difference between *W303 ade2* and both *W303* prototroph and *W303 ADE2* strain trehalose content as statistically significant ($p<0.05$).
Figure adopted from Kokina *et al*., 2014.

We observed, that slowing of growth speed correlates with adenine depletion in rich media. Thus, *ade* strain growth dynamics can give a hint (even without optical clues, like red pigmentation) when adenine is depleted.

When keeping this in mind, we monitored culture growth of adenine mutants (*ade8*) of other strain background – *CEN.PK2*. We observed similar ade- dependent increase in desiccation, results are demonstrated in fig. 3.4.5.

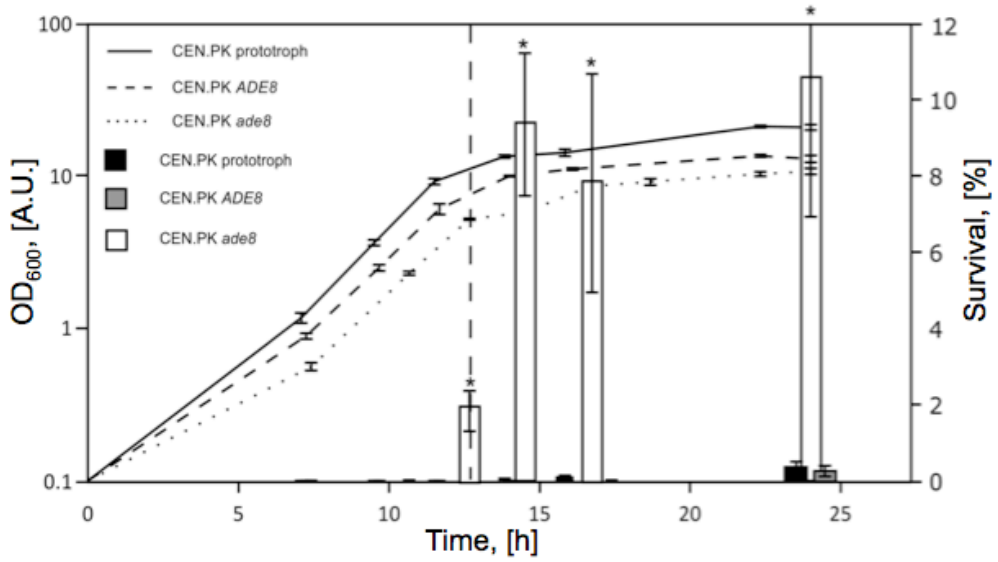


Fig. 3.4.5. Desiccation tolerance of *CEN.PK prototroph, CEN.PK ADE8* and *CEN.PK ade8* when cultivated in YPD media.
Cells were desiccated in +30°C for 10 h. Desiccation tolerance was quantified as in Calahan *et al*., 2011.
Lines denote strain growth curves in logarithmic scale and bars survival rates. Error bars depict standard deviation from biological triplicates. Viability in first two time points (8 and 12 h) is nearly zero for all strains. Vertical interrupted line depicts time of adenine depletion. Asterisks depict difference between *CEN.PK ade8* and both *CEN.PK* prototroph and *CEN.PK ADE8* strains desiccation tolerance as statistically significant ($p<0.05$). Adopted from Kokina *et al*., 2014.

Similar to *W303* series strain cultivation, we did analyses of trehalose dynamics during *CEN.PK* strain series cultivation in rich media. Results are depicted in fig. 3.4.6.

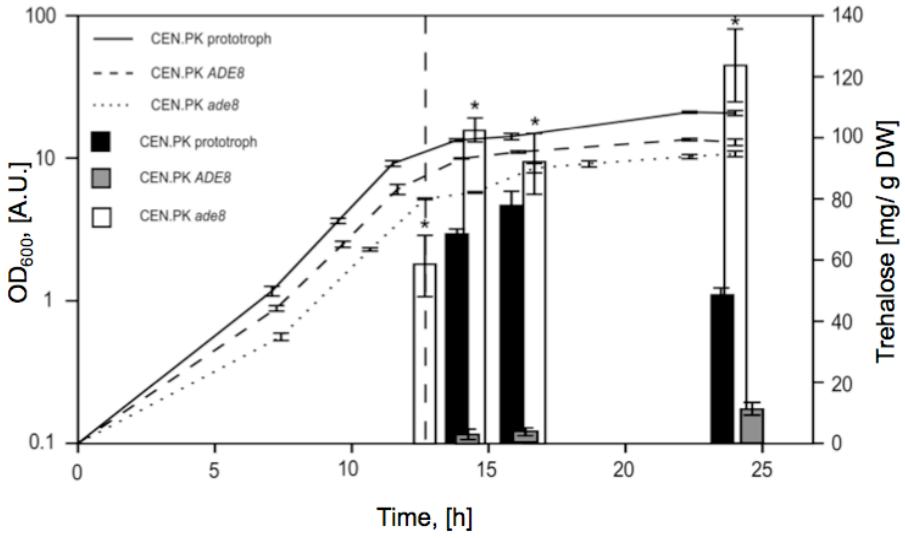


Fig. 3.4.6. Trehalose accumulation during *CEN.PK prototroph, CEN.PK ADE8* and *CEN.PK ade8* cultivation in YPD media. Trehalose was measured by anthrone method.
Lines denote strain growth curves in logarithmic scale and bars trehalose content. Error bars depict standard deviation from biological triplicates. Vertical interrupted line depicts time of adenine depletion. Asterisks depict difference between *CEN.PK ade8* and both *CEN.PK* prototroph and *CEN.PK ADE8* strains strains trehalose content as statistically significant ($p<0.05$). Adopted from Kokina *et al*., 2014.

In the case of *CEN.PK* series, trehalose accumulation before desiccation does not improve survival rates in prototroph and *ADE8* strains.

Carbon source (fermentative or oxidative) changes dynamically when yeast cells are grown in rich, glucose medium (like YPD). First of all, glucose is exhausted and then fermentation end products are oxidized. Depending on media adenine content, adenine starvation might set in different situations: when there is still some glucose left (glucose repression is on) and when there are only oxidative substrates around. Calahan *et al*., 2011 have reported on set of genes essential for desiccation tolerance in BY4741 strain background. Many of them belong to genes critical for mitochondrial metabolism. We decided to model both of those situations and test in which case mitochondrial metabolism is critical to support desiccation tolerance by adding electron transport chain inhibitor antimycin A.

We grew *W303 ade2* yeast in SD media with 2 % of glucose or ethanol as carbon source till exponential phase (OD600 =1-2), then shifted media to fresh one with combination of carbon source and/or adenine with and without antimycin A. Cultivation in the broth was done for 4 h. OD600 was measured; 1 ml of cells at OD600 was harvested, washed with distilled water. Part of cells were serially diluted and spotted on solid YPD media other - centrifuged, supernatant discarded and biomass left to desiccate at desiccator for 6 h +30°C. Cells were resuspended in appropriate volume of water (to bring suspension to OD600=1), serially diluted and spotted on solid YPD media. Survival was calculated as proportion between plate test CFU before and after desiccation. All data were normalized to survival of adenine and carbon source deficient (ade^-C^-) cultivation. Results are depicted in figure 3.4.7 for glucose grown cells and figure 3.4.8 for ethanol grown cells.

Desiccation tolerance of adenine starved cells incubated without C source exhibited 5% and 2 % survival, if precultivated with glucose and ethanol respectively.

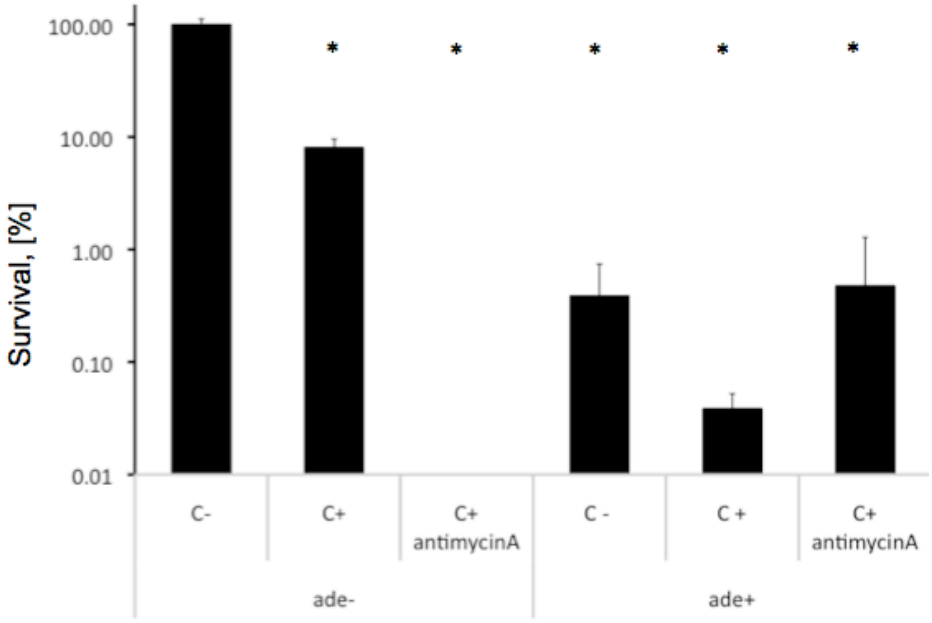


Fig. 3.4.7. Antimycin A effect on desiccation tolerance of *W303 ade2* cells in the presence and absence of adenine.
2% of glucose was used as carbon source. Antimycin A concentration was 20 ug/ml.
Bars depict average, but error bars depict standard deviations from biological triplicates. Asterixs note statistical differences (non parametrical t test, $p<0.05$), between $ade^{-}C^{-}$ and any other glucose/adenine combination.

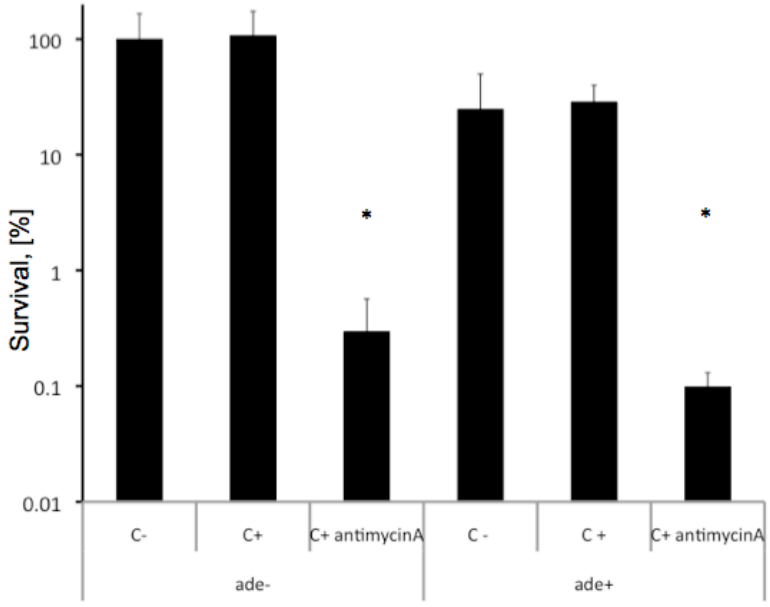


Fig. 3.4.8. AntimycinA effect on desiccation tolerance of *W303 ade2* cells in the presence and absence of adenine if ethanol is used as carbon source.
AntimycinA concentration was 20 ug/ml. Bars depict average, but error bars depict standard deviations from biological triplicates. Asterix note statistically significant differences (non parametrical t test, $p<0.05$), between ade-C- and any other ethanol/adenine combination.

Antimycin A effectively decreases survival rates independent of adenine starvation. Adenine starvation dependent desiccation tolerance effect is most profound in glucose grown cells. In ethanol grown cells desiccation tolerance is determined by presence of oxidative carbon source primarily.

3.4.2. Other auxotrophy effects on yeast desiccation stress tolerance

Many laboratory yeast strains (*W303*, *S288C*, *CEN.PK* and *FY* series) contain common set of auxotrophic markers. Adenine is among typical ones (*ade2* is marker for *W303* strain). Additionally histidine, leucine, uracil, and tryptophan (his, leu, ura, and trp) are the most common auxotrophic markers of *S. cerevisiae* strains used in physiology studies (Pronk, 2002; Da Silva and Srikrishnan, 2012). As demonstrated in previous chapter, starvation for adenine leads to elevated, desiccation tolerance.

Similarly to adenine starvation experiments, we tested if other auxotrophies has any effect on desiccation tolerance in *W303 ade2* and *CEN.PK ade8* strains. Yeasts were cultivated in SD media supplemented with all necessary amino acids till the exponenential phase (OD600 = 1-2). Then cells were harvested, washed with distilled water, resuspended in fresh SD media with particular auxotrophic amino acid omitted. After 4 h of incubation, desiccation and CFU spot test were done similarly as in the case of adenine auxotrophy. Results are depicted in fig. 3.4.9.

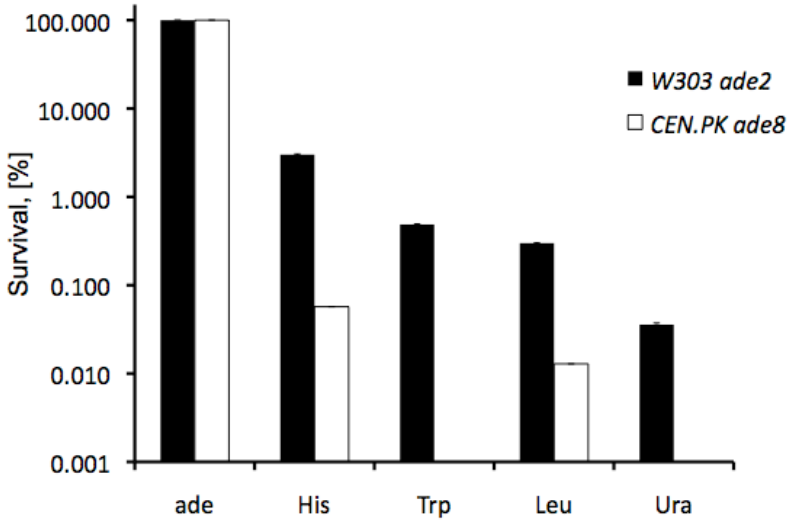


Fig. 3.4.9. Desiccation tolerance of *W303 ade2* and *CEN.PK ade8* cells after incubation of cells in SD, glucose 2 % media without particular auxotrophic agent.
Survival is normalized to adenine auxotrophy (relative survival in adenine starvation was set to 100 %). Bars depict average, but error bars depict standard deviations from biological triplicates.

Desiccation tolerance was depicted as relative to desiccation tolerance of adenine starvation (100 %). In absolute numbers survival after adenine starvation of *W303 ade2* strain and *CEN.PK ade8* were 2 % and 1.2 % respectively. Results demonstrate, that adenine starvation indeed is special, type starvation effectively inducing cell's quiescence.

Welsch *et al*., 2013 identified importance of nutrient signalling pathways to ensure desiccation tolerance. We decided to test if rapamycin treatment would have additive effect on auxotrophy starvation pretreated cells. So, we tested rapamycin effects on *CEN.PK2 ade8* strain if rapidly growing (SD full media) or starved for adenine. Results are depicted in fig. 3.4.10. Rapamycin effectively blocks TOR pathway in growing cell culture and thus increases desiccation tolerance. It does not have similar effect on cultures starved for adenine.

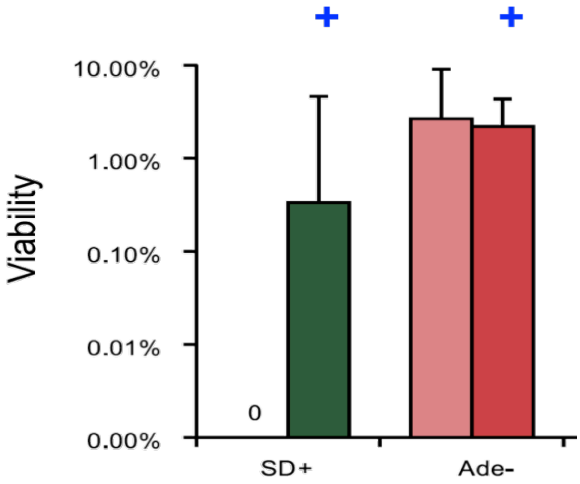


Fig. 3.4.10. TOR inhibitor rapamycin effect on desiccation tolerance in *CEN.PK2 ade8* strain when grown in full media (SD+) or starved for adenine (Ade-) + denotes cultivation with rapamycin.

3.4.3. Nutrient preference effects on S. cerevisiae desiccation tolerance

During yeast growth, yeasts actively take up preferred nutrients first and then shift to "less preferred ones". If several substrates are present, preference towards one or other sarbon or nitrogen source is made by nutrition signalling pathways PKA and TOR. To check if quality of carbon and nitrogen sources have effect on desiccation tolerance – we cultivated *CEN.PK* prototroph in SD media containing 2 % glucose, harvested cells, washed and inoculated in new medias with different carbon (glucose or galactose) and nitrogen (ammonia or urea). We cultivated cells in new media for 6 h and then desiccated cells. Survival was estimated by CFU spot test. Results are represented in fig. 3.4.11. as fold change normalized to cultivation w/o carbon or nitrogen sources.

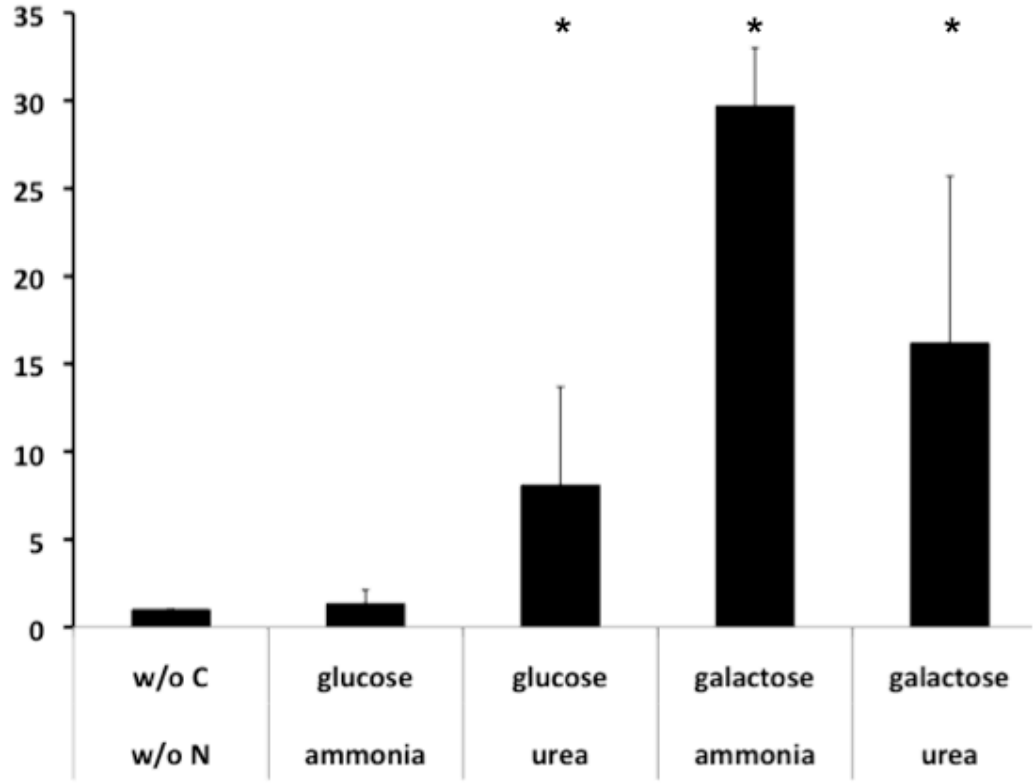


Fig. 3.4.11. Good (ammonia and glucose) and poor (urea and galactose) nitrogen and carbon source effect on *CEN.PK2* prototroph desiccation tolerance.
Results are depicted as fold change if normalised to yeast survival after desiccation when cultivated in broth w/o C or N source. Data are average of three independent cultivations, error bars depict standartdeviations. Asterix denote statistically significant differences (t test, $p<0.05$), between glucose-ammonia and any other N and C source combination.

Alterations in carbon and nitrogen sources affects desiccation tolerance. Results demonstrate, that shift from glucose to galactose gives the most impact desiccation tolerance (more than 25 fold change when compared to rapid growing yeasts cultivated in media with glucose and ammonia). Nitrogen source shift from ammonia to urea gives less increase in desiccation tolerance: 7 or 15 fold change in glucose or galactose media. Most probably, different carbon and nitrogen sources alter signallisation through through PKA and TOR pathways. Our results imply, that PKA pathway could be *master regulator* of desiccation tolerance.

3.5. Desiccation effect on beer yeast cell wall β-D-glucans

Desiccation is one of last step of product (microbial biomass, protein, vitamins, etc.) downstream processing. Among crystallization and lyophilisation, desiccation and spray drying shapes product in the state, which is stable and easily transportable.

Based on literature data, we tried to find out if desiccation *per se* can have additional application by enhancing activity of biological macromolecules. As an example of such application – we explored desiccation effects on spent brewer's yeast β-D-glucan immunoactivity.

Spent brewer's yeast carbohydrates

Starting our study we decided to explore the residual moisture dynamics of spent brewer's yeast biomas during drying process. Bottom fermenting spent yeasts from Bratislava brewery S.t.e.i.n. a/s was used. Data from two separate experiments with spent brewer's yeast samples are pooled together to construct drying dynamics over time (see fig. 3.5.1.).

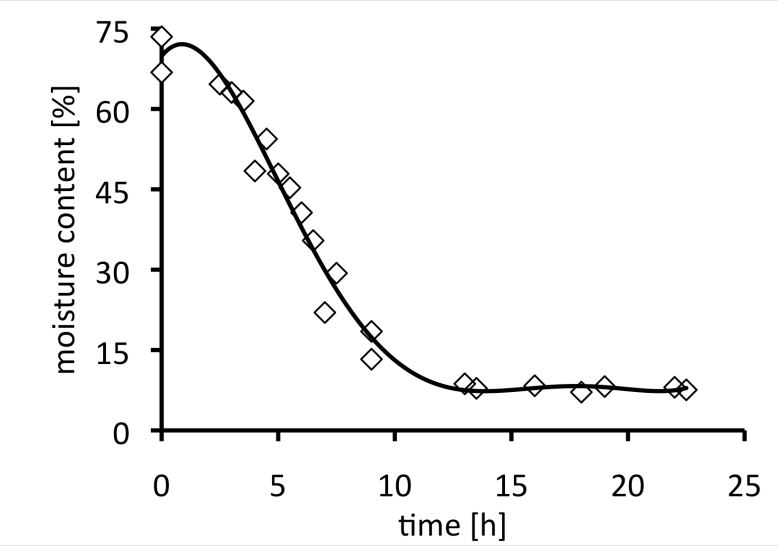


Fig. 3.5.1. Spent brewer's yeast drying dynamics over time.
5th level polinome function approximation is added for representation and not for analysis purposes.

Although variation between moisture content of 'spent yeast cream' exists (fresh spent yeast samples contained 65 % - 75 % of moisture), after 13 h of drying, all samples reached moisture around 8 %. Moisture content did not change when drying was prolonged up till 23 h (see fig. 3.5.1.). Therefore we assumed, that already 15 h is enough to obtain dry preparation with stable moisture content of the spent brewer's yeast. Meanwhile survival rate of spent brewer's yeast dropped from 90 % in fresh to less than 5 % (as estimated with primuline assay) after desiccation.

To further characterize spent brewer's yeast qualities during drying, we estimated the carbohydrate profile dynamics during desiccation process (fig. 3.5.2.).

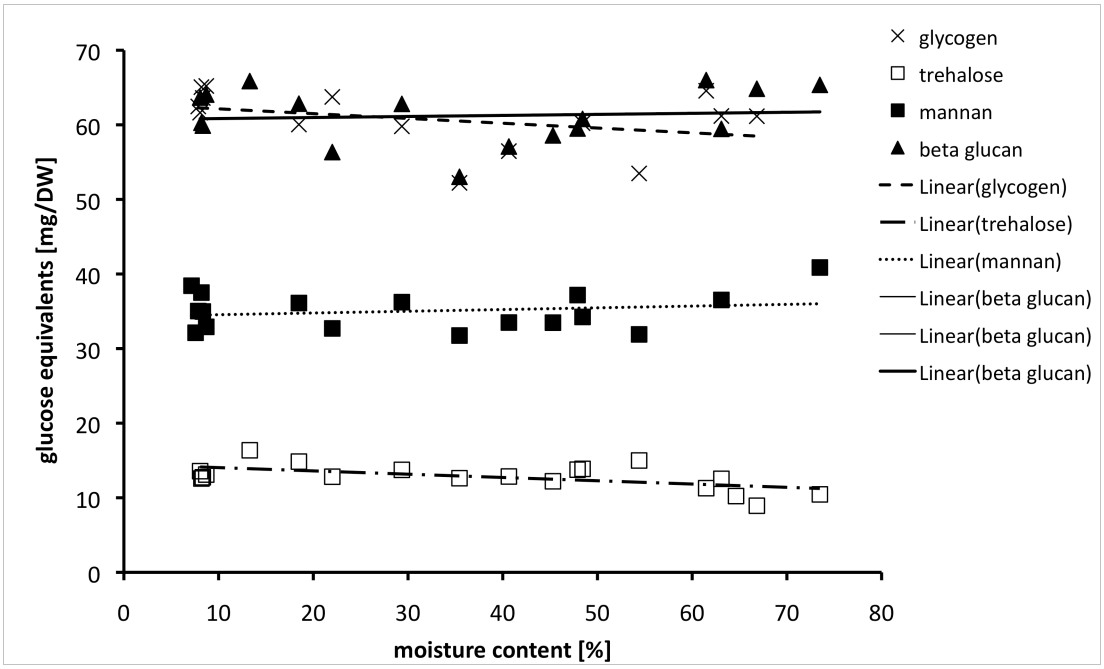


Fig. 3.5.2. Spent brewer's yeast carbohydrate profile dynamics over time.
Linear approximations are added to each carbohydrate to orientate data and not for analysis purposes.

Neither significant loss of β-D-glucans nor other carbohydrate species took place during desiccation. Concentration of different carbohydrate species in glucose equivalents in milligrams per gram of dry weight (mg/ g DW) in spent brewer's yeast biomass were 61.4 +/- 4 for β-D-glucan, 60.7 +/- 3.96 for glycogen, 35.0 +/- 2.5 for mannan and 12.8 +/- 1.75 for trehalose. All carbohydrate quantifications were done with sulphuric acid - anthrone method.

Results obtained were similar to previously published data on carbohydrate content of laboratory (Aguilar-Uscanga and Francois, 2003) and industrial yeast strains (Ouain *et al.*, 1981).

To characterize purified fresh and dried spent brewer's yeast β-D-glucans we chose C^{13} NMR analyses (fig. 3.5.3.).

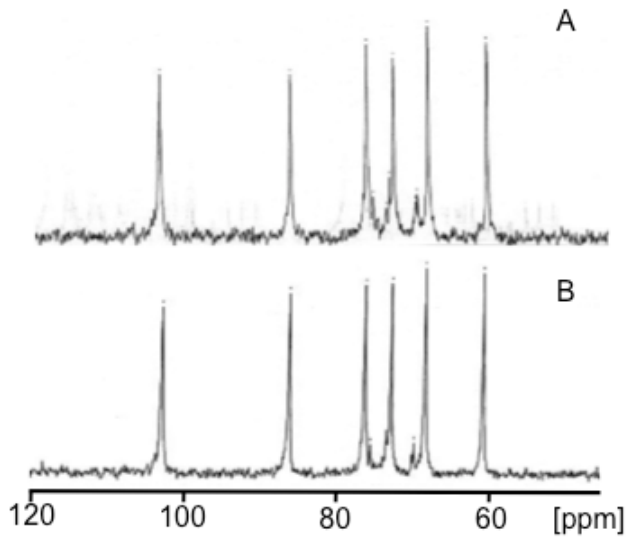


Fig. 3.5.3. Purified (1→3)- β-D-glucan ^{13}C NMR spectra from fresh (A) and dried (B) spent brewer's yeast.

C^{13}NMR spectrum reveals that drying does not affect the carbon backbone of spent brewer's yeast β-D-glucans. Both spectra testify for highly purified 1-3 linked β-D-glucans, similar to that described in Sugawara *et al*., 2004. In addition to (1→3) linked β-D-glucan, a small fraction of (1→6) branching in the extracted glucan is present (as characterized by a small peak at 70.3 ppm, see fig. 4.4.3) (Du *et al*., 2012). Even though cell walls of *S. cerevisiae* might contain up to 8 % of chitin and 60 % of mannan (% of dry cell wall mass) (Aguilar-Uscanga and Francois, 2003), only β-D-glucan specific spectrum was obtained with no glucosamine or mannose-related signals.

β-D-glucans superspiralization (Congo red assay)

Superspiralization is one of the qualitative characteristics of (1→3)- β-D-glucans. Triple helix, single helix and random coils are alternative 3D structures of (1→3)- β-D-glucans (Du *et al*., 2012, Giese *et al.*, 2008). Triple helix forms complexes with Congo red dye and characteristic red shift of maximum absorbance peak occurs. When superspiralized glucans are exposed to increased concentration of alkaline (like NaOH or LiOH), triple helix are disrupted, (1→3)- β-D-glucans remain in single helix or random coil forms and red shift of absorbance maximum disappears (Ogawa *et al*., 1972).

Purified fresh and dried brewer's yeast (1→3)- β-D-glucans demonstrated different degrees of red shift when they were incubated in solutions with increasing alkaline concentration and Congo red dye (fig. 3.5.4.).

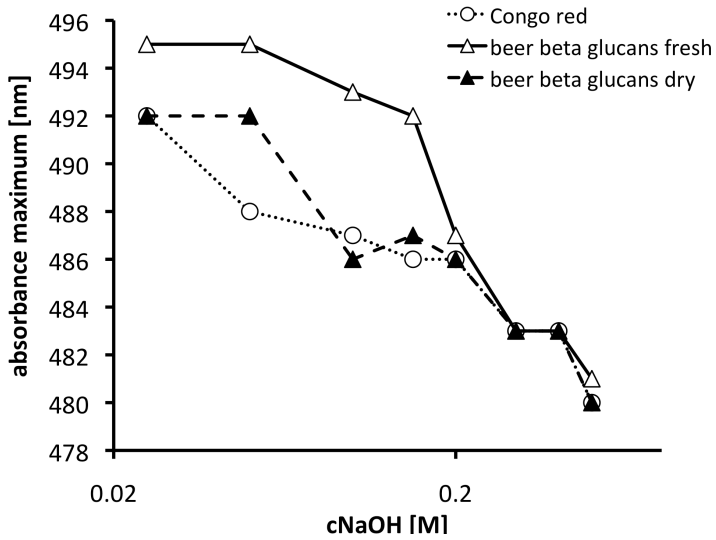


Fig. 3.5.4. Congo red assay of purified 1,3 b-glucans from fresh and dried spent brewer's yeast.

Red shift of maximum absorbance of Congo red and glucan complex is lost rapidly (at lower NaOH concentrations) in the case of β-glucans purified from dried, when compared to β-glucans from fresh, biomass (fig. 3.5.4.). Thus we conclude, that dried spent yeast biomass β-D-glucan preparations contain less triple helixes.

β-glucans immunoactivity (TNF-α induction assay)

Murine peritoneal macrophage cell culture tumour necrosis factor alpha (TNF-α) release assay is a convenient tool to estimate microbial polysaccharide immunogenic activity *in vitro* (Majtan *et al*., 2005, Howard *et al.*, 2008).

Table 3.5.1. Stimulation of peritoneal ICR murine macrophage cell culture TNF-α release (pg/ml) by pleuran, CM-fresh and dried brewer's yeast β-D-glucan.

Colours are added to highlight differences in TNF-α release. Average TNF-α concentrations +/- standard error from technical triplicates are presented.

		Pleuran from *Pleurotus ostreatus*		**CM- glucan from fresh brewer's yeast**		**CM- glucan from dry brewer's yeast**		Released TNF-α (pg/ ml)
		3 h	6 h	3 h	6 h	3 h	6 h	
Doses	12.5	69 ± 14	67 ± 14	7 ± 6	111 ± 17	23 ± 9	144 ± 19	0-100
ug / ml	25	87 ± 15	91 ± 16	6 ± 5	174 ± 20	49 ± 12	152 ± 19	100- 160
	50	90 ± 16	78 ± 15	1 ± 1	52 ± 13	73 ± 14	193 ± 21	160-220

We chose pleuran, β-D-glucan purified from oyster mushroom *Pleurotus ostreatus* (immunoactive ingredient of Imunoglukan®) as a positive control to compare fresh and dried brewer's yeast carboxylmethylated (CM) β-D-glucans. Both fresh and dried spent brewer's yeast CM β-D-glucans revealed TNF-α induction comparable to pleuran (see Table 3.5.1). In addition, spent brewer's yeast CM β-D-glucans revealed higher TNF-α inducing activity than pleuran starting from 6 h. Immunostimulating activity of CM β-D-glucans purified from dry spent brewer's yeast exceeded that of CM β-D-glucan from fresh spent brewer's yeast (compare shaded highlights in Table 3.5.1.).

4. Discussion

4.1. Desiccation tolerance depends on culture growth phase but primarly - on nutritional status.

In this work we aimed to prove the effect of nutritional status on yeast desiccation tolerance. Yeast cultivation in glucose containing media gives an opportunity to sample cells of various nutritional states: during exponential phase – fermentative growth when ample amount of glucose is available, diauxie and postdiauxie – oxidative growth on ethanol and other fermentation products and finally stationary phase – when carbon starvation has set in and no culture growth happens (Werner-Washburne *et al.*, 1993).

In our experimental setup we chose two postdiauxic phase time points (early and late - 24 and 48 h of cultivation) what lead to different values of desiccation and oxidative stress markers. Despite the fact, that both of these points belong to postdaiuxie, distinct desiccation phenotypes was observed.

Many publications dedicated to *S. cerevisiae* desiccation tolerance report results on "stationary phase cells" and "exponential phase cells" without exactly describing cell growth rate or nutritional status at sampling time (Espindola *et al*., 2003, Trofimova *et al.*, 2011). Here our growth dynamics analyses imply, that for typical desiccation model yeast strains (like *SC14* and *BY4741*), more than 50 h is needed to achieve true stationary phase (even at 50 h ample amount of substrate is present). This fact is neglected in many publications where 48 h of cultivation mode is used for "stationary phase" growth. To correctly interpret desiccation tolerance results and to compare them with typical features of "exponential", "diauxic" or "stationary" phase cells – information on culture growth parameters (growth curve, extracellular metabolites) at specific sampling time should be provided.

Results from *SC14* revealed that there is statistically sound difference between desiccation tolerance of early and late postdiauxic phase cells (fig. 3.2.1.). In the same time, we observed high TBARS levels in *SC14* culture of low desiccation tolerance (early postdiauxie phase cells) when compared to desiccation tolerant culture (late postdiauxie phase cells). This pattern repeated also *BY4741* strain background (*BY4741* and corresponding *tps1* deletant). Herdeiro *et al*., 2006, and Espindola *et al.,* 2003 published similar effect in BY4741 strain where TBARS is inversely related to desiccation tolerance.

The core principle of TBARS assay is TBA reaction with malonyldialdehyde (MDA) forming red or purple complexes, which are possible to quantify by spectrophotometry or spectrofluorometry.

MDA is formed due to peroxidation of unsaturated fatty acids. Lipid peroxidation *per se* is "chain reaction" starting from few "reaction centers" in the phospholipid membrane. MDA is product of polyunsaturated fatty acid (having two or more double bonds) peroxidation (Yin *et al*., 2011). MDA is water-soluble and potentially can react with nucleic acids and proteins in any location within the cell. Therefore lipid peroxidation leads to wide cell damages and even cause diseases: lipid peroxidation is central element in neurodegenerative diseases progression, like Parkinson and Alzheimer's disease (Ayala *et al*., 2014).

TBARS assay is routinely used to estimate lipid peroxidation. This reaction is used to estimate *S. cerevisiae* lipid peroxidation after H_2O_2 and heat stress (Steels *et al*, 1994), heavy metal stress (Howlett and Avery, 1997) and desiccation (Franca *et al*., 2005). On contrast to other yeasts, *S. cerevisiae* membranes does not contain polyunsaturated fatty acids (Ejsinga *et al*., 2009). Therefore TBA most probably reacts to different peroxidation products from different sources, like alyl aldehydes from peroxidation of oleic and palmitic acids. Other substances have different reactivity towards TBA than MDA (Kosugi and Kikugawa, 1989). These two facts (lack of polyunsaturated fatty acids and different reactive activity of different peroxidation products to TBA) make TBARS assay in *S. cerevisiae* semi-quantitative with respect to MDA and context dependent.

Proportion of different unsaturated fatty acids in *S. cerevisiae* cell membrane changes with regards to substrate and growth mode. For example, in the presence of ethanol, C18:1 species (oleic acid) dominate while C16:0 and C16:1 species (palmityl and palmitoleic) are more common when fermentative substrate is available. Additionally, lipid saturation increases in stationary phase when carbon source becomes scarce (Henderson *et al*., 2013). Different fatty acids are prone to peroxidation and subsequent autooxidation ("chain reaction") to different degree, polyunsaturated ones are the most vulnerable, followed by sterols and oleic acid. Comparatively high energy should be provided for palmityl acid autooxidation, thus making this reaction in yeast cell unlikely (Pratt *et al.*, 2011, Yin *et al*., 2011).

Zikmanis *et al*., 1982 reported on yeast lipid composition and it's relation to desiccation tolerance. Experiments were done on strain *SC14* when harvested at different time points (exponential growth, early, late postdiauxie, stationary phase) and different carbon sources. Unsaturation degree of membrane fatty acids of *SC14* was inversely correlated to desiccation stress tolerance: as more unsaturated the membranes were, as more desiccation intolerant culture became (Zikmanis *et al*., 1982).

Our TBARS results on early and late postdiauxic *SC14* and *BY4741* cells corroborate idea on presence of peroxidation vulnerable membranes while oxidative carbon sources (ethanol, glycerol, organic acids) are available in large amounts (early postdiauxie) and peroxidation robust membranes when oxidative carbon sources become scarce (late postdiauxic phase). Media metabolite dynamics during *SC14, BY4741* and *tps1* cultivations are depicted in results fig. 3.1.2 A-F. Additionally, TBA reactive species has tendency to increase over cultivation time (when comparing TBARS in *BY4741 tps1* mutants in early and late diauxie), however, their survival, when estimated by primuline, increases. Peroxidised membranes are more leaky than intact (Asayama *et al*., 1992, Shamrock and Lindsey, 2008). If increase of the TBA reactive species would came just from peroxidised (leaky) membranes, then decrease of primuline based survival rates would be expected; however, we saw statistically significant primuline based increase in desiccation tolerance thus underlying existence of lipid unrelated sources of TBARs in *S. cerevisiae* cells (fig. 3.2.5 and 3.2.7).

While TBARS levels were comparatively high in WT yeasts (*SC14* and *BY4741*) in early postdiauxic phase when compared to late postdiauxie phase cells. TBARS level in *tps1* cells did not differ between early and late postdiauxic time points, even though survival rate increased significantly. Herdeiro and colleagues showed proofs on trehalose working as antioxidant and preventing cell membrane peroxidation (Herdeiro *et al.*, 2006). Here we see, that this might be the case for WT cells in late postdiauxic phase, where high trehalose content is accompanied by low TBARS and high survival rate. Nevertheless, comparatively high survival rate is retained in *tps1* mutant strain without significant amount of trehalose. This would indicate, that trehalose might work as antioxidant *in vivo* (if produced), however this activity is not discriminating for yeast to remain desiccation tolerant (viability is significantly higher for *tps1* in late postdiauxic cells with no trehalose and higher TBARS levels).

4.2. Trehalose is not sufficient for desiccation tolerance

Trehalose is one of two major storage carbohydrates of yeast cells (Parrou and Francois, 2001). Thus far trehalose accumulation has been described as necessary prerogative for yeast desiccation tolerance (Crowe *et al*., 1997). Also additional, desiccation tolerance promoting - antioxidative activity of trehalose has been demonstrated (Herdeiro *et al.*, 2006, Shamrock and Lindsey, 2008).

In the case of *BY4741 tps1* mutant we saw increase in desiccation tolerance in the absence of trehalose, meanwhile little increase in GSH level in *tps1* strain cells from late postdiauxie was observed.

Trehalose synthesis starts from glucose-6phosphate by joining it to UDP-glucose thus forming trehalose-6 phosphate. This reaction is catalysed by trehalose synthase, Tps1p (Francois and Parrou, 2001).

There are several reactions competing for glucose-6 phosphate: trehalose synthase (Tps1p), glucose-6 phosphate dehydrogenase (Zwf1p), glucose-6 phosphate isomerase (Pgi1p).

Increase in glucose -6 phosphate concentration in *S. cerevisaie* cells has been reported in *tps1* mutants (Ernandes *et al*., 1998). Increase in glucose-6 phosphate concentration has been reported also for other fungi *tps1* mutants, like plant parasite *Magnaporhe grisea* (Wilson *et al.*, 2007). Besides, Diaz-Ruiz *et al.*, 2008 have demonstrated, that increased concentration of hexose monophosphates (including glucose 6 phosphate) has enhancing effect on mitochondrial oxygen consumption.

Glycolisis in *Fungi* (including *S. cerevisiae*) function not only for energy purposes, but also provide metabolism with reduced cofactors (NADPH) for antioxidative reactions. Oxidative stress induced carbon flux rerouting towards pentose – phosphate pathway (PPP) in the level of triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase has been reported. By this mechanism increased flux through glucose-6 phosphate dehydrogenase reaction is suspected, what in turn provides metabolism with more NADPH (Ralser *et al*., 2007). We hypothesize, that effects alike has happened in *tps1* strain, where in the case of surplus glucose-6 phosphate increase in activity of Zwf1p is observed, see fig. 4.2.1.

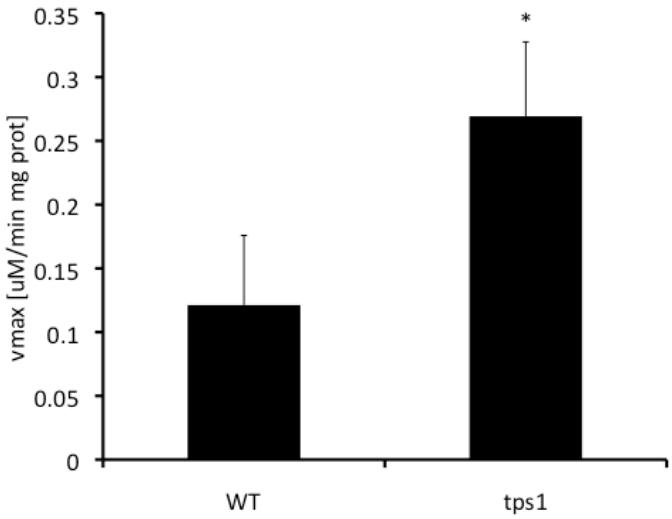


Fig. 4.2.1. BY 4741 (WT) and *tps1* mutant v_{max} for glucose 6 phosphate dehydrogenase reaction. Proteins were purified from yeast cultures at (early postdiauxie 24 h of cultivation).
Enzyme activities are represented by mean values of biological triplicates. Error bars show standard deviations. Star denotes statistically significant difference (t test, $p<0.05$)

Espindola and colleagues have obtained the same results for G6PDH activity in *BY4741* and *gsh1* strain. Their results of increased activity of glucose 6-phosphate dehydrogenase in glutathione deficient strain are in line with carbon rerouting idea in the case of increased oxidative stress (Ralser *et al.,* 2007, Espindola *et al.,* 2003). In our case, increased G6PDH activity would explain increased concentration of GSH in *tps1* strain when compared to BY4741, especially in early postdiauxic phase (fig. 3.2.8.). Similarly, trehalose independent, but growth phase dependent (late postdiauxie phase cells are more desiccation tolerant than early) desiccation tolerance pattern was demonstrated also for *tps1* mutants in other strain backgrounds (*CEN.PK* and *W303*) (Tunnaclife and Ratnakumar, 2006).

I conclude, that *tps1* mutation has other side effects, which might work to support desiccation tolerance (like increased G6PDH and subsequent antioxidative activity).

Trehalose levels in yeast cells are determined as an outcome of dynamic equilibrium of trehalose synthesis and hydrolysis (Hohman and Mager, 2003). Various models have been proposed for coordinated trehalose synthesis, breakdown and accumulation. External signals can induce its breakdown through main nutritional signaling and stress response pathways: cAMP dependent PKA pathway, (Thevelein *et al*., 1999) and *TPS1* transcription regulation by Msn2/4p (Martinez-Pastor *et al.,* 1996, Boy-Marcotte *et al.*, 1998, Zahringer *et al.,* 2000). The activity of trehalase and trehalose synthase is regulated by the PKA pathway, which is up-regulated in the presence of glucose (Winderickx *et al.*, 1996). An increase in trehalose levels in adenine-starved cells would indicate down-regulation of PKA (due to possible AMP depletion) and consistent down-regulation of trehalase activity and up-regulation of

trehalose synthase. Results of other starvations show, that both sides of the trehalose metabolism - synthesis and hydrolysis are stimulated in yeast cells, when starved for carbon, nitrogen, or phosphorous (Klosinska *et al.,* 2011). We have measured trehalase activity in *CEN.PK ade8* strain and it shows the same tendency – trehalose accumulation is accompanied by elevated trehalase activity (results not shown) during adenine starvation. Nevertheless, desiccation tolerance and sharp increase in trehalose content coincides in *ade8 or ade2* mutants (fig. 3.4.1. and 3.4.3. to 3.4.6.). Additionally, in *CEN.PK* prototroph, increase in trehalose content happens at the very end of exponential growth phase, however, its desiccation tolerance is negible (fig. 3.4.5. and 3.4.6.). Obviously desiccation tolerance is primarily determined by other factors, not trehalose content alone.

4.3. *S. cerevisiae* redox engineering for enhanced glutathione turnover.

Many environmental stresses set in during industrial dry yeast preparation process. Salt, osmotic and oxidative stresses are identified to be involved when preparing dry wine yeasts (Garre *et al.*, 2010). A set of oxidative markers (TBARS, drop of GSH, and increase in ROS) is identified as being common to all desiccated organisms (Franca *et al*., 2007). Also our results from *BY4741, tps1* and *SC14* cultivations revealed changes in oxidative markers over cultivation time and depending on desiccation. Our results on trehalose fortified notion, that it is not obligatory necessary for desiccation tolerance, so we attempted to do "oxidative stress engineering". Glycerol metabolism seemed to be attractive point for NADPH engineering, since many fungi posess "glycerol cycle". Existance of "glycerol cycle" in *S. cerevisiae* has not been proven.

Sequence analyses together with literature data on respective enzymatic activities revealed, that also *GLD1* from *T. atroviridiae* could serve as a tool for glycerol cycle construction in *S. cerevisiae.* We propose, that high degree of homology among each of NADP dependent fungal glycerol dehydrogenases might be used to predict the eventual enzyme kinetic properties before *in vitro* characterization (Liepins *et al*., 2006).

NADP dependent glycerol dehydrogenase activities have been attributed also to a set of *S. cerevisiae* gene products (*ARA1, GCY1, GRE3 and YPR1*) (Norbeck and Blomberg 1997, Celton *et al.,* 2012). However, we failed to observe any specific glycerol dehydrogenase activity comparable with introduced Gld2p activity (see zymogram, fig. 4.3.3. B). Some *S. cerevisae* cell extract NADP dependent glycerol dehydrogenase activity is present, however, specificity for glycerol and NADP is still under question. *S. cerevisiae* enzymes cluster together with NADP dependent glycerol dehydrogenases forming DL-

glyceraldehyde and NADPH (fig. 4.3.2.). *In vitro* studies of *YPR1* revealed high specificity towards conversion of NADPH and dl-glyceraldehyde to glycerol. Reaction with DHA to glycerol was 100 fold lower and reaction from glycerol and NADP was even 4000 fold smaller (Ford and Ellis, 2002).

Glutathione has been identified as potential metabolic engineering hot spot to increase lignocellulolytic substrate conversion (Ask *et al*., 2013). Also yeast glutathione metabolism has been identified as engineering spot for oxidatively more stable wine production (Mezzetti *et al.,* 2014). Our redox cofactor engineering was succsesfull – GSH: GSSG ratio was increased in yeast strains with introduced glycerol cycle. Unexpectedly, this effect did not affect strains desiccation tolerance. Therefore – we conclude, that improvement of yeast oxidative stress tolerance via enchancing GSH: GSSG ratio, does not help to improve cell'c desiccation tolerance. However, glutathione engineering might have other applications for nonconventional substrate fermentation or wine production.

4.4. Cell nutritional effects on desiccation tolerance

For free living yeasts in their natural habitats (sugar rich fruits and saps) times of famine are interrupted with short intervals of feast: long lasting starving is interrupted by relatively short intervals of nutritional abundance. Usually "natural" starving occurs because of carbon, nitrogen, phosphorous or sulphur shortages. In "natural habitats", microorganisms spend their life in scarcity, where one or several simultaneous nutritional shortages are present. Term "quiescent state" is used to describe yeast cuture during starvation (De Virgilio, 2012).

Similar to "natural conditions", laboratory cultivations can lead to starving. When batch cultivation starts, surplus amount of nutrients is present, which gradually gets depleted. Cell proliferation ceases when one of the nutrients become limiting and starvation sets in. If "basic" nutrients are depleted (C, N, S or P sources), cell cycle gets effectively stopped, culture enters stationary growth phase and obtains phenotype of multiple stress resistance (Werner-Washburne *et al*., 1993, De Virgilio, 2012, Boer *et al.*, 2008, Klosinska *et al.,* 2011).

Laboratory yeast strains are often auxotrophs, carrying one or several auxotrophic markers. Lack of auxotrophic agent leads to stop of cell proliferation. When working with auxotrophic strains, additional care should be taken in order to provide sufficient amount of auxotrophic agents to sustain cell growth (Pronk, 2002).

Depending on broth composition – a distinction can be made between synthetic and full (rich) media. The former usually are composed of defined set of chemicals essential for microbial growth whereas latter contains bacterial, plant, animal cell hydrolisates or extracts. While synthetic broth provide enough of necessary factors for microbial growth, rich medias exact composition might have large variations depending on component's vendor and lot. Typical adenine supplement to complement adenine auxotrophy for *S. cerevisiae* in synthethic media is between 20 and 40 mg/L (Kokina *et al*., 2014). Rich, yeast extract based medias have variable adenine content, depending on yeast extracts vendor and lots they might contain sub-optimum (from 3 – 50 mg/L) amount of adenine (Van Dusen *et al*., 1997). Due to variances in adenine content of "rich" medium, sooner or later adenine depletion sets in and adenine starvation phenotype can override other cell effects of interest. The same applies for other auxotrophic markers – also their specific phenotype might set in when medium adenine content depletes.

On contrary to other authors, which have described auxotrophic starvations (uracil and leucine) as burden for cell's viability. (Boer *et al.*, 2008), our results imply, that adenine starvation is different in many aspects. When compared to other auxotrophic starvations, adenine auxotropy leads to elevated starvation stability, effective halt of cell cycle and increased desiccation (fig. 3.4.1. and fig. 3.4.3. – 3.4.6.) tolerance (Kokina *et al.*, 2014). Besides, different auxotrophies lead to different level of stress tolerance.

Interestingly, chronological life span (or half life) of leucine auxotrophs during leucine starvation is prolonged if active mitochondrial metabolism is present (cultivation on glycerol and ethanol) (Boer *et al*., 2008). Calahan *et al.*, 2011 has demonstrated, that functional mitochondria are important to sustain desiccation tolerance of the yeast. This was tested by two methods – genetical *petit* mutants and respiratory chain blocking by cytochrome complex inhibitor myxotiazol. We have seen similar effects on adenine starved when adding antimycin A (complex III blocker).

It turned out, that adenine auxotrophy induced desiccation tolerance is pronounced just in the case of cultivation with glucose as carbon source (fig. 3.4.2., 3.4.7. and 3.4.8.). Besides, desiccation tolerance appeared to be strongly respiration dependent, as demonstrated by antimycin A addition and independent of substrate (fermentative or oxidative).

Although we used *CEN.PK* and *W303* strain backgrounds to test effect of starvation for different auxotrophic agents on desiccation tolerance, there might be additional auxotrophic starvation in place. Methionine, leucine, histidine and uracil are auxotrophic

markers for *BY4741* strain. There are literature data on elevated methionine consumption by *BY4741* strain already after 24 h (Mülleder *et al.*, 2012), therefore methionine starvation might set in already in early postdiauxic phase when sampling of *BY4741* and *tps1* were done. Methionine starvation has been characterized by: increased longevity, decreased ROS generation (Sanz *et al.,* 2006). Methionine auxotrophs posess increased viability during prolonged starvation (10 days or more); similar physiological and transcriptional response as in phosphate and sulphate starvation (Unger and Hartwell, 1976, Petti *et al.,* 2011). In this way, increase of BY4741 and it's *tps1* mutant desiccation tolerance over time can be interpreted as gradual set in of single or even multiple auxotrophic starvations.

After 24 h of BY4741 cultivation in YPD media, we checked auxotrophic agent content of spent media qualitatively. We sterile- filtrated spent media and supplemented it with one of auxotrophic markers: histidine, leucine, methionine, uracil and glucose. The results are depicted in fig. 4.4.1.

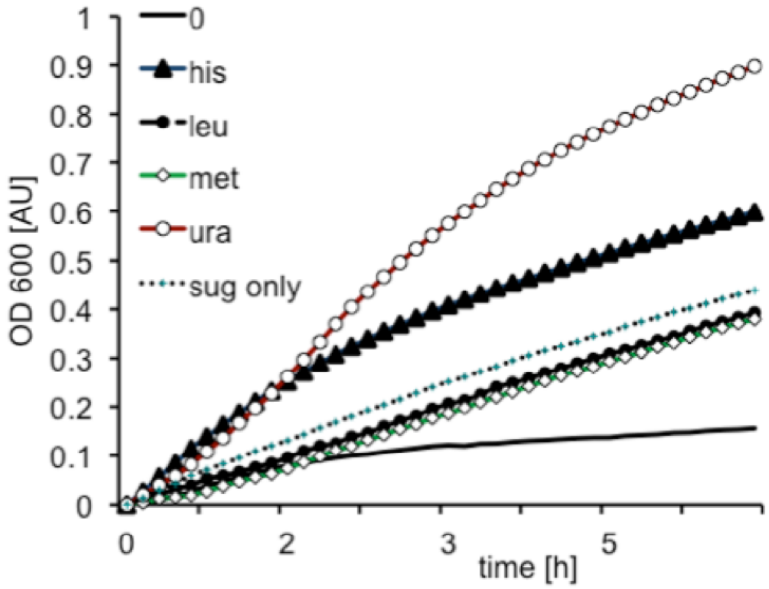


Fig. 4.4.1. BY4741 growth on spent media supplemented with auxotrophic agents.

Each cultivation plot is average of 5 paralel cultivations. Standart deviation is max 10 % of measured values.

For our YPD media (contaning yeast extract and bacteriological peptone from Biolife, Italy), uracil and histidine are auxotrophic agents depleted after 24 h of *BY4741* growth. Similar situation might happen with other strains in rich media, therefore one cannot relay only on yeast extract and peptone composition, especially in prolonged batch cultivations.

Welsch *et al*., 2013 identified desiccation stress uniquely related to yeast nutrient signalling. PKA and TOR inhibition or effector deletion leads to increased desiccation tolerance. On another hand, TOR (mammalian rapamycin sensitive TOR complex) has been related to sensing of intracellular amino acids and purines (Dennis *et al.,* 2001).

Rim15p which is identified as common activator for postdiauxic (PDS) and stress responsive (STRE) genes is repressed both from PKA and TOR pathways. In this way repression of any of those pathways (due to change in carbon or nitrogen substrates or in the presence of specific inhibitors) would lead to activation of one or both groups of genes.

When we tested effect of rapamycin on desiccation tolerance of adenine or uracil starved cells, it revealed, that adenine starved cells are insensitive to rapamycin. We propose, that this effect coud be related to decrease PKA signalling due to decreased cAMP supply.

When doing substrate shifts – presence of any of poor nitrogen or poor carbon sources enchanced desiccation tolerance, most probably through decreased PKA and TOR signalling. Our results demonstrate that nutrient signalling related effects (rapid media shift, adenine starvation) on desiccation tolerance ar far more profound than prolonged cultivation effects (late versus early postdiauxie).

In large scale genetic screens more than 10 fold difference in desiccation tolerance is evaluated as significant signal, while less than 2 times difference is perceived as poor and nonimportant (Calahan *et al.*, 2011, Welch *et al.*, 2013). Auxotrophy and nutrient quality effects gives 20 and more fold increase in yeast desiccation tolerance.

We conclude, that auxotrophic starvation and carbon/ nitrogen source quality remarkably contribute to yeast population's desiccation tolerance and thus should not be overlooked.

4.5. Immunostimulating activity of dried spent yeast beta glucans

Spent brewer's yeasts are characterized as old, large cells, usually flocculant. In the case of bottom fermenting brewer's yeasts, especially during maturation in tanks, we deal with senescent, anaerobic yeasts. One of the mechanisms responsible for flocculation induction is nitrogen starvation sensed and mediated through TOR pathway (Ogata, 2012). In this way flocculent lager yeasts are another example of yeasts under specific nutrient starvation.

Similarly to bacterial cell wall polysaccharides or lipopolysaccharides (LPS), yeast β-D-glucans possesses immunostimulatory and anti-tumour properties (Vannucci *et al*., 2013).

Differences in TNF-α induction between pleuran and CM β-D-glucans might be related to different affinity to macrophage receptors: large, insoluble β-D-glucans (like pleuran) attach to a group of Toll-like receptors and Dectin-1 inducing rapid (within minutes) TNF-α secretion. Meanwhile macrophages ingest these β-D-glucans. Relatively small, soluble β-D-glucans (like CM β-D-glucans) attach to few Dectin-1 receptors and induce TNF-α secretion; these molecules probably are not imported into macrophages (Goodridge *et al*., 2011, Batbayar *et al.*, 2012). Thus, we conclude, that spent brewer's yeast CM β-D-glucans might reside outside macrophages for longer time and eventhough it's TNF-α induction is comparatively weak at low concentrations, it saturates receptors and works effectively in higher concentrations outcompeteing pleuran. Zymosan derived soluble β-D-glucans could be used in applications, where slow and prolonged immunostimulation is required.

Treatments such as freeze-drying, air air-drying, and alkali treatment affect *S. cerevisiae* β-D-glucan immunogenic activity (Hromadkova *et al.*, 2003). Immunogenic activity of β-D-glucans is related to their 3D supercoil structure (Kogan, 2000). It is noted, that side-chain modifications (carboxylation or phosphorylations) do not affect β-D-glucan superspiralization (Williams *et al*., 1991).

Small alterations in the degree of β-D-glucan superspiralization can lead to vast differences in their biological activity, as demonstrated by fluorescence resonance energy transfer (FRET) analyses with laminarin (Young *et al*., 2000). Our results from the Congo red assay demonstrate that β-D-glucans from dried brewer's yeast are less superspiralized than fresh brewer's yeast β-D-glucans, nevertheless their immunostimulating activity when tested in murine macrophage TNF-α induction model is the same or even higher (see highlights in Table 3.5.1.). This observation is in line with previous results published by Young *et al.,* 2003 on relaxed triple helix as being an immunologically more active form of β-D-glucan than it's strictly superspiralized form.

The microstructure of *S. cerevisiae* cell surface changes during slow, gradual desiccation (Beker and Rapoport 1987). Also changes in the cell wall-related gene transcription pattern during the slow desiccation of yeast are observed (Singh *et al*., 2005). Our results demonstrate that desiccation does not affect carbohydrate content of brewer's yeast and improves immunogenic properties of cell wall β-D-glucans, when compared to fresh biomass β-D-glucans or pleuran (already commercialized β-D-glucan purified from oyster mushroom, *Pleurotus ostreatus*, Jesenak *et al.*, 2013). Since spent brewer's yeast is

characterized as being a by-product of low added value (if recycled in biogas tanks or livestock feed), our results suggest to use this yeast as a potential and cheap source of pharmaceutical-grade products (immunoactive substance CM β-D-glucan).

In human cells, microbial cell wall polysaccharides (including β-D-glucan) induce immunoresponse by attaching to toll-like receptor 4 (TLR4). These receptors respond to a variety of endo- and exo-ligand molecules (glucans, LPS, etc.). TNF-α secretion is an effect of TLR4 activation (Oblak and Jerala, 2011). Historically TNF-α was characterized as a unique factor responsible for tumour necrosis induction. However, it turned out to be one of several cytokines with various functions both in promoting and attenuating tumourogenesis (Waters *et al.*, 2013). Interestingly, TNF-α dependent tumour necrosis is possible in a sarcoma model, but tumour-promoting effects are observed in other cancer *in vitro/in vivo* systems (Balkwill, 2009). Our results demonstrate that desiccation pretreatment is a way to increase CM β-D-glucan mediated TNF-α induction. However, additional research should be done to specify the application range (tissues, cancer types) sensitive to spent brewer's yeast β-D-glucans mediated TNF-α secretion. If successful, this would promote a specific usage of spent brewer's yeast CM β-D-glucans as a feasible by-product of the beer industry.

4.6. Conclusions and future prospects

As previously demonstrated, yeast desiccation tolerance relates to nutrient status:

- prolonged cultivation in postdiauxie increases desiccation tolerance approximately 2 times,
- shift of carbon and or nitrogen sources can affect desiccation tolerance up to 25 times,
- adenine starvation increase desiccation tolerance by more than 100 times.

Starvation leads to TOR and/or PKA pathway derepression, these effects can be simulated by PKA and TOR "common target" *sch9* deletion (Welsh *et al.*, 2013). Our observations corroborate these results by proving, that nutrition source dependent desiccation tolerance is wide spread: we have seen starvation and carbon source related effects in diploid and haploids, prototrophs and auxotrophs of different strain backgrounds (*SC14*, *W303*, *CEN.PK* and *BY4741*). We have proved, that independent of strain background adenine auxotrophy cause sharp increase in desiccation tolerance. Our core findings are summarised in simple model describing gradual decrease of PKA and TOR pathway activity during prolonged cultivation of *S. cerevisiae* (fig. 4.5.1.). Maximum desiccation tolerance is

achieved when preferred carbon and nitrogen sources are depleted (thus PKA and TOR pathways derepresses stress response genes and "quiescence phenotype" prevails).

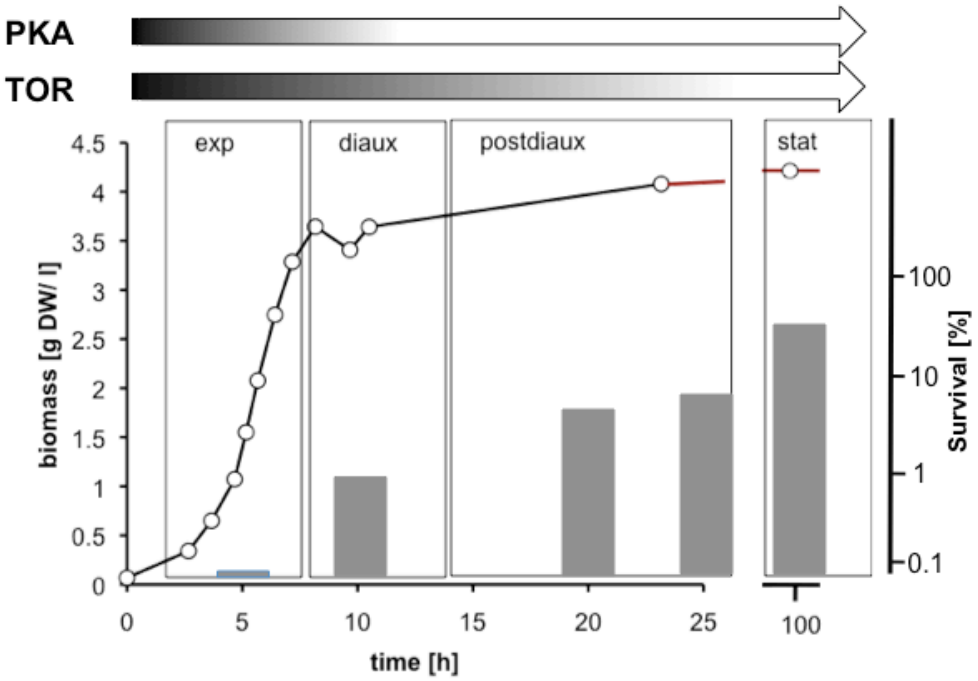


Fig. 4.5.1. Suggested relation of interplay among protein kinase A (PKA), target of rapamycin (TOR) pathway's activities throughout *S. cerevisiae* cultivation and desiccation tolerance.
Arrows on the top depict activity of TOR and PKA pathways during batch cultivations. Dark colour indicates strong activity (in the case of good nitrogen and carbon source), while pale colour indicates derepression of particular nutrient signalling pathway.
Bars represent desiccation tolerance (%); here exact time, survival, biomass growth values are of illustrative value only.

Nowadays, growing baker's yeast of industrial scale, shift from N rich to N poor media is used to effectively stop yeast cell cycle, increase it's trehalose content and stress (including desiccation) tolerance. We propose, that similar strategies can be applied to reach the same goal by inducing starvation for other nutrients. Auxotrophy could be one of the engineering targets. Besides, parts of nutritional signalling pathways have proven to be sensitive spots to control desiccation tolerance (Welch *et al.,* 2013, Calahan *et al*., 2011).

There are, however, open question regarding mechanisms how signal of lack of particular nutrient (adenine) is translated into stress resistant quiescent phenotype ready for many abiotic challenges. Indeed, in opposite to basic nutrients, there is not a specific "adenine sensor" inside the yeast cell. We propose two mechanisms which might play a role here – decrease of PKA signalling due to drop of cAMP concentration in cytoplasm; or if disrupted, adenine synthesis pathway accumulates metabolic intermediates, which might work as inhibitors for some kinases in PKA pathway. Interestingly, AICAR, an adenine and

histidine *de novo* synthesis pathway intermediate is used in animal cAMP signalling cascade studies as cAMP analogue (Kwon *et al.*, 2010).

Another challenge would be to find out if there is specific expression profile related to adenine starvation. If yes – gene expression pattern would give a hint on master regulators responsible for set in of adenine dependent stress resistant phenotype.

Recent results on transcriptional responses of various starvations (carbon, phosphorous, nitrogen) show, that they overlap poorly. Moreover, transcriptional response relation to starved cell's phenotype is vague because additional effects from nutrient availability set in (Klosinska *et al*., 2011). Based on previous reports on starvation physiology, we conclude, that, transcriptomics alone would not be sufficient to understand observed phenotypic effects.

The aim of this study was to identify nutritional (including adenine) related effects on desiccation resistance. The quest for specific transcriptional and metabolomic signature specific for adenine starved cells would be logical continuum of this study. Combining several –omics (transcriptomcs, metabolomics, etc.) approaches would have explanatory power to clarify interesting physiological effects of adenine starvation.

Additionally, our data warn, that rich media is not "safe solution" for auxotrophic cell cultivation unless precise content of each media component is known. Especially for prolonged cultivations - auxotrophic starvation can set in and mask other physiological effects.

5. Conclusions

1. Prolonged cultivation in postdiauxic phase enhances *S. cerevisiae* desiccation tolerance; it's indepenent to strain genetical background or trehalose synthesis.

2. Growth conditions strongly affect oxidative stress markers (GSH and TBARS) levels before desiccation.

3. "Glycerol cycle" can be used as a tool for glutathione engineering; however, it does not affect desiccation tolerance.

4. Adenine starvation increases desiccation tolerance via TOR/ PKA derepression.

5. Brewer's yeast beta glucans derieved from dehydrated cells posess higher immunoactivity as commercialised β-D-glucan (pleurean, Immunoglukan©).

6. Thesis for defense

Cell nutritional status restricts metabolical options for yeast desiccation stress tolerance.

A dynamic change in substrate concentrations and character during cultivation governs *S. cerevisiae* desiccation tolerance.

Starvation for particular nutrient as well as nutrient signalling pathways can be targets to engineer cells desiccation tolerance.

Desiccation improves β-D-glucan immunoactivity by decreasing superspiralization.

7. List of original publications

Liepins J, Kovačova E, Shvirksts K, Grube M, Rapoport A, Kogan G, 2015. Drying enhances immunoactivity of spent brewer's yeast cell wall b-D-glucans. Journal of Biotechnology, [accepted] doi: 10.1016/j.jbiotec.2015.03.024

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8. Approbation of the research

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Liepins J., Richard P., Rapoport A.,Yeast *Saccharomyces cerevisiae* response to desiccation stress – proteome study, 2nd Federation of European Microbiology Society congress (2nd FEMS congress), 4.-8. July, 2006, Madrid, Spain,

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Liepins J, Gavare M, Kokina A, Grube M, Smits K, FT-IR based *Saccharomyces cerevisiae* biomass quantification and it's application in ZrO_2 nanoparticle toxicity studies, proof of principle, 1st Congress of Baltic Microbiologists (1st CBM), 31.10-2.11. 2012. Riga, Latvia.

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8.2. National

A. Kokina, **J. Liepiņš**, V. Brusbārdis, N. Rostoks, A. Rapoports, E. Stalidzāns „Glicerola cikla izveide un raksturojums raugā S. cerevisiae", 2011, Mikrobioloģijas un biotehnoloģijas sekcijas sēdē. LU 69. konference, Rīga, 9. Februāris, 2011

J. Liepiņš, A. Kokina, V. Brusbārdis, E. Stalidzāns, NADPH metabolisma inženierija maizes raugā Saccharomyces cerevisiae, Mikrobioloģijas un biotehnoloģijas sekcijas sēdē. 68. LU konference, Rīga, 10. Februāris, 2010

I. Dirnēna, **J. Liepiņš**, A. Rapoports, Oksidatīvais stress rauga Saccharomyces cerevisiae žāvēšanas laikā, Mikrobioloģijas un biotehnoloģijas sekcijas sēdē. 67. LU konference, Rīga, 19. Februāris, 2009

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Annex I

	time [h]	Glucose (consumed)	pyruvate	succinate	glycerol	acetate	ethanol	biomass
BY4741	10	173,00	1,36	5,86	10,84	14,66	110,00	25,74
	11	273,33	1,17	5,45	14,10	16,17	173,77	41,18
	25	675,33	0,42	5,15	20,28	16,73	332,75	153,85
	33	675,33	0,36	5,82	13,04	11,40	270,72	189,00
	47	675,33	0,38	5,91	8,58	0,00	196,23	225,56
	55	675,33	0,38	6,58	7,79	0,00	176,09	237,71
tps1	10	113,78	0,61	6,02	5,42	14,73	74,49	19,75
	11	192,44	0,41	5,67	6,18	15,37	108,41	32,22
	25	675,33	0,30	5,62	14,04	14,62	335,51	171,43
	33	675,33	0,98	6,08	11,66	4,61	280,72	198,94
	47	675,33	2,52	7,56	23,93	0,00	179,71	231,48
	55	675,33	1,59	8,05	26,13	0,00	159,71	234,64
SC14	8	285,89	1,06	5,19	6,54	15,32	164,20	50,87
	9	526,00	0,89	4,98	9,52	15,84	305,51	116,87
	25	675,33	0,26	6,25	7,93	0,00	305,36	248,61
	33	675,33	0,27	6,25	7,25	0,00	242,61	293,30
	47	675,33	0,00	5,99	6,18	0,00	102,46	363,62
	55	675,33	0,26	5,20	5,65	0,00	61,74	406,55

Metabolite analyses of *SC14, BY4741* and *tps1* mutant batch cultivations in YPD medium. Results are expressed as mili c-moles, numbers are average of biological triplicates.