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Utilization of α-glucosidic sugars by *Ogataea* (*Hansenula*) *polymorpha*

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Dissertation was accepted for the commencement of the degree of Doctor of Philosophy (in Microbiology) on 18.06.2018 by the Council of the Institute of Molecular and Cell Biology, University of Tartu.

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Commencement: Room No 105, 23B Riia Street, Tartu, Estonia
on September 28th, 2018, at 10:15.

Publication of this thesis is granted by the Institute of Molecular and Cell Biology, University of Tartu.

ISSN 1024-6479
ISBN 978-9949-77-832-4 (print)
ISBN 978-9949-77-833-1 (pdf)

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University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on following original publications that are referred to by Roman numerals in the text:

I. **Viigand, K.,** Tammus, K., Alamäe, T. (2005). Clustering of *MAL* genes in *Hansenula polymorpha*: Cloning of the maltose permease gene and expression from the divergent intergenic region between the maltose permease and maltase genes. FEMS Yeast Research, 5: 1019–1028.

II. **Viigand, K**., Alamäe, T. (2007). Further study of the *Hansenula polymorpha MAL* locus: characterization of the α-glucoside permease encoded by the *HpMAL2* gene. FEMS Yeast Research, 7: 1134–1144.

III. Suppi, S., Michelson, T., **Viigand, K.,** Alamäe, T. (2013). Repression vs. activation of *MOX*, *FMD*, *MPP1* and *MAL1* promoters by sugars in *Hansenula polymorpha*: the outcome depends on cell's ability to phosphorylate sugar. FEMS Yeast Research, 13: 219–232.

IV. **Viigand, K**.*, Visnapuu, T.*, Mardo, K., Aasamets, A., Alamäe, T. (2016). Maltase protein of *Ogataea* (*Hansenula*) *polymorpha* is a counterpart to resurrected ancestor protein ancMALS of yeast maltases and isomaltases. Yeast 33(8): 415–432.
*Equal contribution

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My contribution to the articles referred to in this dissertation is following:

Ref. I Carried out majority of the experiments, participated in data analysis and interpretation. Participated in writing the manuscript.

Ref. II Carried out the experiments. Analyzed the data and participated in writing the manuscript.

Ref. III Designed and performed the experiments, analyzed the data, participated in writing the manuscript.

Ref. IV Constructed mutants Asp199Ala (D199A) and Thr200Val (T200V) of the *MAL1* gene, expressed the proteins in *E. coli*, investigated and compared the substrate specificity and kinetics of mutant and wild-type enzymes. Studied growth properties of *MAL1* and *MAL2* deletion mutants on α-glucosidic sugars. Designed and performed the experiments, analyzed the data, participated in writing the manuscript.

ABBREVIATIONS

aa	–	amino acids
α-MG	–	α-methylglucoside/methyl-α-D-glucopyranoside
bp	–	base pairs
CAZy	–	Carbohydrate-Active enZYmes Database
CCCP	–	carbonyl cyanide-*m*-chlorophenylhydrazone
DSF	–	differential scanning fluorimetry
GH	–	Glycoside Hydrolase (Family)
IMOs	–	isomalto-oligosaccharides
K_i	–	inhibition constant
K_m	–	Michaelis constant
NCBI	–	National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/)
Op	–	*Ogataea polymorpha* (previous name *Hansenula polymorpha*)
OpMAL1	–	*O. polymorpha* maltase encoded by *OpMAL1* (previous designation HpMAL1)
OpMAL2	–	*O. polymorpha* α-glucoside permease encoded by *OpMAL2* (previous designation HpMAL2)
PDB	–	Protein Data Bank (https://www.rcsb.org/)
PNPG	–	*p*-nitrophenyl-α-D-glucopyranoside
Sc	–	*Saccharomyces cerevisiae*
SGD	–	*Saccharomyces* Genome Database (https://www.yeastgenome.org)
UAS	–	upstream activating sequence

INTRODUCTION

Sugars are extremely abundant in nature and are the preferred energy sources for yeasts. Yeasts prefer glucose to more complex sugars (sucrose, maltose and many others) down-regulating their utilization if glucose is present at sufficiently high concentration. Respective regulatory mechanism is called glucose repression. Utilization of α-glucosidic disaccharides such as maltose and sucrose has been extensively studied in baker's yeast *Saccharomyces cerevisiae* because baking, brewing and production of bioethanol mostly relies on these sugars as fermentation substrates. Repression of maltose and sucrose utilization by glucose in *S. cerevisiae* was among the first models in yeast glucose repression studies (Gancedo, 1998; Zimmermann and Scheel, 1977). About 20 years ago, Tiina Alamäe's research group chose a non-conventional methylotrophic yeast *Ogataea polymorpha* (earlier *Hansenula polymorpha*) as an alternative yeast model to study glucose repression mechanisms. Methanol utilization in yeasts is very strongly repressed by sugars (Sibirny *et al.*, 1988). As *O. polymorpha* also assimilates disaccharides maltose and sucrose, study of this yeast was expected to show light on glucose repression mechanisms in this yeast by addressing regulation of these two specific glucose-repressed metabolic processes. As *O. polymorpha* diverged from the main evolution line of yeasts much earlier than *S. cerevisiae* (see Ref IV), glucose repression mechanisms of this yeast were expected to differ from those shown for *Saccharomyces*.

These studies led to assay of genetics, genomics and biochemistry of disaccharides metabolism in *O. polymorpha.* Most of the results of this work is presented in this dissertation. The obtained data was compared with those available for *S. cerevisiae* and some other yeasts and a hypothesis on regulation of *MAL* (maltose-related) genes in *O. polymorpha* was proposed. Though this work mostly contributes to basic science, some of the results have a biotechnological value as well. So, the bidirectional promoter in the *MAL* gene cluster can be used for regulated co-expression of two genes (proteins) of interest. It is also interesting that *O. polymorpha MAL1* promoter is perfectly recognized not only in another yeast *S. cerevisiae*, but also in a bacterium *Escherichia coli*, and has already been used for heterologous overexpression in *E. coli* of a biotechnologically relevant protein – levansucrase.

I OVERVIEW OF THE LITERATURE

1.1. Overview of methylotrophic yeasts with emphasis on *Ogataea polymorpha*

Methylotrophic yeasts are able to grow on methanol as a sole carbon source. Currently the following five yeast genera belong to methylotrophs: *Candida*, *Pichia*, *Ogataea*, *Kuraishia* and *Komagataella* (Limtong *et al.*, 2008; Yurimoto *et al.*, 2011 and references therein).

Methylotrophic yeasts are found in decaying fruits, juice and other vegetable products, on plant leaves, in plant exudates, soil and insect gut (Limtong *et al.*, 2008; Morais *et al.*, 2004; Negruţă *et al.*, 2010) as these habitats provide methanol. Methanol that results from the turnover of cell-wall pectin is emitted by living plant leaves (Keppler *et al.*, 2006; Nemecek-Marshall *et al.*, 1995), it is also released in the soil at degradation of pectin and lignin of plant residues (Nakagawa *et al.*, 2005). Kawaguchi *et al.* (2011) have shown for *A. thaliana* that methanol concentration in the phyllosphere of plant leaves is ~25 mM and rises up to 250 mM in wilting plants allowing growth of methylotrophs.

Methylotrophic yeasts have been used as a model to study the biology of peroxisomes – the intracellular eukaryotic organelles harboring the key enzymes (methanol oxidase, dihydroxyacetone synthase) of methanol metabolism. Growth of yeasts on methanol is accompanied by massive proliferation of peroxisomes – they can occupy up to 80% of the cell mass. Transfer of methanol-grown cells to glucose or ethanol medium triggers transcriptional repression of methanol-specific enzymes and rapid degradation of peroxisomes – pexophagy (Stasyk *et al.*, 2007). Aside of peroxisome studies, methylotrophic yeasts have been used for the study of glucose repression, stress response, mating type switching, protein glycosylation and nitrate assimilation (see Wolf, 1996 and references therein). Importantly, methylotrophic yeasts, especially *Komagataella phaffii* (formerly *Pichia pastoris*) and *Ogataea polymorpha* have been and are used for heterologous large-scale production of biotechnologically relevant proteins using strong regulatable promoters from methanol pathway (Löbs *et al.*, 2017 and references therein). For example *O. polymorpha* has been used as a gene host in producing pharmaceuticals such as insulin for treatment of diabetes, hepatitis B vaccines or IFN α-2a for the treatment of hepatitis C and many enzymes such as the feed additive phytase, anticoagulants hirudin and saratin (reviewed in Ramezani-Rad *et al.*, 2003).

O. polymorpha is thermotolerant (can grow at temperatures up to 50 °C) and belongs to phylum Ascomycota, family Saccharomycetaceae. *O. polymorpha* was initially isolated from orange juice and described by Wickerham in 1951 (NRRL Y-1798 (=ATCC14754)). At this time it was named *Hansenula angusta*. *H. angusta* was formally decsribed by Teunisson *et al.* (1960) and by Morais & Maia (1959) as *H. polymorpha* and in 1970 Wickerham considered *H. angusta* and *H. polymorpha* as synonyms (Naumov *et al.*, 1997). Later,

H. polymorpha was renamed as *Pichia angusta* (Kurtzman, 1984). The new genus *Ogataea* was proposed for nitrate-assimilating methylotrophic yeasts by Yamada *et al.* (1994) and *H. polymorpha* as a nitrate-assimilating species was transferred to the new genus as *O. polymorpha*. In scientific literature, the names *Pichia angusta*, *Hansenula polymorpha* and *Ogataea polymorpha* are all used to designate the same species.

Genomes of three independently isolated *O. polymorpha* strains: CBS 4732, NCYC 495 and DL-1 are sequenced. However, the DL-1 strain was re-classified as *O. parapolymorpha* in 2013 (Naumova *et al.*, 2013). The first genome of *O. polymorpha* (CBS 4732) was sequenced 15 years ago (Ramezani-Rad *et al.*, 2003), but it is not yet released to the public domain. The genome sequences of *O. polymorpha* strain NCYC 495 and *O. parapolymorpha* strain DL-1 are publicly available in MycoCosm portal: http://genome.jgi.doe.gov/programs/fungi/index.jsf (Grigoriev *et al.*, 2014). The genomes of *O. polymorpha* and *O. parapolymorpha* are approximately 10% divergent in sequence (Ravin *et al.*, 2013; Riley *et al.*, 2016).

1.2. α-Glucosidic sugars in nature

Yeasts prefer sugars over other carbon sources and therefore they thrive in sugar-rich habitats. Many plants (such as sugar cane and sugar beet) and berries contain lots of sucrose – a disaccharide of glucose and fructose (for monomeric composition of α-glucosidic sugars and linkages see Figure 1). Sucrose is also synthesized by cyanobacteria and proteobacteria (Lunn, 2002). Importantly, sucrose can be converted to other sugars by isomerizing enzymes of many organisms including plants, yeasts, filamentous fungi, bacteria and even insects (Lee *et al.*, 2011 and references therein). Turanose, palatinose and maltulose (Fig 1) are present for example in honey and are isomerization products of sucrose (Sawale *et al.*, 2017). Importantly, palatinose is currently enzymatically produced from sucrose at large scale and advertised as a novel healthy sugar with low glycemic index and no cariogenic effect (Sawale *et al.*, 2017). A trisaccharide melezitose (Fig 1) is a main constitute of aphid honeydew and is also found in honey (Daudé *et al.*, 2012).

α-Glucosidic sugars comprised of only glucose: maltose, isomaltose, maltotriose and panose (Fig 1) are resulting from starch and glucogen degradation by amylases (Janecek, 2009). For example, the beer wort contains 50–60% of maltose, 15–20% maltotriose and 10–15% glucose as major sugars (Stewart, 2016). A trisaccharide panose is also considered an isomalto-oligosaccharide (IMO) as it contains an isomaltose moiety. IMOs are also considered as novel prebiotics – they stimulate growth of probiotic bacteria such as *Bifidobacterium* in the gut (Mäkeläinen *et al.*, 2009). *Ogataea* species have been isolated from spoiled orange juice, leaf surfaces, plant exudates and insect guts (Limtong *et al.*, 2008; Morais *et al.*, 2004 and references therein). Both methanol and α-glucosidic sugars are available in these habitats and should enable the growth

of these yeasts. Tiina Alamäe's research group was the first to deal with genetics and biochemistry of assimilation of α-glucosidic sugars in *Ogataea* – the first paper on this subject was published in 1998 (Alamäe and Liiv, 1998).

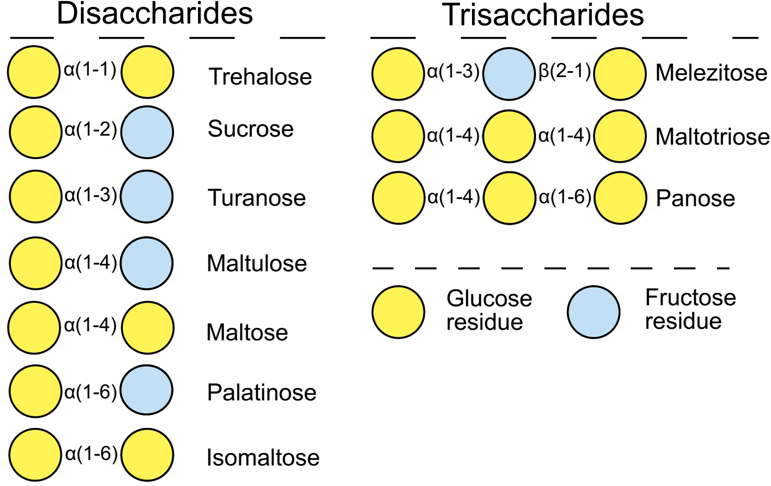

Figure 1. α-Glucosidic di- and trisaccharides and the linkages within these sugars.

1.3. *MAL* genes and clusters in yeasts

In *Saccharomyces cerevisiae*, the genes required for maltose metabolism are genomically clustered forming so-called *MAL* clusters (loci) at subtelomeric regions of the chromosomes. Subtelomeres are gene-poor regions proximal to the telomeres. The length of subtelomeric region varies from 20 kb in some yeasts to several hundred kb in higher eukaryotes (Brown *et al.*, 2010). Subtelomeric gene families show typical patterns of rapid expansion and evolution – frequent duplication events are followed by functional divergence of the genes yielding novel alleles that may allow for example metabolism of new carbohydrates (Brown *et al.*, 2010). Genomic clustering of functionally related genes is not very common in eukaryotes. In addition to *MAL* clusters, genomic clusters have been characterised also for utilization of galactose, allantoine and nitrate in yeasts and filamentous fungi (Ávila *et al.*, 2002; Kunze *et al.*, 2014; Slot and Rokas, 2010; Wong and Wolfe, 2005).

S. cerevisiae has five *MAL* clusters: *MAL1*, *MAL2*, *MAL3*, *MAL4* and *MAL6* situated near the telomeres of chromosomes VII, III, II, XI and VIII (Brown *et al.*, 2010; Charron *et al.*, 1986; Needleman, 1991; Vanoni *et al.*, 1989). Each *MAL* cluster in *S. cerevisiae* consists of three genes (Fig 2): *MALx1* (maltose permease gene), *MALx2* (maltase gene) and *MALx3* (regulatory *MAL*-activator gene) (Chang *et al.*, 1988; Charron *et al.*, 1986; Dubin *et al.*, 1985; Needleman, 1991). The „x" refers to the number of the cluster.

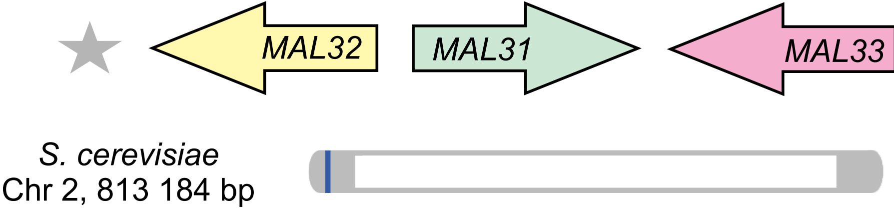

Figure 2. Composition of the *S. cerevisiae MAL3* cluster. *MAL31* – α-glucoside permease; *MAL32* – maltase; *MAL33* – *MAL*-activator. A star marks the location of the telomere. Lower panel depicts position of the *MAL* cluster (in blue) in chromosome 2 of *S. cerevisiae* S288C. Subtelomeric regions (50 kbp from the chromosome end) are shown in grey. Data on *S. cerevisiae* strain S288C was taken from the MycoCosm portal (https://genome.jgi.doe.gov/programs/fungi/index.jsf; Grigoriev *et al*. 2014). The chromosome was visualised using a web-based program PhenoGram (http://visualization.ritchielab.psu.edu/phenograms/plot).

Maltase and maltose permease genes are next to each other also in the genome of several other yeasts, for example *Kluyveromyces lactis* (Fairhead and Dujon, 2006; Goffrini *et al.*, 2002; Leifso *et al.*, 2007) and *Torulaspora delbrueckii* (Alves-Araújo *et al.*, 2004). Multiple *MAL* clusters are present in the genome of *Scheffersomyces* (*Pichia*) *stipitis* which is exceptionally rich in gene clusters having at least 35 clusters of functionally linked genes (Jeffries *et al.*, 2007; Jeffries and Van Vleet, 2009). No *MAL* clusters have been described in a phylogenetically ancient yeast *Schizosaccharomyces pombe*.

1.4 Transport of maltose and other α-glucosidic sugars into a yeast cell

α-Glucosidic sugars can be hydrolyzed extracellularly by secreted or membrane-bound enzymes or intracellularly after the sugar has been transported into the cell. *S. cerevisiae* hydrolyzes maltose intracellularly. Yet, there are some other yeasts (*Lipomyces starkeyi*, *Saccharomycopsis fibuligera* and *Malbranchea sulfurea*), filamentous fungi (for example *Aspergillus* species) and bacteria (*Lactobacillus acidophilus*, *Thermococcus* sp. and *Bacillus* sp.) that can hydrolyze maltose outside the cell (Jansen *et al.*, 2006 and references therein). Both possibilities have been shown for *S. pombe* (Chi *et al.*, 2008; Jansen *et al.*, 2006).

For those yeasts that hydrolyze α-glucosidic sugars inside the cell, the first step in metabolism of these sugars is their transport across the plasma membrane into the cytosol. So, maltose is transported into the cell unchanged by maltose permease, and only then the disaccharide is hydrolyzed into two glucose molecules by cytoplasmic maltase and further metabolized (Klein *et al.*, 1998; Needleman, 1991). Maltose is transported into *S. cerevisiae* (and other yeasts) cells actively in 1:1 symport with protons while glucose is transported only

using downhill facilitated diffusion (Van Leeuwen *et al.*, 1992). Active uphill transport is reasonable as the intracellular maltase has low affinity for maltose (see Table 2) – therefore maltose has to be concentrated into the cell to allow the further hydrolysis (Alamäe and Liiv, 1998; Needleman *et al.*, 1978).

A high-affinity maltose transport system was initially discovered in *S. cerevisiae* (see Novak *et al.*, 2004 for a review). A high-affinity maltose permease gene *MAL61* was cloned by Cheng and Michels in 1989 and kinetic properties of the permease protein were studied by the same authors in 1991 (Cheng and Michels, 1991). This permease has K_m of 4 mM for maltose and its expression is maltose-induced (Cheng and Michels, 1991). The **MAL61** protein is 614 aa long, has twelve transmembrane domains and the N- and C-termini of the permease are located at the cytoplasmic side (Cheng and Michels, 1989; Table 1). Aside of transcriptional control by glucose, the maltose permease of *S. cerevisiae* is inactivated in the presence of glucose – addition of glucose into the medium causes inactivation of the maltose transport system within 90 minutes and transfer of the cells back to maltose triggers its fast (within 1 h) regeneration (see references in Novak *et al.*, 2004). Inactivation of the maltose permease starts with ubiquitination of the permease and thereafter the protein is degraded in the vacuole (Medintz *et al.*, 1998, 1996). It has been also shown that glucose-caused catabolite inactivation of maltose permeases is related to the presence of PEST sequences (which are rich in proline, glutamate, serine and threonine) in the N-terminal cytoplasmic domains, but these sequences have not been found in AGT1 and MPHx transporters (Day, Higgins, *et al.*, 2002; Dietvorst *et al.*, 2005; Medintz *et al.*, 2000). Differently from maltose permease, maltase protein is not inactivated when glucose is added into the medium – regulation of maltase by glucose occurs only at transcriptional level (Federoff *et al.*, 1983).

The α-glucoside transporter **AGT1** (*MAL11* according *to Saccharomyces* Genome Database) has wider substrate range than MAL61 that transports only maltose and turanose, yet it plays a role also in maltose entry (Cheng and Michels, 1991; Table 1). The *AGT1* gene is a mutant allele of the permease gene of the *MAL1 l*ocus situated in subtelomeric region of chromosome VII (Han *et al.*, 1995; Needleman, 1991). The AGT1 permease (as MAL61 and other α-glucoside transporters) is a member of the Sugar Porter family (TCDB 2.A.1.1) of the Major Facilitator Superfamily (MFS) (http://www.tcdb.org) and has similarly to MAL61 twelve transmembrane domains. Its sequence identity to MAL61 permease is 57% (Han *et al.*, 1995). The AGT1 is a maltose/proton symporter with relaxed substrate specificity. It has high affinity for trehalose and sucrose (K_m 8 mM), medium affinity for maltose (K_m 5–17.8 mM), maltotriose (K_m 18.1 mM) and *α*-methylglucoside (K_m 20–35 mM), and low affinity for isomaltose, melezitose and palatinose (Han *et al.*, 1995; Stambuk *et al.*, 1999; Table 1). The preferred substrate for the AGT1 permease is trehalose while MAL1 and MPHx permeases cannot transport this disaccharide (Vidgren *et al.*, 2005). The *AGT1* expression is transcriptionally induced by maltose as of *MALx1* and the induction is mediated by the MALx3 (Mal-activator) protein.

This is explained by identical UAS_{MAL} region in the promoters of the both genes (Han *et al.*, 1995). Gallone *et al.* (2016) found that beer yeast strains have significantly higher capacity to consume maltotriose compared to wine strains which lack this ability. Efficient metabolism of maltotriose has been explained by the presence of *MAL1* locus (contains the *AGT1* gene) in several copy numbers (Gallone *et al.*, 2016).

A new α-glucoside transporter was independently characterized in 2005 by two groups (Dietvorst *et al.*, 2005; Salema-Oom *et al.*, 2005) in industrial strains of brewer's, baker's and distiller's yeasts. These new transporters are coded by *MTT1* (also called *MTY1*) genes, which are 90% and 54% identical to *S. cerevisiae MALx1* and *AGT1* genes, respectively (Vidgren *et al.*, 2009; Table 1). The **MTT1** transporters have lower K_m values for maltotriose (16–27 mM) than for maltose (61–88 mM), being therefore different from all other α-glucoside transporters (Dietvorst *et al.*, 2005; Salema-Oom *et al.*, 2005; Table 1). The MTT1 permease also transports trehalose and turanose (Dietvorst *et al.*, 2005; Salema-Oom *et al.*, 2005). The ability to efficiently transport maltotriose is very important in brewing where maltotriose makes up about 20% of the wort sugars and is usually the most abundant sugar at later stages of fermentation (Magalhães *et al.*, 2016).

Day *et al.* (2002) characterized two additional maltose permease genes in *S. cerevisiae*: *MPH2* ja *MPH3* (Table 1). These genes encode two identical proteins with 75% identity to *S. cerevisiae* permeases MAL31 and MAL61 and 55% identity to AGT1. Day *et al.* (2002) showed that **MHPx** permease can transport maltose (K_m ~4.4 mM), maltotriose (K_m ~7 mM), turanose and methyl-α-D-glucopyranoside (α-MG) (Table 1). Interestingly, general α-glucoside permeases such as AGT1 and MPH2 were also able to mediate glucose transport while overexpressed in a hxt1-17 gal2-deletion strain (Wieczorke *et al.*, 1999).

Maltose transport has also been studied in *Torulaspora delbrueckii* (Alves-Araújo *et al.*, 2004) and *S. pombe* (Reinders and Ward, 2001). Alves-Araújo *et al.* (2004) described a *T. delbrueckii* gene *TdMAL11* (Table 1), which shares similarity with genes of maltose permeases from *S. cerevisiae* (identity 71%) and *Klyuveromyces lactis* (identity 57%). Disruption of the *TdMAL11* gene indicated that there are at least two maltose transporters in this yeast (Alves-Araújo *et al.*, 2004). Reinders and Ward (2001) described α-glucoside transporter SUT1 (Table 1) from *S. pombe* which is most similar to sucrose transporters of plants. SUT1 was expressed in *S. cerevisiae* and assayed for kinetics and range of transported sugars. Differently from plant sucrose transporters, the affinity of the transporter for maltose (K_m 6.5 mM) was higher than for sucrose (K_m 36 mM) (Reinders and Ward, 2001; Table 1). Expression of *TdMAL11* and *SUT1* are both regulated by carbon source as of maltose transporters of *S. cerevisiae* – induced by maltose and repressed by glucose (Alves-Araújo *et al.*, 2004; Reinders and Ward, 2001). A summary of the more thoroughly characterized α-glucoside permeases is presented in Table 1. No data was available in the literature on maltose transport in *O. polymorpha.*

Table 1. Maltose and α-glucoside permeases in yeasts.

Yeast	Permease	Substrates	K_m (mM)			Reference
			PNPG	Maltose	Sucrose	
S. cerevisiae	MAL21	maltose, sucrose, turanose	No transport	5	120	Hollatz and Stambuk, 2001; Stambuk *et al.*, 2000; Stambuk and de Araujo, 2001
S. cerevisiae	MAL31	maltose, turanose, maltotriose	–	4.2±1.1	–	Day, Higgins, *et al.*, 2002; Day, Rogers, *et al.*, 2002
S. cerevisiae	MAL61	maltose, turanose, maltotriose	No transport	2–4	No transport	Han *et al.*, 1995; Vidgren *et al.*, 2005
S. cerevisiae	AGT1	maltose, sucrose, turanose, PNPG, trehalose, α-MG, maltotriose, isomaltose, palatinose, melezitose	3	18	5.1–17.8	Day, Higgins, *et al.*, 2002; Han *et al.*, 1995; Hollatz and Stambuk, 2001; Stambuk and de Araujo, 2001
S. cerevisiae	MPH2/ MPH3	maltose, turanose, maltotriose, α-MG	–	4.4	No transport	Day, Higgins, *et al.*, 2002
S. pombe	SUT1	maltose, sucrose	–	6.5	36	Reinders and Ward, 2001
S. carlsbergensis	MTY1 (MTT1)	maltose, maltotriose	–	61–88	–	Dietvorst *et al.*, 2005; Salema-Oom *et al.*, 2005
T. delbrueckii	TdMAL11	maltose	–	2–3	–	Alves-Araújo *et al.*, 2004
C. utilis	–	maltose	0.83	0.2 (K_i)	–	Sims *et al.*, 1984

– no data available

The role of specific amino acid residues in α-glucoside permeases has been studied by few groups. Trichez (2007) identified four charged amino acid residues in transmembrane domains of the AGT1 permease of *S. cerevisiae*: Glu120, Asp123, Glu167 and Arg504 which are conserved within α-glucoside transporters of several yeast species. The importance of these amino acids was investigated by generating AGT1 mutants at Glu120, Asp123 and Arg504 and testing the growth of *S. cerevisiae* on maltotriose and *p*-nitrophenyl-α-D-glucopyranoside (PNPG, a specific substrate for AGT1 and similar permeases) uptake in mutant strains having only this permease variant (Trichez, 2007). The strain with an Arg504Ala variant of AGT1 lost the ability to grow on maltotriose while strains with AGT1 variants Asp123Gly and Glu120Ala had only reduced maltotriose transport (Trichez, 2007). Trichez (2007) suggested that Glu120 and Asp123 residues of AGT1 are involved in proton translocation and Arg504 is responsible for binding of the sugar. Ten years later, Henderson and Poolman (2017) performed site-directed mutagenesis of key acidic residues in the membrane-embedded domain of MAL11 (AGT1) and showed that the transmembrane acidic residues Glu120, Asp123 addressed also by Trichez (2007) and Glu167 are all essential for effective binding of maltose and proton co-transport. Notably, triple mutants of the three acidic residues were completely deficient in uphill maltose transport, but maintained full downhill efflux and exchange activity (facilitated diffusion process), and mutation of any or all of these three acidic residues introduced substrate leakage from the cell (Henderson and Poolman, 2017).

Maltose transport in *O. polymorpha* was first studied in Tiina Alamäe's research group – respective data are included in current theses. Earlier, two kinetically different glucose transport systems were described by this group for *O. polymorpha*: a low-affinity transport system (K_m for glucose 1.75 mM) present in glucose-repressed cells and a high-affinity transport system (K_m for glucose ~0.05 mM) detected in glucose-derepressed cells (Karp and Alamäe, 1998). The first hexose transporter gene *HXT1* of *O. polymorpha* was cloned and successfully expressed in a hexose transporterless mutant of *S. cerevisiae* by Stasyk *et al*. in 2008. Low-affinity glucose transport was strongly reduced in *hxt1* mutants of *O. polymorpha* indicating that HXT1 protein is a low-affinity glucose transporter (Stasyk *et al.*, 2008). According to Stasyk *et al.* (2008) there are at least six glucose transporters and two fructose transporters encoded in the genome of *O. polymorpha.* There are 20 hexose transporter-related genes in *S. cerevisiae* and 34 sugar permease genes in total (Wieczorke *et al.*, 1999).

1.5. Yeast α-glucosidases and their evolution

Yeast α-glucosidases belong to a group of glycoside hydrolases (EC 3.2.1.–). Glycoside hydrolases (GHs) are a widespread group of enzymes which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Lombard *et al.*, 2014). For example, α-glucosidase of *O. polymorpha* can hydrolyze an α-glycosidic bond

not only in maltose and sucrose, but also in α-MG (methyl-α-D-glucopyranoside) and PNPG liberating methanol and *p*-nitrophenol from respective substrates (Liiv *et al.*, 2001). Hydrolysis of a chromogenic substrate PNPG is widely used for quantitating of catalytic activity of α-glucosidases (Zimmermann *et al.*, 1977). Yeast α-glucosidases degrade di- and oligosaccharides rapidly, large polysaccharides (starch) are hydrolyzed slowly or not at all (Deng *et al.*, 2014; Needleman *et al.*, 1978; Table 2).

The most extensively studied yeast *S. cerevisiae* has two types of α-glucosidases for the hydrolysis of α-glucosidic sugars: maltases (EC 3.2.1.20) and isomaltases (EC 3.2.1.10) (Table 2). Enzymes of the latter group have also been named oligo-1,6-glucosidases, sucrase-isomaltases and α-methylglucosidases.

There are 146 Glycoside Hydrolases (GH) Families in the CAZY database (http://www.cazy.org). Maltases and isomaltases belong to GH13 family which in turn contains 42 subfamilies (Lombard *et al.*, 2014). In addition to maltases and isomaltases, the GH13 family includes for example α-amylases, cyclodextrin glucantransferases, pullulanases, isoamylases, trehalose synthases, trehalose-6-phosphate hydrolases, branching enzymes, neopullulanases and some others (http://www.cazy.org). The similarity of the amino acid sequences within the GH13 family proteins is low, however they all share four highly conserved regions and three acidic catalytic residues located in conserved regions (Yamamoto *et al.*, 2010). α-Glucosidases have been found in a variety of organisms and short overview of most thoroughly characterized α-glucosidases from yeasts and other organisms are found in Tables 3 and 4 respectively. Many putative α-glucosidases have also been disclosed through genome mining. However the enzymes deduced from the genomes mostly remain uncharacterized.

Maltases have a quite narrow substrate range – they degrade maltose and maltotriose (both α-1,4 linked) while cannot degrade isomaltose, an α-1,6 linked starch degradation product (Needleman *et al.*, 1978; Voordeckers *et al.*, 2012; Tables 2 and 3). For isomaltose degradation, *Saccharomyces* yeasts have specific enzymes – isomaltases IMA1 to IMA5 (Naumoff and Naumov, 2010; Teste *et al.*, 2010; Table 2). *IMA1–IMA5* genes are located in *S. cerevisiae* at subtelomeric regions of chromosomes VII, XV, IX, X and X, respectively.

The first glycoside hydrolases were crystallized in 1980s. The first crystal structures resolved for glycoside hydrolases were of TAKA-amylase A (Brzozowski and Davies, 1997; Matsuura *et al.*, 1984) and porcine pancreatic α-amylase (Buisson *et al.*, 1987; Qian *et al.*, 1993). By now, there are quite many α-glucosidases with solved structures (Table 3): human maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) (Sim *et al.*, 2010), oligo-1,6-glucosidase (malL) from *Bacillus cereus* (Watanabe *et al.*, 1997), malL of *Bacillus subtilis* (Hobbs *et al.*, 2013), α-glucosidase GSJ of *Geobacillus* sp. (Shirai *et al.*, 2008), α-glucosidase (HaG) of *Halomonas* sp. (Shen *et al.*, 2015). The common structure of GH13 family enzymes consists of three domains: an N-terminal catalytic domain (domain A) folded into a $(\beta/\alpha)_8$-barrel, an additional domain (domain B) extending out of the barrel, and a β-sheet-rich domain in the C-terminus (domain C) (Yamamoto *et al.*, 2010).

Table 2. Comparison of biochemical properties of selected α-glucosidases with known substrate specificity from yeasts.

Organism; enzyme; extra/intracellular	Protein mass (kDa)	Confirmed substrates / linkages	K_m (mM)				Reference
			PNPG	Maltose	Sucrose	Isomaltose	
S. cerevisiae, MAL62 (intracellular)	63	PNPG, maltose, sucrose, turanose	0.31	16.6	15	No activity	Needleman *et al.*, 1978; Krakenaĭte and Glemzha, 1983
S. cerevisiae, IMA1 (intracellular)	68.5	PNPG, isomaltose, α-MG, sucrose, palatinose, isomaltotriose, panose	0.58 ± 0.16 / 2.13	No activity	144 ± 26	17 ± 4	Yamamoto *et al.*, 2004; Deng *et al.*, 2014; SGD
S. cerevisiae, IMA2 (intracellular)	68.6	PNPG, isomaltose, α-MG, sucrose, palatinose, isomaltotriose, panose	0.89 ± 0.13	No activity	147 ± 24	17 ± 2	Deng *et al.*, 2014; SGD
S. cerevisiae, IMA3/4 (intracellular)	68.6	PNPG, isomaltose, α-MG, sucrose, palatinose	0.35 ± 0.09	No activity	116 ± 12	37 ± 9	Deng *et al.*, 2014; SGD
S. cerevisiae, IMA5 (intracellular)	67.5	PNPG, isomaltose, sucrose, palatinose, isomaltotriose, panose	0.48 ± 0.11	No activity	191 ± 24	13 ± 2	Deng *et al.*, 2014; SGD
Schizosaccharomyces pombe, mal1 (intracellular)	67.75	PNPG, maltose, sucrose, dextrin, soluble starch	20.3 ± 0.3	14.5 ± 0.2	3.1 ± 0.1	–	Chi *et al.*, 2008
S. pombe, agl1 (extracellular)	108.72	maltose	–	7.0 ± 1.8	–	–	Jansen *et al.*, 2006; Kato *et al.*, 2013; PomBase (https://www.pombase.org/)

Organism; enzyme; extra/intracellular	Protein mass (kDa)	Confirmed substrates / linkages	K_m (mM)				Reference
			PNPG	Maltose	Sucrose	Isomaltose	
Ogataea (Hansenula) polymorpha, MAL1 (intracellular)	65.3	PNPG, sucrose, maltose, α-MG	0.51	95 (K_i)	42 (K_i)	–	Liiv *et al.*, 2001
Candida albicans, CAMAL2 (intracellular)	66.1	maltose, sucrose, α-MG	–	20	13	No activity	Geber *et al.*, 1992
Torulaspora pretoriensis α-glucosidase (intracellular)	60–69	PNPG, maltose, isomaltose, maltotriose, sucrose, α-MG	0.15	150	29	17	Oda *et al.*, 1993
Xanthophyllomyces dendrorhous (*Phaffia rhodozyma*), α-glucosidase (extracellular)	115 (2 mono-mers)	α-1,4 glycosidic bonds in soluble starch and malto-oligosaccharides	No activity	2.71	No activity	No activity	Marín *et al.*, 2006
Lipomyces starkeyi, α-glucosidase (extracellular)	35	PNPG, maltose, isomaltose, maltotriose, isomaltoriose, amylopectin, starch, panose, amylose	High activity*	High activity*	No activity	High activity*	Kelly *et al.*, 1985

"–" data not available
* values not available

Table 3. Comparison of biochemical properties of well characterized α-glucosidases from other organisms.

Organism; enzyme	Protein mass (kDa)	Confirmed substrates / linkages	K_m (mM)				Reference
			PNPG	Maltose	Sucrose	Isomaltose	
Bacillus (*Geobacillus*) *stearothermophilus* α-1,4-glucosidase	–	α-1,4 glycosidic linkages: maltose, malto-oligosaccharides, α-glucans	0.63	5.6	–	–	Tsujimoto *et al.*, 2007
Bacillus (*Parageobacillus*) *thermoglucosdasius* oligo-1,6-glucosidase	–	α-1,6 linkages: isomaltose, IMOs, α-limit dextrin	0.24	–	–	3.3	Tsujimoto *et al.*, 2007
Geobacillus thermodenitritrificans α-glucosidase	–	α-1,3 and α-1,4 linkages: PNPG, maltose, dextrin, turanose, maltotriose, maltotetraose, maltopentaose, maltohexaose, xylose, soluble starch	2.68	–	–	–	Cihan *et al.*, 2011
B. subtilis α-glucosidase MalL *(sucrase-isomaltase-maltase)*	66	α-1,4, and α-1,6 glycosidic linkages: PNPG, sucrose, maltose, isomaltose, maltotriose, maltotetraose, maltopentaose, maltohexaose	0.21	0.135	10.2	0.45	Schönert *et al.*, 1998, 1999
Bifidobacterium longum subsp. longum α-1,4-glucosidase (BLAG)	67	α-1,2, α-1,3, α-1,4, and α-1,6 glycosidic linkages	1.8	43.5	–	129.6	Kim *et al.*, 2017
Staphylococcus xylosus maltase MalA	62.5	PNPG, maltose, maltotriose, maltopentaose, sucrose	6.9	0.9	–	–	Egeter and Brückner, 1995

Organism; enzyme	Protein mass (kDa)	Confirmed substrates / linkages	K_m (mM)				Reference
			PNPG	Maltose	Sucrose	Isomaltose	
Sulfolobus solfataricus maltase (a-glucosidase)	80 (subunit)	PNPG, maltose, maltotriose, maltotetraose, maltoheptaose, isomaltose, dextrin	3.2	0.91	–	–	Rolfsmeier and Blum, 1995
Homo sapiens sucrase-isomaltase (SI) (intestinal membrane-bound)	209	α-1,2, α-1,4 and α-1,6 glycosidic linkages	1.3 ± 0.1	7.1 ±1.3	–	–	Gericke *et al.*, 2016; Sim *et al.*, 2010; http://www.uniprot.org/
Homo sapiens maltase-glucoamylase (MGAM) (intestinal membrane-bound)	209.9	linear α-1,4 and branched α-1,6 oligosaccharides	12.1 ± 1.0	4.3 ± 1.2	–	–	Sim *et al.*, 2010; http://www.uniprot.org/
Apis mellifera L. α-glucosidase III (secreted)	68	PNPG, sucrose, maltose, maltoriose, maltotetraose, maltopentaose, maltohexaose, kojibiose, nigerose, isomaltose, turanose	13	11	30	–	Kubota *et al.*, 2004; Nishimoto *et al.*, 2001

"–" data not available

From yeast α-glucosidases, three-dimensional structure has been determined only for *S. cerevisiae* isomaltase 1 (IMA1; PDB ID 3AJ7 and 3A4A) (Yamamoto *et al.*, 2010). Its structure in complex with a competitive inhibitor maltose has uncovered the active site bordering (signature) amino acids (Y158, V216, G217, S218, L219, M278, Q279, D307, E411) the Val216 being crucial for selective binding of the substrate (Yamamoto *et al.*, 2010; Table 4).

Table 4. Signature amino acids of α-glucosidases with known substrate specificity. The table is mostly based on the data from Figure 4 in Voordeckers *et al.*, 2012. Background coloring in the table is as follows: maltases (pink), isomaltases (green) and maltase-isomaltases (blue). The position corresponding to Val216 of *Sc* IMA1 is of key importance in determination of substrate specificity of α-glucosidases and is shown in red frame. AncMALS – a resurrected hypothetical ancestor protein of *Sc* maltases/ isomaltases (Voordeckers *et al.*, 2012); *Le* – *Lodderomyces elongisporus* (GenBank accession XP_001526531.1; Voordeckers *et al.*, 2012); *Sc* – *S. cerevisiae*; *Sp* – *Schizosaccharomyces pombe*; *Bs* – *Bacillus stearothermophilus*; *Bt* – *Bacillus thermoglucosidasius.*

α-glucosidase	**Signature amino acids (numbering as in *Sc* IMA1)**									**Substrate specificity**
	158	**216**	**217**	**218**	**219**	**278**	**279**	**307**	**411**	
ancMALS	F	**T**	A	G	L	V	G	D	E	**maltase-isomaltase**
Le α-glucosidase	H	**T**	A	G	M	V	G	D	N	**maltase-isomaltase**
Sc MAL12	F	**T**	A	G	L	V	A	E	D	**maltase**
Sc MAL32	F	**T**	A	G	L	V	A	E	D	**maltase**
Sp Mal1	Y	**A**	I	N	M	M	P	D	E	**maltase**
Bs α-1,4-glucosidase	I	**A**	I	S	H	A	N	G	A	**maltase**
Bt oligo-1,6-glucosidase	V	**V**	I	N	M	T	P	D	E	**isomaltase**
Sc IMA1	Y	**V**	G	S	L	M	Q	D	E	**isomaltase**
Sc IMA2	Y	**V**	G	S	L	M	Q	D	E	**isomaltase**
Sc IMA3/4	Y	**V**	G	S	L	M	R	D	E	**isomaltase**
Sc IMA5	F	**V**	G	S	M	V	G	S	E	**isomaltase**

The structures of the IMA1 mutant E277A of *S. cerevisiae* in complex with isomaltose and maltose were also determined by Yamamoto *et al.* (2011). The signature amino acids from *Sc* IMA1 compared to other α-glucosidases with known substrate specificity are shown in Table 4. Analysis of the amino acid sequences of α-glucosidases with known function showed that α-glucosidases hydrolyzing the α-1,6-glycosidic linkage have a Val residue following the catalytic nucleophile in region II. The corresponding residue of α-glucosidases

acting on the α-1,4-glycosidic linkage is Thr (Yamamoto *et al.*, 2010; see also residues inside the red frame in Table 4). Another important position in IMA1 is Gln279 (see Table 4) which is located in the vicinity of catalytic acid/base residue (Yamamoto *et al.*, 2010). Isomaltase mutants Val216Thr and Gln279Ala gained the ability to hydrolyze maltose, so the amino acid residues at these positions are certainly responsible for determining the substrate specificity of α-glucosidases (Yamamoto *et al.*, 2010). Tsujimoto *et al.* (2007) have shown the importance of the position Ala/Val200 (corresponds to Val216 in *Sc* IMA1) also in *Bacillus stearothermophilus* α-1,4-glucosidase and *Bacillus thermoglucosidasius* oligo-1,6-glucosidase (Tables 3 and 4).

Voordeckers *et al.* (2012) predicted *in silico* and resurrected *in vitro* a hypothetical ancestor protein ancMALS (Table 4) of maltases and isomaltases of *Saccharomyces* yeasts (see also Figure 4 in Voordeckers *et al.*, 2012). The ancMalS was predicted as bifunctional being primarily active on maltose-like substrates, but also having a minor activity on isomaltose-like sugars. The present-day α-glucosidases of *S. cerevisiae* preferentially hydrolyze either isomaltose-like sugars (IMA1, IMA2 and IMA5) or maltose-like sugars (MAL12, MAL32, MAL62) (Voordeckers *et al.*, 2012). The authors speculate that it is difficult to fully optimize these two activities in one protein – so the catalytic activity of the promiscuous ancestral protein stays quite low. Gene duplication and subfunctionalization of the gene product resolved this adaptive conflict optimizing the subfunctions separately in different paralogs (Voordeckers *et al.*, 2012). Gabriško (2013) has studied the evolutionary origin of GH13 α-glucosidases and pointed out that respective fungal enzymes are always closely related to the prokaryotic group. Gabriško hypothesised that fungal α-glucosidases may originate from bacterial ancestors and current enzymes retain certain similarity with ancestral ones, but he also considers (ancient) horizontal gene transfer from bacteria as a possibility (Gabriško, 2013). Herein it should be noted that the maltase Mal1 of an 'ancient' yeast *S. pombe* and a bacterial maltase (from *B. stearothermophilus*) both have an Ala and Ile at positions corresponding to Val216 and Gly217 of *S. cerevisiae* IMA1 protein (Table 4). *S. cerevisiae* maltases have Thr and Ala at this position. Interestingly, the *MAL1* gene of *O. polymorpha* has also a property of a bacterial gene – its promoter region is perfectly recognized in a bacterium *Escherichia coli* – it possesses two pairs of sigma 70-like sequences (Alamäe *et al.*, 2003).

1.6. Regulation of the *MAL* genes and proteins in *S. cerevisiae* and some other yeasts

The preferred carbon sources for yeasts are monosaccharides glucose and fructose. In the presence of these sugars, the enzymes required for utilization of other carbon sources are synthesized at a low level or not at all. This phenomen is known as glucose repression. Glucose repression in yeasts has mostly been

studied using *S. cerevisiae* as a model organism and invertase and maltase genes as model genes (Gancedo, 1998; Ronne, 1995; Zimmermann and Scheel, 1977).

1.6.1. *S. cerevisiae:* glucose sensing, inactivation and repression by glucose

To sense low and high concentrations of glucose, two specific transporter-like transmembrane proteins SNF3 and RGT2 with long C-terminal cytosolic extensions are present in *S. cerevisiae* (Gancedo, 1998; Fig 3). In the presence of glucose, maltose utilization in *S. cerevisiae* is prevented at three levels: transcriptional, translational and posttranslational (Klein *et al.*, 1996). Firstly, if glucose is present, maltose transporter is inactivated and degraded as described in chapter 1.4. Thereby maltose does not reach the cell and transcription from *MAL* genes is not induced (Brondijk *et al.*, 1998; Hicke *et al.*, 1997; Lucero *et al.*, 1993; Medintz *et al.*, 2000). Secondly, adding glucose to induced cells will result in mRNA lability of the glucose repressed genes and that has also an effect on translational efficiency. For example, the functional half-life of *MAL62* mRNA decreases from 25 to 6 min in the presence of glucose (Federoff *et al.*, 1983; Gancedo, 1998).

The main repressive effect of carbon source is executed at transcriptonal level (Gancedo, 1998) and it is described below in more detail. The key components of the glucose repression are shown on Figure 3. It is accepted that the signal for glucose repression is transmitted to transcriptional machinery *via* hexokinases in *S. cerevisiae*, with hexokinase PII (HXK2) playing a major role (Ahuatzi *et al.*, 2004; Gancedo, 2008; Mayordomo and Sanz, 2001; Moreno and Herrero, 2002; Zimmermann and Entian, 1997). Transcriptional repression is executed by a repressor protein MIG1. At high glucose concentrations, HXK2 and MIG1 enter the nucleus, where MIG1 leads corepressors TUP1 and CYC8 to target promoters whereas HXK2 stabilizes the repressor complex (Ahuatzi *et al.*, 2007; Gancedo, 1998; Kayikci and Nielsen, 2015; Santangelo, 2006). MIG1 is a C_2H_2 zinc finger protein which binds to several promoters of genes repressed by glucose such as *SUC2* and most genes of the *MEL-GAL* regulon (Nehlin *et al.*, 1991; Nehlin and Ronne, 1990). When glucose concentration reduces, the SNF1 protein kinase will inactivate MIG1 by phosphorylation and MIG1 will be exported from the nucleus, allowing transcriptional activation of the promoters (Ahuatzi *et al.*, 2004, 2007; Gancedo, 2008; Kayikci and Nielsen, 2015; Moreno and Herrero, 2002). The recognition sequence in promoters for MIG1 is (G/C)(C/T)GGGG, an AT-rich sequence is needed at the 5' end of the GC-box (Lundin *et al.*, 1994; Needham and Trumbly, 2006; Santangelo, 2006).

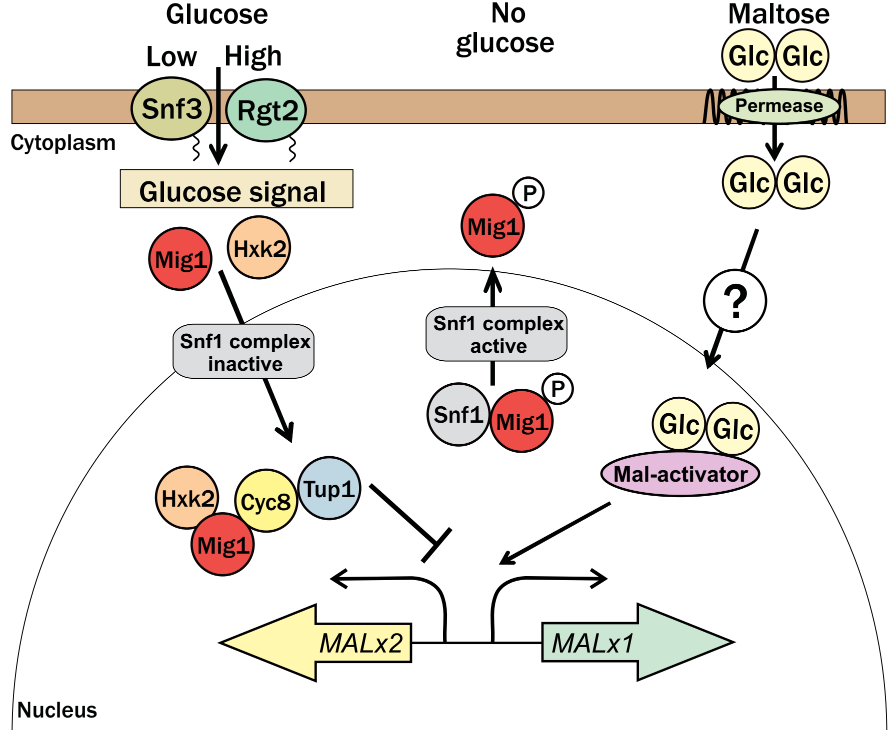

Figure 3. A simplified scheme of glucose-repression and maltose-induction in *S. cerevisiae.* The arrows indicate inducing effects and a hammerheaded line indicates repressing effects.

1.6.2. *S. cerevisiae:* induction by maltose

The presence of maltose in the medium induces the transcription of the *MALx2* (maltase) and *MALx1* (maltose permease) genes in *S. cerevisiae* (Jiang *et al.*, 2000; Wang *et al.*, 1997; Wang and Needleman, 1996; Yao *et al.*, 1994). Maltose permease and maltase genes share a 700 bp bi-directional promoter region that coordinate transcription of both genes (Bell *et al.*, 1995; Dubin *et al.*, 1985; Meurer *et al.*, 2017; Needleman *et al.*, 1984). In the presence of maltose, the transcription of both genes is induced *via* binding of the MAL-activator (MALx3) to the bi-directional promoter (Chang *et al.*, 1988); Kim and Michels, 1988; Sirenko *et al.*, 1995; Meurer *et al.*, 2017; Fig 3). Glucose represses expression of both genes through binding of the MIG1 repressor even if the inducer maltose is present (Klein *et al.*, 1998). So, the activation/ repression of the *MALx1* – *MALx2* bi-directional promoter may be explained by competition between MIG1 and MAL63 for GC-binding boxes (Gancedo, 1998).

MAL63 is a 470 aa zinc finger protein belonging to C_6 zinc cluster proteins and it binds to the DNA as a dimer (Chang *et al.*, 1988; Kim and Michels, 1988; Sirenko *et al.*, 1995). Mutation in *MAL63* gene unables the induction of maltase

and maltose permease genes (Novak *et al.*, 2004). Some laboratory strains of *S. cerevisiae* (for example S288C and W303-1A) fail to grow on maltose and other α-glucosidic sugars because of a nonfunctional *MAL*-activator allele (Brown *et al.*, 2010; Meurer *et al.*, 2017). The DNA sequence recognized by MALx3 is not yet clear. Initially it was suggested that motif GAAA(A/T)TTTCGC is important, but later on sequences $CGGN_9CGG$, $CGCN_9CGC$ and $CGGN_9CGC$ (N_9 is AT rich) were proposed as crucial for maltose-induced activation of promoters (Gancedo, 1998). It has been hypothesized that binding of maltose to MAL63 causes confirmational changes in the activator and leads to transcriptional activation (Danzi *et al.*, 2000; Wang and Needleman, 1996), but the exact mechanism is unknown (Fig 3). Constitutive alleles of *MAL63* encode proteins with several mutations in C-terminal regions which may lead to active confirmation even if no maltose is present (Wang and Needleman, 1996). Expression of *MAL63* is repressed by glucose *via* MIG1 repressor protein – removal of MIG1 binding sites in *MAL63* promoter and in the *MIG1*-disruption mutant the *MAL63* is expressed in the presence of glucose (Wang and Needleman, 1996).

1.6.3. Regulation in *O. polymorpha* and some other yeasts

In 2004, Stasyk *et al*. characterized a hexose transporter homologue GCR1 in *O. polymorpha* that was similar to glucose sensors SNF3 and RGT2 of *S. cerevisiae*, but lacked the C-terminal cytosolic „tail" (Stasyk *et al.*, 2004) which has been shown essential in SNF3 for glucose sensing (Santangelo, 2006; Vagnoli *et al.*, 1998). The GCR1-deletion mutant grew well on high glucose and the authors suggested that GCR1 could be involved in high-affinity glucose transport or its regulation (Stasyk *et al.*, 2004). In 2008, Stasyk *et al*. characterized another transporter-like protein – HXS1 – with similarity to *S. cerevisiae* RGT2 and SNF3. The *HXS1* gene did not complement the hexose transporter-less mutant of *S. cerevisiae* indicating that HXS1 is not a functional transporter, but rather a sensor (Stasyk *et al.*, 2008).

Induction of maltase synthesis by maltose and sucrose, and repression by glucose was shown for *O. polymorpha* already in 1998 (Alamäe and Liiv, 1998). Growth of *O. polymorpha* on glycerol and ethanol allowed derepression of maltase synthesis (Alamäe and Liiv, 1998). Literature data show that glucose repression mechanisms of *O. polymorpha* differ from those described for *Saccharomyces*. Differently from *S. cerevisiae*, both hexokinase and glucokinase can mediate glucose repression in *O. polymorpha* whereas fructose repression is mediated only by hexokinase (Kramarenko *et al.*, 2000; Laht *et al.*, 2002). Therefore, phosphorylation of the sugar seems to be important for initiation of the repression. Stasyk *et al*. (2007) described *S. cerevisiae* MIG1 and MIG2 homologues of *O. polymorpha*. The identity of OpMIG2 and OpMIG1 to ScMIG1 is up to 76% and 80% when N-terminal conserved regions are compared (Stasyk *et al.*, 2007). The OpMIG1 and OpMIG2 are respectively

480 aa and 204 aa long and they are 33% identical to each other (Stasyk *et al.*, 2007). Disruption of *MIG1* and *MIG2* genes in *O. polymorpha* had only a minor effect on glucose repression of alcohol oxidase (Stasyk *et al.*, 2007) and no effect on glucose repression of maltase (unpublished data). As hexokinase and MIG1 proteins have no specific role in glucose repression in *O. polymorpha*, some mechanism alternative to that of *S. cerevisiae* should be involved. *O. polymorpha MAL1* gene was expressed in *S. cerevisiae* maltase-negative mutant 100-1B and it was regulated the same way as the *S. cerevisiae* native maltase (Alamäe *et al.*, 2003). It may allow to hypothesize that regulator proteins from *S. cerevisiae* are able to bind the promoter of *O. polymorpha MAL1*. The analysis of the promoter region of *O. polymorpha MAL1* also showed that there are potential MIG1 and MAL63 binding sites (Alamäe *et al.*, 2003).

MAL63 homolog a zinc finger protein CaSUC1 has been found in *C. albicans* and it complements the *MAL63* mutant of *S. cerevisiae* (Kelly and Kwon-Chung, 1992). The CaSUC1 shares 28% identity with *S. cerevisiae* MAL63. CaSUC1 is required to up-regulate expression of *C. albicans* α-glucosidase by maltose and sucrose (Kelly and Kwon-Chung, 1992).

S. cerevisiae MIG1 homologs have been found from *C. albicans* (Zaragoza *et al.*, 2000), *Kluyveromyces lactis* (Cassart *et al.*, 1995), *C. utilis* (Delfin *et al.*, 2001), *S. pombe, Scwanniomyces occidentalis* (Carmona *et al.*, 2002) and *Aspergillus* sp. (Gancedo, 1998; Klein *et al.,* 1998). MIG1 homologs from *K. lactis* and *K. marxianus* complement the *S. cerevisiae* MIG1 mutant restoring glucose repression (Cassart *et al.*, 1995, 1997). *C. albicans CaMIG1* was also able to complement the MIG1 deficiency in *S. cerevisiae*, but disruption of *CaMIG1* did not relieve glucose repression (Zaragoza *et al.*, 2000). So, the mechanism of MIG1 functioning in *C. albicans* is not yet known.

Alves-Araujo *et al*. (2004) cloned *T. delbueckii* maltose permease *TdMAL11* and further sequencing revealed the presence of maltase gene *TdMAL12* transcribed from the opposite strand. The expression of both of the genes is regulated by glucose. Analysis of the intergenic region of *TdMAL11-TdMAL12* genes revealed the presence of two potential MAL-activator and MIG1 binding sites and one of these site was overlapping as in *S. cerevisiae* (Alves-Araújo *et al.*, 2004). This finding is in good correlation as the expression of maltose permease *TdMAL11* is regulated the same way as in *S. cerevisiae*. No *MAL*-activator has been found in this yeast (Bussereau *et al.*, 2006; Fairhead and Dujon, 2006).

II AIMS OF THE STUDY

The main aim of the study was to characterize the genes and proteins responsible for metabolism of α-glucosidic disaccharides in *O. polymorpha*.

In more detail, the aims of my work are as follows:

- To identify the genomic clustering of *MAL* genes in *O. polymorpha* (Refs I and II)
- To characterize transport of α-glucosidic sugars and respective transporter OpMAL2 in *O. polymorpha* (Refs I, II and III)
- To characterize substrate specificity of the α-glucosidase OpMAL1 and significance of Thr200 in its substrate selection (Ref IV)
- To characterize the regulation of expression of α-glucosidase OpMAL1 and α-glucoside permease OpMAL2 by carbon sources (Refs I, II, III, IV)

III RESULTS AND DISCUSSION

3.1. The *MAL* cluster of *O. polymorpha* (Refs I and II)

The maltase structural gene (*MAL1*) of *O. polymorpha* was isolated from a genomic library by Tiina Alamäe's research group in 2001 (Liiv *et al.*, 2001). An open reading frame of 1695 bp encoding a 564 aa protein with calculated molecular weight of 65.3 kD (see Table 2) was characterized in the genomic insert of the library plasmid p51 (Liiv *et al.*, 2001).

Inspection of several Génolevures project (Blandin *et al.*, 2000; Feldmann, 2000) library clones of *O. polymorpha* CBS 4732 showed that genomic inserts of the clones BB0AA021D05, BB0AA011B12 and BB0AA003C10 (Fig 4) contained fragments of different *MAL* genes. Further analysis of these clones disclosed composition of the *O. polymorpha MAL* locus. In Ref 1 a bidirectional promoter region was identified between the *MAL1* and *MAL2* genes of *O. polymorpha* (see scheme of BB0AA011B12 in Figure 4) and regulation from this promoter by carbon sources was studied (will be discussed in chapter 3.3). In addition to N-terminal fragment of the maltase gene and full-length sequence of the permease gene, the clone BB0AA011B12 contained the N-terminal fragment of a putative Zn-finger *MAL*-activator gene (Fig 4). From analysis of sequences of library clones BB0AA011B12 and BB0AA021D05 present in the Génolevures database, it was considered that *O. polymorpha* has a three-gene *MAL* locus (Ref 1). However, sequencing of the genomic insert of the clone BB0AA021D05 showed that sequence AL434102.1 belongs to the *MAL*-activator 2 gene instead of *MAL*-activator 1 gene as was erroneously reported in Ref I (see Fig 1a of Ref I). In fact, the genomic library clone BB0AA021D05 contains the complete sequence of the *MAL*-activator 1 gene and a sequence coding a C-terminal fragment of the putative *MAL*-activator 2 (see Fig 4, BB0AA021D05 correct). To conclude, *O. polymorpha* has two hypothetical Zn-finger transcription factor genes next to the *MAL2* gene which were named *MAL*-activator 1 (*MAL-ACT 1*) and *MAL*-activator 2 (*MAL-ACT 2*). Full-length *MAL*-activator 2 gene is present in the library clone BB0AA003C10 (Fig 4).

As shown in Figures 4 and 5, the composition of the *O. polymorpha MAL* locus is almost the same as of *MAL*-loci of *S. cerevisiae* except for the number of *MAL*-activator genes (two *vs* one) and the transcriptional direction of the first *MAL*-activator gene. For composition of a *MAL* locus of *S. cerevisiae* see Figure 2.

GenBank accession numbers of the genes from the *MAL* locus of *O. polymorpha* CBS 4732 are given in Figure 5. Full-length sequence of the *MAL* locus of *O. polymorpha* CBS 4732 is accessible under the number MH252366 (shown also on Fig 5). Comparison of genomic sequences of *MAL* clusters from *O. polymorpha* strains NCYC 495 leu1.1 and CBS 4732 showed their identity.

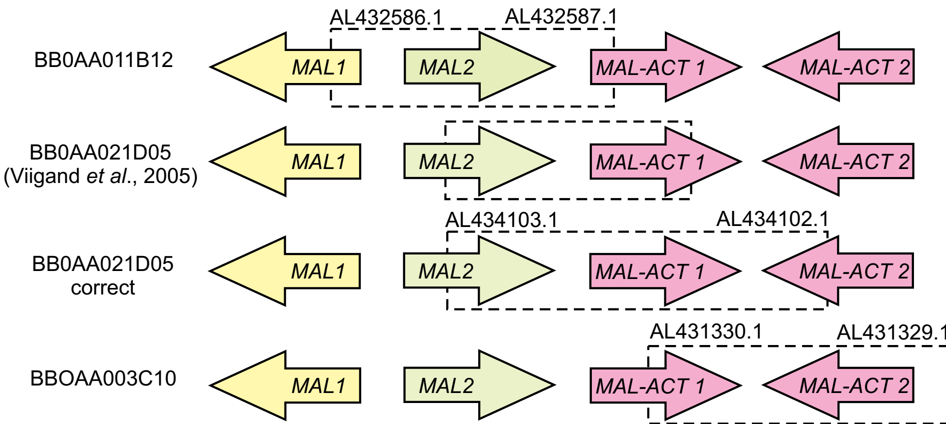

Figure 4. Composition of the *O. polymorpha MAL* locus. Genomic inserts in the Génolevures library clones are shown within the frame. Respective GenBank numbers of sequences belonging to *MAL* genes are shown on top of the frames. *MAL1* gene encoding a maltase is shown in yellow, *MAL2* gene encoding the permease is shown in green and the putative *MAL*-activator genes are shown in pink. The corrected scheme of the insert in clone BB0AA021D05 is also shown.

As *MAL* loci of *S. cerevisiae* are positioned subtelomerically, the chromosomal location of the *O. polymorpha MAL* locus was inspected. The first *O. polymorpha* genome – of strain CBS 4732 – was sequenced in 2003 (Ramezani-Rad *et al*. 2003), but the sequence is not yet public. Therefore, the genome of *O. polymorpha* strain NCYC 495 leu1.1 (Grigoriev *et al.*, 2014; MycoCosm portal https://genome. jgi.doe.gov/programs/fungi/index.jsf) was used instead to illustrate the chromosomal position of the *MAL* locus in *O. polymorpha*. Figure 5 (bottom) shows that in *O. polymorpha* the *MAL* cluster is not subtelomeric.

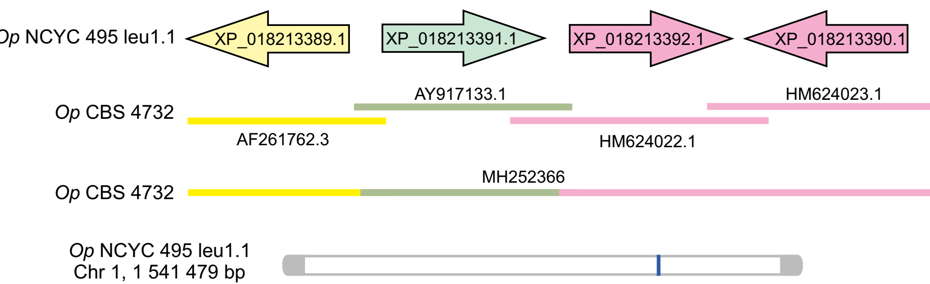

Figure 5. Composition of *MAL* clusters in *O. polymorpha* CBS 4732 and NCYC 495 leu1.1. Maltase gene is yellow, permease gene is green and *MAL*-activator genes are pink. Accession numbers of CBS 4732 *MAL* gene sequences and the full-length *MAL* cluster deposited to GenBank are given next to coloured line referring to the respective gene sequence. The lower panel depicts position of the *MAL* cluster (in blue) in chromosome 1 of *O. polymorpha* NCYC 495 leu1.1. Subtelomeric regions (50 kbp) are shown in grey. The chromosome was visualised using a PhenoGram program (http://visualization.ritchielab.psu.edu/phenograms/plot).

Analysis of the N-terminal fragment of the putative MAL-activator 1 (*Op* MALACT 1) also encoded in the Génolevures clone BB0AA011B12 (Fig 4) revealed presence of a Zn-finger motif containing six conserved cysteins (Ref I) as in the case of MAL-activators MAL63 from *S. cerevisiae* and CaSUC1 from *Candida albicans* (Chang *et al.*, 1988; Kelly and Kwon-Chung, 1992). Alignment of the N-terminal parts of yeast MAL-activators is shown in Figure 6. At this time there was information on only one putative *MAL*-activator gene (*MAL*-activator 1). Further analysis of the *MAL* cluster revealed the presence of another putative *MAL*-activator next to it (Figs 5 and 6).

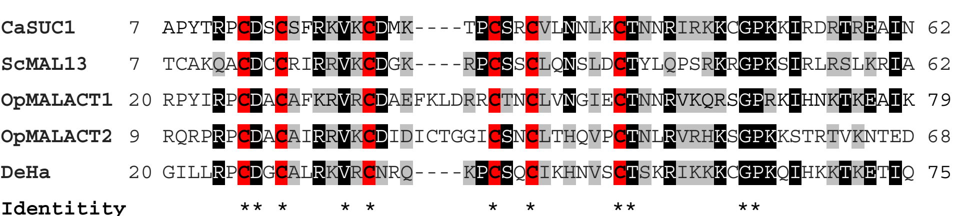

Figure 6. Alignment of the N-terminal parts of the putative MAL-activator proteins. The figure was adjusted from Ref I, Figure 2b, by adding the sequence of the *Op*MALACT 2. CaSUC1 from *Candia albicans* (P33181); MAL13 from *S. cerevisiae* (P53338); DeHa, a hypothetical MAL-activator from *Debaryomyces hansenii* (Q6BYN4). Six cysteine residues of zinc fingers are shown in red. The crosses above the alignment designate the predicted nuclear transport motif of the CaSUC1 protein (Blandin *et al.*, 2000).

The N-terminal part of both hypothetical Mal-activators of *O. polymorpha* aligns well with yeast Mal-activators and contains six conserved cysteins (Fig 6). Both putative *MAL*-activator genes of *O. polymorpha* have been sequenced and submitted to the GenBank under accession numbers HM624022.1 and HM624023.1 (Fig 5). Proteins deduced from respective gene sequences are 570 aa (MAL-activator 1) and 628 aa (MAL-activator 2) long. The identity between these two proteins is only 26%, their identity to *C. albicans* CaSUC1 is 23-24%, and the identity to the *S. cerevisiae* MAL63 is even less – 15–16% (unpublished data). Despite the low identity (only 28%) between the CaSUC1 and MAL63 proteins of *S. cerevisiae*, CaSUC1 of *C. albicans* can replace the function of the MAL-activator of *S. cerevisiae* (Kelly and Kwon-Chung, 1992).

Southern blot has confirmed presence of a single maltase gene in *O. polymorpha* (Liiv *et al.*, 2001). The fact that disruptants of *O. polymorpha MAL1* and *MAL2* genes lose the ability to grow on α-glucosidic sugars (Refs II and IV) confirms that these genes encode sole functional proteins for the transport and hydrolysis of α-glucosidic sugars. On the basis of information available it can be concluded that the *O. polymorpha* has a single *MAL* locus that consists of four genes: maltase (maltase-isomaltase), maltose (α-glucoside) permease and two putative *MAL*-activators. The published experimental data confirm that *MAL1* and *MAL2* genes are indispensable for utilization of α-glucosidic sugars

by *O. polymorpha*. Functionality and role of two putative *MAL*-activators still have to be proven. There is indirect support of functionality for at least *MAL*-activator 1 gene. Namely, the promoter of this gene was regulated by carbon sources the same way as the promoters of *MAL1* and *MAL2* genes (Table 4 in Ref II).

MAL clusters have been previously identified in *Scheffersomyces stipitis* (Jeffries and Van Vleet, 2009) and *Kluyveromyces lactis* (Fairhead and Dujon, 2006; Leifso *et al.*, 2007) though no *MAL*-locus identical to *O. polymorpha* has been found.

In addition to the *MAL* cluster, the nitrate cluster has been described in *O. polymorpha*. It consists of five genes: a nitrate transporter *YNT1*, a nitrite reductase *YNI1*, a nitrate reductase *YNR1* and transcription factors *YNA1* and *YNA2* (Silvestrini *et al.*, 2015; Siverio, 2002). Genes required for nitrate assimilation are also clustered in *B*. (*Arxula*) *adeninivorans* (Böer *et al.*, 2009), *Aspergillus fumigatus* (Amaar and Moore, 1998), *A. nidulans* (Johnstone *et al.*, 1990) and *A. oryzae* (Amaar and Moore, 1998; Johnstone *et al.*, 1990; Kitamoto *et al.*, 1995). Recently a gene cluster involved in MEL (mannosylerythritol lipids) biosynthesis was described in basidiomycetous yeast *Pseudozyma tsukubaensis* (Saika *et al.*, 2016). *S. stipitis* is exceptionally rich in gene clusters – at least 35 clusters of functionally related genes were discovered after sequencing of the genome (Jeffries and Van Vleet, 2009). As noted by Jeffries and Van Vleet (2009), genes coding proteins with physiologically related functions may have a survival advantage when coinherited. Hurst *et al.* (2004) have proposed the "coregulation" model – metabolic gene clustering through selection for more precise coordination of gene regulation pathway.

3.2. The α-glucoside permease of *O. polymorpha* (Refs I, II and IV)

The putative maltose permease gene *MAL2* from the *MAL* cluster of *O. polymorpha* was sequenced and the deduced protein sequence was aligned with other permeases (Ref I). Comparison of *O. polymorpha* maltose permease protein (582 aa) deduced from the *MAL2* gene revealed 39–57% identity with yeast maltose permeases (Table 1 in Ref I) with the hypothetical maltose permeases of *D. hansenii* and *C. albicans* being the closest homologs but these transporters have not yet been biochemically characterized. These yeasts are also neighbors on the phylogenetic tree and their maltase proteins are also highly similar (Liiv *et al.*, 2001; Ref I). The closest homolog of *O. polymorpha* MAL2 from *S. cerevisiae* is an experimentally characterized permease – the general α-glucoside transporter AGT1 (identity 41%; Ref I).

To investigate functionality of the MAL2 protein, the *MAL2* gene was disrupted in *O. polymorpha* genome using homologous recombination (Fig 1b in Ref II). The gene disruption was highly (95%) efficient hinting that this is the

only copy of maltose permease gene as previously shown also for the *MAL1* gene (Liiv *et al.*, 2001). The *MAL2* disruptant of *O. polymorpha* mutant lost the ability to grow on maltose, sucrose, trehalose, maltotriose and turanose. Even though the MAL2 permease of *O. polymorpha* is responsible for the transport of trehalose, the MAL1 protein does not hydrolyze this sugar (Liiv *et al.*, 2001) and the *MAL1* disruption mutant of *O. polymorpha* grows on trehalose (Fig 6 in Ref II). Though internalized through the MAL2 permease, trehalose is hydrolyzed in *O. polymorpha* cell not by a maltase but a specific enzyme – trehalase (Ishchuk *et al.*, 2009). Even though MAL2 was responsible for trehalose uptake, its expression was not induced during growth on trehalose (Ref II). Complementation of the permease disruption mutant with the *MAL2* gene on a plasmid restored the growth on maltose and sucrose showing that the MAL2 permease is solely responsible for the transport of these sugars (Ref II). The MAL2 permease was also functional in a *S. cerevisiae* maltose permease-negative mutant restoring its growth on maltose (Ref I).

In addition to natural α-glucosidic sugars the MAL2 permease (and for example the AGT1 permease of *S. cerevisiae*) also transports a synthetic chromogenic α-glucosidic substrate PNPG (Hollatz and Stambuk, 2001; Ref II). PNPG transport is very convinient to measure (Hollatz and Stambuk, 2001) and this is why this method was applied to characterize the properties of the MAL2 permease.

Study of energization of the MAL2 permease indicated that similarly to α-glucoside transporters of other yeasts (Stambuk *et al.*, 2000; Hollatz and Stambuk, 2001; Reinders and Ward, 2001), transport by MAL2 of *O. polymorpha* was characterized as energy-dependent proton-symport. In good accordance with that, PNPG transport in *O. polymorpha* was dependent on pH (with pH optimum of 5.0; Fig 4 in Ref II). The pH optimum 5.0 for PNPG transport has previously been shown also for *S. cerevisiae* (Stambuk, 2000). The transport by MAL2 in *O. polymorpha* was sensitive to protonophores-energy uncouplers carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and sodium azide (NaN_3) (Table 2 in Ref II).

Inhibition of PNPG transport by various α-glucosidic substrates was used to reveal substrate specificity of the MAL2 permease. The K_i values calculated from inhibition studies reflect the affinity of the MAL2 permease for these sugars. The MAL2 has a high affinity (K_m 0.51 mM) for PNPG (Table 3 in Ref II). The K_m value of the AGT1 permease of *S. cerevisiae* for PNPG is ~3 mM whereas maltose permeases MAL21 and MAL61 of *S. cerevisiae* do not transport PNPG (Table 1 and references therein). Sucrose, maltose, trehalose, maltotriose, turanose and α-MG competitively inhibited the transport by MAL2 in *O. polymorpha* (respective K_i values were between 0.23 and 1.47 mM; Table 3 in Ref II). α-MG is probably able to bind the MAL2 permease and also the MAL1 (inhibits respectively PNPG transport and hydrolysis), but *O. polymorpha* does not grow on this synthetic substrate. High affinity maltose transport system has also reported for *C. utilis* with the K_m for maltose 0.4 mM (Peinado *et al.*, 1987). *S. cerevisiae* AGT1 has much lower affinities for its

substrates (Table 1): K_m for maltose is 5.1–17.8 mM (Day, Higgins, *et al.*, 2002; Stambuk and de Araujo, 2001), K_m for sucrose is ~ 8 mM (Stambuk *et al.*, 2000), K_m for trehalose is 7 mM (Stambuk and de Araujo, 2001) and for maltotriose 4–18.1 mM (Day, Rogers, *et al.*, 2002; Stambuk *et al.*, 2000). Glucose also inhibited PNPG transport by *O. polymorpha* MAL2 (K_i ~1 mM, but in noncompetitive manner; Table 3 in Ref II).

To conclude, considering a wide range of substrates transported by the MAL2 permease, it should be defined as an α-glucoside permease rather than a maltose permease (Ref II). It was shown using the *MAL2*-disruption mutant that this permease is responsible for the transport of a at least maltose, sucrose, turanose, maltotriose, maltulose, melezitose, isomaltose, palatinose and IMOs (Ref IV).

The affinity of the MAL2 permease for maltose and sucrose is much higher (respective K_i values were 0.23 and 0.38 mM; Table 3 in Ref II) than that of the maltase for these sugars (respective K_m values 51.8 and 25.1 mM; Table 2 in Ref IV). This indicates that these substrates must be concentrated into the cell to enable their efficient hydrolysis. Also, transport is most probably a limiting step in the utilisation of α-glucosidic sugars by *O. polymorpha* as also shown for *S. cerevisiae* (Chang *et al.*, 1989). Transport of α-glucosidic sugars is also crucial for the induction of *MAL* genes. It has been proven for *S. cerevisiae* that intracellular maltose is required for the induction of maltase gene expression (Wang *et al.*, 2002). The same was shown by us for *O. polymorpha* by using a *MAL2*-disruptant mutant – though maltose was present in the growth medium, no maltase gene induction was seen (Table 4 in Ref II).

3.3. Regulation of the *MAL* genes in *O. polymorpha* (Refs I, II and III)

Regulation of the expression from the *MAL1* promoter in *O. polymorpha* and *S. cerevisiae* has been investigated previously (Alamäe *et al.*, 2003). *S. cerevisiae* maltase gene *MAL62* was repressed by glucose and induced by maltose and sucrose when expressed from its native promoter in maltase-negative mutant of *O. polymorpha* (Alamäe *et al.*, 2003). The same was true *vice versa* – the *O. polymorpha MAL1* promoter was recognized and correctly regulated by the carbon source in a *S. cerevisiae* maltase-negative mutant (Alamäe *et al.*, 2003). As the promoters of the maltase genes of these two yeast species were crosswise recognized, it was hypothesized that transcriptional regulators of *S. cerevisiae MAL* genes (MAL-activator and MIG1 repressor) probably participate in the regulation of the expression of the *O. polymorpha* maltase gene (Alamäe *et al.*, 2003). Potential *S. cerevisiae* MAL-activator and MIG1 binding sites in *O. polymorpha* have been discussed by Alamäe *et al.* (2003). It is also interesting that the promoter of the *MAL1* gene of *O. polymorpha* is perfectly recognized in a prokaryote *Escherichia coli* (Alamäe *et al.*, 2003).

Regulation of expression from the bidirectional *MAL1*-*MAL2* promoter region of *O. polymorpha* was investigated in Ref I by using a single- and a two-reporter test system. The *MAL1-MAL2* bidirectional promoter was coordinately regulated by carbon sources in both directions: repressed by glucose and induced by maltose, while the basal expression was higher in the direction of the permease gene (Fig 4 in Ref I). It is reasonable, because the permease activity is first required to provide intracellular maltose that is needed for induction of the *MAL* genes (Ref II). Coordinated expression by carbon sources has been also described for *S. cerevisiae MAL61-MAL62* bi-directional promoter, except that for *MAL61–MAL62* the basal expression was higher in the maltase direction (Bell *et al.*, 1995; Levine *et al.*, 1992). As *S. cerevisiae* strains usually have several *MAL* loci, they most probably have sufficiently high basal activity of maltose transport to ensure the *MAL* genes induction. It is also noteworthy that induction of the *O. polymorpha* bidirectional *MAL1*-*MAL2* promoter is stronger in the maltase direction (Table 4 in Ref II) and induced strength of the *MAL1* promoter (induced by maltose or sucrose) constitutes up to 70% of that of the MOX promoter (Alamäe *et al.*, 2003). This knowledge can be used in biotechnological applications. The *MAL1* promoter has already been successfully used to overexpress and purify a biotechnologically relevant levansucrase protein from *E. coli* (Visnapuu *et al.*, 2008).

When testing the *O. polymorpha MAL2* permease functionality in a *S. cerevisiae* permease-negative mutant, no growth complementation on maltose was seen when the *MAL2* was expressed from its own promoter (Table 2 in Ref I). Maltose growth appeared only after replacement of the native promoter with that of the *S. cerevisiae* maltose permease gene (Table 2 in Ref I). Thus, the *MAL1*-*MAL2* promoter of *O. polymorpha* is functional in *S. cerevisiae* only in the direction of the maltase gene. Potential *MAL*-activator and MIG1 repressor binding sites were searched from the *MAL2* promoter region. In *S. cerevisiae* the consensus sequence for *MAL*-activator binding is proposed to be CGG/CN$_9$CGG/C where N$_9$ region is AT-rich (Gancedo, 1998). As matching binding sites were not found in *O. polymorpha MAL2* promoter region (unpublished data), it allows to conclude that *S. cerevisiae* MAL-activator most probably cannot bind the *MAL2* promoter and induce the transcription from the *MAL2* gene.

In Ref II the regulation of the *MAL*-activator 1 promoter was also assayed. The intergenic region between the *MAL2* and putative *MAL*-activator 1 gene is AT-rich and rather short (238 bp) (Ref I). The reporter gene assay showed that expression from that promoter region was regulated similarly to *MAL1*-*MAL2* promoter: induced by maltose and sucrose, repressed by glucose and derepressed during glycerol growth (Table 4 in Ref II). Therefore, the *MAL*-activator 1 gene may encode a functional regulator.

The signal for glucose repression is mediated by hexokinases in *S. cerevisiae*, with the main role of hexokinase PII (HXK2) (see subchapter 1.6.1 for review). *O. polymorpha* has two hexose kinases: a hexokinase phosphorylating both glucose and fructose, and a glucose-specific glucokinase (Kramarenko *et*

al., 2000). It has been shown in *S. cerevisiae* that hexokinase-negative mutants lack glucose repression (Moreno and Herrero, 2002; Zimmermann and Scheel, 1977). In contrast to *S. cerevisiae*, hexokinase has no specific role in glucose repression in *O. polymorpha* – the absence of both glucose phosphorylating enzymes is required to abolish glucose repression. In hexokinase-negative mutants only fructose repression is lost whereas glucose repression is retained (Kramarenko *et al*., 2000). Study of hexose kinase mutants of *O. polymorpha* showed that monosaccharides glucose and fructose repressed the *MAL1* promoter only if these monosaccharides were phosphorylated by the cell, whereas if the cell could not phosphorylate these sugars, activation of the *MAL1* promoter was observed (Table 4 and Fig 8 in Ref III). For example, if a double kinase-negative mutant of *O. polymorpha* (has no glucose phosphorylating enzymes and cannot grow on glucose) was cultivated on glycerol in the presence of glucose, a high maltase activity was recorded in the cells (Fig 8 in Ref III). This phenomenon allowed to raise a hypothesis according to which phosphorylated glucose (glucose-6-phosphate, Glc6P) acts as a signalling metabolite for sugar repression in *O. polymorpha* (Fig 7) whereas glucose that stays unphosphorylated acts as an activator of *MAL* genes (Ref III). Hypothesis on Glc6P as a repressing metabolite is supported by the fact that 2-deoxy-D-glucose (2DG; a glucose analogue) also causes glucose repression even though not metabolized further the phosphorylation step (Ref III).

The proposed scheme of regulation of the expression from *MAL1-MAL2* bidirectional promoter and the role of hexokinase and glucokinase proteins is shown in Figure 7.

It is presumed that *O. polymorpha* senses by yet unknown mechanism intracellular concentration of Glc6P and in response down-regulates the transcription from *MAL1-MAL2* promoters (Ref III). In double kinase-negative mutants of *O. polymorpha* unphosphorylated glucose accumulates in the cell and activates the transcription of *MAL1-MAL2* promoters which are trivially considered glucose-repressible (Fig 7; Ref III). Glc6P also signals for fructose repression as the Fru6P will be isomerized to Glc6P after phosphorylation by hexokinase (Ref III). Considering this hypothesis, growth of yeasts on disaccharides is complicated and metabolism must be well balanced – intracellular hydrolysis products of disaccharides cause initial derepression of *MAL* genes, but later on may cause *MAL* promoter repression if phosphorylated glucose accumulates. It has been shown for *S. cerevisiae* that if transport, intracellular hydrolysis and further catabolism of hydrolysis products is not balanced, the cells lyse due to accumulation of a toxic amount of sugar in the cell (Henderson and Poolman, 2017). Involvement of two potential *MAL*-activators of *O. polymorpha* in regulation disaccharides utilization still needs to be investigated.

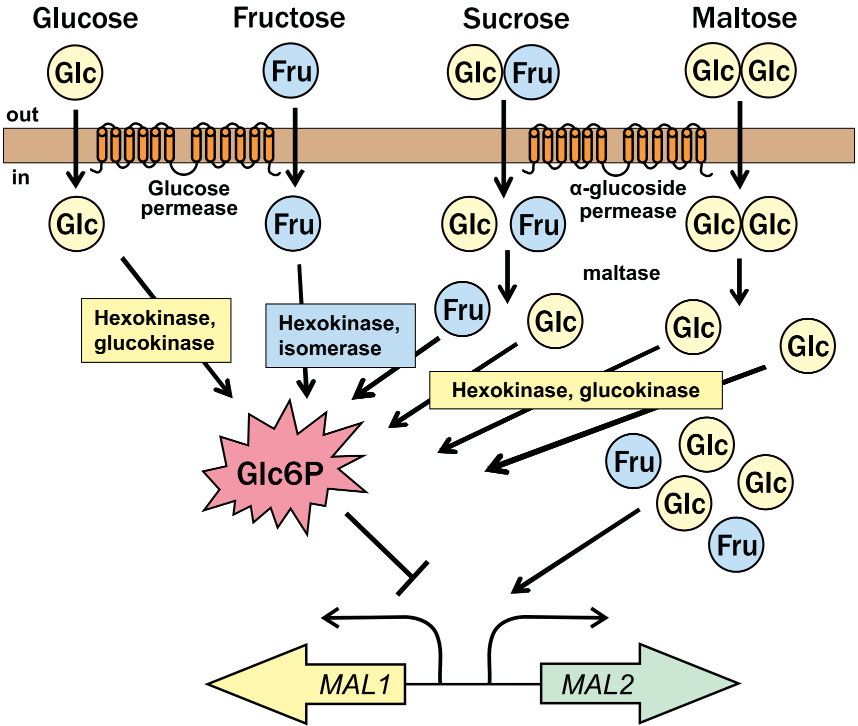

Figure 7. A hypothetical scheme of regulation of sugar metabolism *via* hexose kinases in *O. polymorpha*. Modified from Figure 5 in Ref III.

3.4. Substrate specificity of *O. polymorpha* maltase protein and its similarity to a hypothetical ancestor of yeast maltases and isomaltases (Ref IV)

Disruption of *MAL1* in the *O. polymorpha* genome and further complementation with a *MAL1* gene on a plasmid confirmed that MAL1 is responsible for the utilization of maltose and sucrose in this yeast (Alamäe *et al.*, 2003; Liiv *et al.*, 2001). Substrate specificity assay of the MAL1 protein in crude extract of *E. coli* expressing the MAL1 showed that it can also hydrolyze α-MG (α-methylglucoside) but cannot hydrolyze trehalose, melibiose and cellobiose concluding that MAL1 is active on α-1,4 (as in maltose) and α-1,2 (as in sucrose) glycosidic linkages (Liiv *et al.*, 2001). So, quite interestingly, *O. polymorpha* MAL1 can hydrolyze both maltose-like (maltose and sucrose) and isomaltose-like (α-MG) substrates. In Ref IV the substrate specificity of MAL1 was investigated in more detail and it was shown that it could hydrolyze the following maltose-like substrates with affinities decreasing in the order: maltulose, maltotriose, sucrose, turanose, maltose and melezitose (Table 2 in Ref IV). From isomaltose-like substrates palatinose was the most suitable substrate, followed by isomaltose and α-MG (Table 2 in Ref IV). It was also

showed that MAL1 hydrolyzed also fructooligosaccharides (FOS) 1-kestose and 6-kestose, this property has not been shown before for α-glucosidases (Fig 2 in Ref IV). Respective products inulo- and levanbiose are short fructooligosaccharides which act as prebiotics for beneficial gut bacteria (Adamberg *et al.*, 2014; Visnapuu *et al.*, 2015). Trisaccharides melezitose and panose are also substrates for MAL1, the preferred linkage type is α-1,3 and α-1,6 respectively over α-1,2 linkage (Ref IV; for monomeric composition of α-glucosidic sugars and linkages see Figure 1). Phylogenetic analysis of α-glucosidase sequences shows that MAL1 of *O. polymorpha* clusters together with maltase proteins from *S. stipitis*, *L. elongisporus*, *C. albicans* and *D. hansenii* (Figure 6 in Ref IV). From those yeasts maltase has been characterized only from *C. albicans* and it hydrolyses maltose, sucrose (maltose-like substrates) and α-MG (an isomaltose-like substrate), but not isomaltose (Geber *et al.*, 1992; Table 2). α-Glucosidase from *Torulaspora pretoriensis* (phylogenetically close to *S. cerevisiae*) can use PNPG, maltotriose, isomaltose, α-MG, sucrose and maltose (Oda *et al.*, 1993; Table 2). It was not possible to analyze the substrate specificity-related amino acids of the *T. pretoriensis* α-glucosidase as respective protein sequence is not available. α-Glucosidases have been also studied in phylogenetically „old" yeast *S. pombe*. The intracellular MAL1 of *S. pombe* hydrolyzes PNPG, maltose, sucrose and also dextrin and soluble starch (Chi *et al.*, 2008; Table 2). In addition, *S. pombe* has extracellular maltase AGL1 which is specific for maltose and does not hydrolyze maltose-like sugars maltotriose and turanose (Jansen *et al.*, 2006; Table 2).

Malt extract and IMOs were also tested as substrates for *O. polymorpha* MAL1 and it can be concluded that the DP4 oligosaccharide is the longest substrate for MAL1 (Fig 2 in Ref IV). From this aspect, MAL1 is different from some other yeast α-glucosidases/maltases. For example, the *S. pombe* extracellular α-glucosidase uses maltooligosaccharides with size up to maltoheptaose (Okuyama *et al.*, 2005). Bacterial α-glucosidases also hydrolyze longer oligosaccharides (up to maltoheptaose) and also polysaccharides starch and dextrin in some cases (Table 3). In older publications, the *O. polymorpha* MAL1 has been defined as a maltase, but according to the substrate specificity it should be rather considered as maltase-isomaltase.

It was also shown that glucose released from maltose and maltotriose has inhibitory effect on the enzyme (Table 3 in Ref IV). As in living yeast cells glucose released from di- and trisaccharides will be further metabolized in the glycolysis, the *in vivo* inhibitory effect of glucose is probably lower than that recorded *in vitro*.

Substrate specificity of the MAL1 and MAL2 proteins can be illustrated by a simple growth ability assay (Table 5).

Table 5. Growth of wild-type (wt) *O. polymorpha* and mutants with deleted maltase-isomaltase (*ΔMAL1*) or α-glucoside permease genes (*ΔMAL2*) on solid medium supplemented with different α-glucosidic substrates. Table is modified from Table 4 in Ref IV. Maltose-like substrates are on pink backround and isomaltose-like substrates on green backround.

Substrate	Monomers/linkage	*O. polymorpha*		
		wt	*ΔMAL1*	*ΔMAL2*
Maltose	Glc α(1-4) Glc	+	–	–
Sucrose	Glc α(1-2) Fru	+	–	–
Turanose	Glc α(1-3) Fru	+	–	–
Maltotriose	Glc α(1-4) Glc α(1-4) Glc	+	–	–
Maltulose	Glc α(1-4) Fru	+	–	–
Melezitose	Glc α(1-3) Fru β(2-1) Glc	+	–	–
Trehalose	Glc α(1-1) Glc	+	+	–
α-MG	Glc — CH_3	–	–	–
Isomaltose	Glc α(1-6) Glc	+	–	–
Palatinose	Glc α(1-6) Fru	+	–	–
Isomaltooligo-saccharides	$[$Glc α(1-6) Glc$]_n$	+	–	–

The data on growth confirmed that wild-type *O. polymorpha* does not grow on α-MG. Reason for this is unclear as the MAL1 protein hydrolyzes α-MG and MAL2 is able to transport this substrate. Both proteins, MAL1 and MAL2, are required for growth on maltose, sucrose, turanose, maltotriose, maltulose, melezitose, isomaltose, palatinose and isomaltooligosaccharides (Table 4 in Ref IV). Only α-glucoside permease is needed for growth on trehalose as this substrate is hydrolyzed in the cell by a trehalase and not by MAL1 (Liiv *et al.*, 2001; Ref II).

As described in subchapter 1.5, *S. cerevisiae* maltases use maltose, maltulose, turanose and maltotriose, isomaltases use isomaltose, α-MG and palatinose, and both use sucrose (see also substrate specificity pattern in Fig 8). Voordeckers *et al.* (2012) raised a hypothesis that modern maltases and isomaltases as those present in *S. cerevisiae* have evolved from a common promiscuous ancestor.

Comparison of the amino acids bordering the active site pocket of α-glucosides from different yeasts showed that maltases have a Thr and isomaltases have a Val at the position corresponding to Val216 in *S. cerevisiae* isomaltase IMA1 (Voordeckers *et al.*, 2012; Table 4). If Val216 was replaced with a Thr in IMA1, the enzyme gained the ability to hydrolyze maltose (Yamamoto *et al.*, 2004). The *O. polymorpha* MAL1 has a Thr at respective position and substitution of Thr200 with a Val in MAL1 reduced the hydrolysis of maltose-like substrates by the enzyme significantly. Thus, the mutant enzyme became more similar to isomaltases (Fig 8).

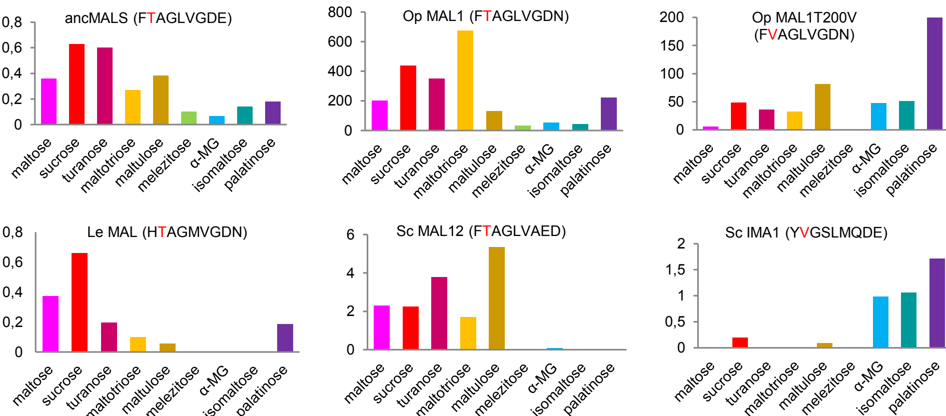

Figure 8. Catalytic efficiencies, k_{cat}/K_m (mM/min) of *Op* maltase-isomaltase MAL1, ancient maltase ancMALS (G279), maltase of *Lodderomyces elongisporus* (*Le*), maltase MAL12 of *S. cerevisiae* (*Sc*), isomaltase IMA1 of *Sc* and the T200V mutant of the *Op* MAL1 (Fig 3 in Ref IV). Data on other proteins, except for the *Op* MAL1 and its mutant, are taken from Voordeckers *et al.* (2012). Signature amino acid sequence (see Figure 3 in Ref IV and Table 4 of current theses) is presented with the residue corresponding to V216 (Val216) of *Sc* IMA1, shown in red.

A catalytically inactive mutant Asp199Ala (D199A) of MAL1 was constructed and differential scanning fluorimetry (DSF) was performed to evaluate binding of α-glucosides and some selected monosaccharides as ligands to the enzyme (Fig 5 in Ref IV). DSF showed that a trisaccharide maltotriose increased the thermostability of the Asp199Ala protein most significantly suggesting that it binds most strongly to the protein and that substrate binding pocket of MAL1 most probably has two plus-subsites for substrate binding (see also Fig 1 in Ref IV). Three monosaccharide binding subsites have been also shown for the *S. cerevisiae* maltase protein (Yao *et al.*, 2003).

Intriguingly, considering the substrate range and the signature amino acids of the substrate-binding pocket, the OpMAL1 protein is highly similar to ancMALS – a resurrected hypothetical ancestor of *Saccharomyces* maltases and isomaltases (Fig 3 in Ref IV). Even though Voordeckers *et al.* (2012) claimed

that both maltase and isomaltase activities cannot be fully optimized in a single ancestral enzyme, it was shown that MAL1 is a good example of a promiscuous enzyme with perfect catalytic ability to hydrolyze a wide range of substrates.

Gabriško has analyzed putative α-glucosidase proteins from yeasts and suggested that (i) a common ancestor of the Ascomycota had two α-glucosidase genes, (ii) in the subphylum Saccharomycotina the gene coding for isomaltase was lost during the evolution and the gene coding for maltase had further lineage-specific duplication (Gabriško, 2013). Gabriško (2013) suggested that in Saccharomycotina evolution, isomaltase-type specificity of α-glucosidases evolved independently and repeatedly in distinct lineages. Brown *et al*. (2010) studied the evolution of subtelomeric gene families in yeasts focusing on *MAL* gene families and suggested that the common ancestor of yeasts had only few *MAL* genes which have completely disappeared in some yeast lineages whereas in the others multiple recent duplication events occurred. With regard to early ancestry of fungal α-glucosidases, phylogenetic analysis has shown that the α-glucosidase mal1 of an "ancient" yeast *S. pombe* shares similarity with α-glucosidases of *Bacillus* bacteria (see Table 4 for signature amino acids) referring that yeast maltases may have bacterial ancestry. Gabriško (2013) has also hypothesized that fungal α-glucosidases may originate from bacterial ancestors.

CONCLUSIONS

The main results of this work are summarized as follows:

1. *MAL* genes are genomically clustered in *O. polymorpha* similarly to *S. cerevisiae*, but the cluster is not subtelomeric. *O. polymorpha* has a single genomic *MAL* cluster that consists of four genes coding for maltase (maltase-isomaltase), maltose (α-glucoside) permease and two putative *MAL*-activators. *MAL1* and *MAL2* genes are indispensable for utilization of α-glucosidic sugars by *O. polymorpha*. Functionality and role of two putative *MAL*-activators still have to be proven.
2. The MAL2 transporter of *O. polymorpha* is a proton symporter with pH optimum of 5.0. The MAL2 permease is responsible for the transport of maltose, sucrose, trehalose, turanose, maltotriose, maltulose, melezitose, isomaltose, palatinose and isomaltooligosaccharides.
3. The MAL1 protein hydrolyzes maltose-like substrates maltulose, maltotriose, sucrose, turanose, maltose and melezitose and isomaltose-like substrates palatinose, isomaltose and α-methylglucoside. The MAL1 hydrolyzes also fructooligosaccharides 1-kestose and 6-kestose and a trisaccharide panose. Maltotetraose is the longest oligosaccharide hydrolyzed by MAL1.
4. *O. polymorpha* MAL1 has a Thr at the key position determining binding of α-glucosidic sugars. Substitution of Thr200 with Val reduces the hydrolysis of maltose-like substrates significantly, making the MAL1 enzyme more similar to isomaltases. Differential scanning fluorimetry (DSF) performed with catalytically inactive mutant Asp199Ala of MAL1 showed that a trisaccharide maltotriose increases the thermostability of the Asp199Ala protein most significantly suggesting that substrate binding pocket of MAL1 has two plus-subsites.
5. The MAL1 has a wide-substrate specificity similar to the promiscuous ancestor of maltases and isomaltases predicted by Voordeckers *et al*. (2012). *O. polymorpha* MAL1 has been defined as a maltase (Liiv *et al*., 2001), but according to currently revealed substrate specificity it should be considered as maltase-isomaltase.
6. The *MAL1-MAL2* bidirectional promoter is coordinately regulated in both directions: repressed by glucose and induced by maltose, while the basal expression is higher in the direction of the permease gene. Induction of the bidirectional *MAL1-MAL2* promoter is stronger in the maltase direction. The promoter of *MAL*-activator 1 gene is regulated the same way as *MAL1-MAL2* promoter: induced by maltose and sucrose, repressed by glucose and derepressed during glycerol and trehalose growth. It can be suggested that the gene encodes a functional regulator.
7. Monosaccharides glucose and fructose repress the *MAL1* promoter only if phosphorylated in the cell, whereas unphosphorylated monosaccharides activate expression from the *MAL1* promoter. It can be proposed that glucose-6-phosphate is a sugar repression signalling metabolite for *O. polymorpha*.

SUMMARY IN ESTONIAN

α-glükosiidsete suhkrute kasutamine pärmil *Ogataea* (*Hansenula*) *polymorpha*

Suhkrud on looduses laialt levinud ning paljudele mikroorganismidele eelistatuim energiaallikas. Suhkrute transporti ning rakusisest kasutamist on põhjalikult uuritud pagaripärmis *Saccharomyces cerevisiae* tema laialdase kasutuse tõttu pagaritööstuses, alkohoolsete jookide kääritamisel ning ka bioetanooli tootmisel. Õllevirde põhilise komponendi maltoosi (kahest glükoosi molekulist koosnev α-1,4 sidemega seotud disahhariid) kasutamises osalevaid geene nimetatakse *MAL*-geenideks ning need paiknevad pagaripärmil *MAL*-lookuses. Pagaripärmi *MAL*-lookustes on reeglina klasterdunud kolm geeni, mis kodeerivad maltaasi, maltoosi transporterit ning nende geenide aktivaatorit. Need lookused paiknevad genoomis telomeeride lähedal ning pärmil on vaja vähemalt ühte lookust, et maltoosi kasutada.

Tiina Alamäe töögrupis on uuritud suhkrute kasutamist metülotroofsel pärmil *Ogataea* (*Hansenula*) *polymorpha* juba aastast 1998. Metülotroofsed pärmid on pärmide hulgas erandlikud võime poolest kasvada ühesüsinikulisel inimesele väga mürgisel alkoholil – metanoolil. Seetõttu on metülotroofsetes pärmides peamiselt uuritud metanooli metabolismiks vajalike spetsiaalsete organellide – peroksisoomide – biogeneesi ning ka tugevaid metanooliga indutseeritavaid promootoreid, mida saab kasutada biotehnoloogias võõrvalkude tootmiseks. Kuna *O. polymorpha* suudab kasvada ka disahhariididel, näiteks maltoosil ja sahharoosil, saab teda kasutada ka disahhariidide metabolismi geenide, valkude ja regulatsiooni uurimiseks. See annab hea võimaluse võrrelda disahhariidide kasutamist ja selle regulatsiooni pagaripärmil ja temast evolutsiooniliselt palju 'vanemal' pärmil *O. polymorpha*.

Käesoleva töö eesmärgiks oli iseloomustada disahhariidide kasutamiseks vajalikke geene ja valke pärmil *O. polymorpha*. Valkudest oli põhitähelepanu suunatud α-glükosiidide transporterile MAL2 ja maltaas-isomaltaasile MAL1.

O. polymorpha genoomse DNA sekveneerimine näitas, et *MAL1* geenil on ' head naabrid'– tema kõrval paikneb α-glükosiidide transporteri geen *MAL2* ning kaks hüpoteetilist *MAL*-aktivaatorgeeni. Seega paiknevad *O. polymorpha MAL*-geenid genoomse klastrina nagu pagaripärmilgi, erinev on vaid *MAL*-aktivaator geenide arv (1 *vs* 2) ja *MAL*-aktivaator 1 geeni suund. Erinevalt pagaripärmist ei paikne *O. polymorpha MAL*-lookus subtelomeerselt. Lookuses paiknevatest geenidest tõestasin funktsiooni *MAL1* ja *MAL2* geenidel ning iseloomustasin vastavaid valke, kuid oletatavate *MAL*-aktivaator geenide funktsioon vajab veel tõestamist.

Maltaasi MAL1 substraadispetsiifilisuse uurimine näitas, et see ensüüm ei ole tüüpiline maltaas ega ka isomaltaas, sest on võimeline hüdrolüüsima väga paljusid erinevaid α-glükosiidseid suhkruid: maltoosi, maltuloosi, maltotrioosi, sahharoosi, turanoosi ja meletsitoosi (maltoosi-tüüpi suhkrud) ning palatinoosi,

isomaltoosi ja α-metüülglükosiidi (isomaltoosi-tüüpi suhkrud). Seetõttu oleks tema korrektne nimetus maltaas-isomaltaas. Oma substraadivaliku poolest on *O. polymorpha* MAL1 valk väga sarnane tänapäevaste pagaripärmis leiduvate maltaaside ja isomaltaaside hüpoteetilise eellasega – nn ‘ürgmaltaasiga’, mis oli Voordeckers’i jt. (2012) hüpoteesi kohaselt vähevaliv. Seega võiks *O. polymorpha* maltaas-isomaltaasi pidada ürgse valgu tänapäevaseks esindajaks ja sellise valgu olemasolu pagaripärmist evolutsiooniliselt ‘vanemal’ pärmil *O. polymorpha* toetab maltaaside ja isomaltaaside evolutsioneerumist vähevalikulisest eellasest.

Uurisin *O. polymorpha* MAL1 valgu substraadivalikut ka mutatsioonanalüüsiga. *S. cerevisiae* maltaasid ja isomaltaasid erinevad substraadi sidumistasku ümbruses paiknevate aminohapete poolest. Pärmide maltaasidest ja isomaltaasidest on kristallstruktuur lahendatud pagaripärmi isomaltaasil 1 (IMA1), mille valgu 216. positsioonis on valiin (Val). Maltaasidel on vastavas positsioonis treoniin (Thr). Näitasin, et *O. polymorpha* MAL1 mutant Thr200Val hüdrolüüsib maltoosi-tüüpi substraate oluliselt kehvemini kui metsiktüüpi MAL1, muutudes seega isomaltaasile sarnasemaks.

O. polymorpha α-glükosiidide permeaas MAL2 on kõrge afiinsusega prootonsümporter. Nii nagu MAL1 valku, iseloomustab ka MAL2 transporterit väga lai substraadivalik. *MAL2* geeni katkestamine genoomis tõestas, et see transporter on vajalik maltoosi, sahharoosi, trehaloosi, turanoosi, maltotrioosi, maltuloosi, meletsitoosi, isomaltoosi, palatinoosi ja isomaltooligosahhariidide transpordiks.

O. polymorpha MAL1 ja *MAL2* geenidel on ühine kahesuunaline promootorala, millelt ekspressioon on mõlema geeni suunas koordineeritult indutseeritud maltoosi ja sahharoosiga ning represseeritud glükoosiga. Induktsioon on tugevam *MAL1* suunal ning basaalne ekspressioon on tugevam permeaasi geeni *MAL2* suunas. *O. polymorpha* mutantide uurimisel selgus, et *MAL1* promootori represseerimiseks glükoosiga on vajalik glükoosi fosforüülimine rakus. Samas on fosforüülimata glükoos võimeline aktiveerima promootori ekspressiooni, mis on väga üllatav tulemus. Püstitasin hüpoteesi, mille kohaselt toimib represseeriva signaalina glükoos-6-fosfaat.

O. polymorpha maltaas-isomaltaasi saaks kasutada valkude evolutsiooni uurimisel mudelina – muteerides teda võiks temast konstrueerida kitsa substraadivalikuga ensüüme, mis on sarnased pagaripärmi maltaasidele ja isomaltaasidele. Kahesuunaline *MAL1*-*MAL2* promootorala võimaldab samaaegselt ja koordineeritult ekspresseerida kahte erinevat geeni või siis ühe ja sama geeni kahte koopiat. Viimane variant peaks suurendama sünteesitava valgu hulka. Kuna *O. polymorpha MAL1* geeni promootor on äratuntav ka soolekepikeses *E. coli*, on seda juba edukalt kasutatud biotehnoloogilise potentsiaaliga võõrvalgu levaansukraasi ekspresseerimisel ja puhastamisel sellest bakterist.

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ACKNOWLEDGEMENTS

First of all, I would like to express my enormous gratitude to my supervisor Tiina Alamäe for giving me the opportunity to be part of her fantastic team and complete my bachelor and master studies in her group and continue with my doctoral studies. Thank you Tiina for your support, patience, motivation and knowledge during all these years! Thank you for pushing me and believing in me even if I did not believe myself anymore.

I am also grateful to the members of our small research group. Special thanks to Karin for her positive attitude and for thinking with me when I was totally stuck with my experiments. Thank you, Tiina Michelson, for motivating me!

My sincere and special thanks to my past and present colleagues, labmates and students in the chair of genetics. I would also like to thank all the co-authors of the publications presented in this thesis.

I am also thankful to Tiina Tamm for proofreading my thesis. Her useful suggestions and improvements adjusted the quality of the thesis.

Last but not least, I would like to thank my family. Thank you Merilin and Mariette for being the best daughters in the world. Those deep discussions with you have kept me motivated through all these years. My special thanks goes to my lovely parents for understanding and believing in me and supporting me through the years of my studies in the university.

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