Detailed Investigation of Quantitative Trait Loci (QTLs) Involved in Superior Very-High-Gravity (VHG) Fermentation Performance

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Abstract

Bioethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels [1]. One versatile technology in process optimization for bioethanol production is Very-High-Gravity (VHG) fermentation which offers great savings in water and energy requirements through fermentation of substrate containing higher sugar concentrations which results in increased final ethanol concentration. [2]. However, the high concentrations of sugar in the beginning of the fermentation expose yeast cells to osmotic stress leading to sluggish fermentations [3]. The goal of this work is to identify superior alleles that can improve the fermentation performance of industrial strains under VHG conditions. Previously, an osmotolerant strain (X6003) was selected as superior strain and Ethanol Red was selected as the inferior strain to be improved for VHG fermentation. Quantitative trait locus (QTL) mapping was achieved by Pooled-segregant whole-genome sequencing whereby 10 QTLs that might contain genes that play a role in superior osmotolerance were identified. In this thesis, two of those QTLs were studied in detail. With this purpose, strains were constructed to be used in ‘bulk’ reciprocal hemizygosity analysis (bRHA). For this, two isogenic strains with the background from both parent strains were constructed in which a particular DNA fragment was deleted in either the chromosome coming from the superior, or from the inferior strain. These strains were subjected to VHG fermentation in order to compare their phenotype and evaluate if the genes from one genetic background are advantageous over those from the other. DCK1 was identified as a causative gene, since its allele from X6003 conferred a better fermentation performance in VHG conditions than the one from Ethanol Red.

Keywords: Bioethanol production, Saccharomyces cerevisiae, Osmotic stress, VHG fermentation, QTL mapping, bRHA analysis.

1. Introduction

1.1. Bioethanol Production and Bioethanol Industry

Nowadays, one of the most relevant drivers globally is climate protection with its ecological, economic, social and political implications [4]. However, energy consumption is still increasing rapidly and it is still highly dependent on fossil fuels with 81% of all energy production coming from fossil fuels [5]. Ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels [1].

It is usually produced by sugar fermentation, where a series of chemical reactions performed by microbes convert sugars to ethanol. The overall chemical formula for alcoholic fermentation is expressed in equation 1.1.\n
\[ C_nH_{2n}O_n \text{(sugar)} \rightarrow \frac{n}{3} C_2H_5OH \text{(Ethanol)} + \frac{n}{3} CO_2 + \text{Heat} \] (1.1)

Fermentation can be performed by diverse microbes, but in industrial bioethanol settings robust species of microorganisms are selected based on compatibility with desired product, processes and equipment [6]. The yeast Saccharomyces cerevisiae is the dominant organism owing to its high rate of fermentation of hexose sugars, high tolerance to ethanol, inhibitors, acidity and other industrial process conditions, well-established production, storage and transport systems at commercial scale, comprehensive physiological and molecular knowledge and its genetic tractability [7].

To be considered a viable substitute for a fossil fuel, ethanol should have superior environmental benefits over the fossil fuel it displaces, be economically competitive with it, be producible in sufficient quantities to make a meaningful impact on energy demands and it should also provide a net energy gain over the energy sources used to produce it [8]. Therefore, process optimization is a crucial tool employed within the framework of process design, not only during the experimentation steps, but also during the design steps [1]. Optimizing the conditions used for production of bioethanol has been studied over the past decades. In particular, very-high-gravity (VHG) fermentation is a versatile technology among the new tendencies in process optimization for ethanol production [2].
1.2. Very-High-Gravity (VHG) Fermentation

The concept of VHG fermentation was proposed by Casey and Ingledew in the 1980’s and has been developed since then, allowing drastic increase of ethanol concentration from the previous level of 7–8% (v/v) [9][10]. VHG fermentation involves the preparation and fermentation of mashes containing 27g or more dissolved solids per 100g mash [11]. VHG fermentation offers innumerable advantages such as significantly increased ethanol titer in the fermentation broth, which not only saves energy consumption for ethanol distillation, but also reduces waste distillate discharged from the distillation system, which can significantly reduce costs associated with it [12]. Furthermore, risk of bacterial contamination could be minimized due to the fact that bacteria cannot thrive well under increased osmotic conditions; therefore, costs associated with antibiotics use are also lowered [2].

However, during industrial VHG fermentation, yeast cells are exposed to several stresses including osmotic stress (resulting from the high sugar concentration at the beginning of fermentation) and ethanol stress (resulting from high concentration of ethanol at the end of fermentation) which leads to a sluggish fermentation [3]. In order to reduce the adverse effects induced by the high density medium, much research has been made towards the optimization of this process. This research can be divided into two major fields: optimization of the fermentation medium and breeding and engineering of yeast strains that can tolerate these adverse effects.

1.3. Osmotic Stress and Osmoadaptation in Yeast

Osmotic stress greatly interferes with yeast fermentation performance. More particularly, yeast cells under hypersmotic stress shrink due to dehydration by osmosis resulting in significant alteration of the intracellular density of macromolecules and of the concentration of intracellular inorganic ions (especially Na⁺) [13]. To counteract these effects, yeasts start a series of adjustments in order to survive these adverse conditions. Osmoadaptation in yeast is mainly governed by the high osmolarity glycerol (HOG) pathway. The HOG pathway is one of the best understood and most intensively studied MAPK systems. The HOG signaling system consists of two branches that converge on the MAPKK Pbs2: the Sln1 branch and the Sho1 branch [14]. Once activated, Pbs2 phosphorylates Hog1 on a threonine and a tyrosine residue [15]. Subsequently, a substantial fraction of Hog1 is transported into the nucleus where it regulates transcription and the cell cycle [16].

In order to cope with high osmotic stress, yeast produces glycerol via a short branch of glycolysis consisting of two enzymatic steps [17]. Hog1 appears to control glycerol accumulation at several steps, such as the expression of the genes encoding Gpd1, Gpp1 and Gpp2 [18]; the activity of Pfk26, which appears to increase the rate of glycerol production [19]; and control of the activity of the glycerol export channel Fps1 [20].

In addition to the HOG pathway, other pathways have been associated with response to an osmotic upshift, such as the general environmental stress response (ESR) in yeast [21]. ESR refers to the common changes observed in gene expression upon stress, regardless of the nature of the stress itself [22]. Osmotic balance is also maintained by the regulated opening and closing of channel proteins in both the cell membrane and intracellular organelles such as the vacuole. Osmotic shock also induces intracellular calcium signaling [21]. Thus, osmotolerance is a complex trait. Due to such complexity, the development of osmoto tolerant yeast has proven to be a difficult task to achieve. Nevertheless, different approaches have been used over the past decades [3][23][24][25][26]. The approach used in the background of the present work is a whole genome approach called quantitative trait locus (QTL) mapping.

1.4. Quantitative Trait Locus (QTL) analysis

A QTL refers to an individual locus that explains a specific part of the phenotypic expression of a quantitative trait and it can contain a single gene or a cluster of closely linked genes that contribute to the quantitative trait. The identification of genes that contribute to quantitative traits has proven to be difficult. This is because of the complex genetic architecture of quantitative traits, which is dictated by factors such as variable QTL contribution, epistasis, genetic heterogeneity, and gene–environment interaction [27]. A method used for studying QTLs is QTL mapping, which aims at the simultaneous genomic localization of all loci determining a quantitative trait. QTL mapping in S. cerevisiae is typically performed by crossing two strains that differ in the trait of interest: a haploid parental strain possessing the trait is mated with another haploid parental strain lacking the trait [27]. During QTL mapping, the allocation of the genetic
determinants to regions in the genome relies on their co-segregation with genetic loci of known positions, which are called genetic markers. The most widely used genetic markers are DNA polymorphisms such as single nucleotide polymorphisms (SNPs), which are usually plentiful in number and thus enable complete genome coverage [27][28].

Over the past decade, different approaches have been developed that allow identification of QTLs responsible for complex traits [27]. Pooled-segregant whole-genome sequence was the approach employed by Mukherjee [6]. QTL mapping by pooled-segregant whole-genome sequencing is a fast, reliable and robust methodology for the simultaneous identification of superior genes involved in polygenic traits [28]. Previously, pooled-segregant whole-genome sequence analysis has been used to identify QTLs responsible for high ethanol tolerance [29], capacity for high ethanol accumulation [30], thermotolerance [31], and low glycerol and high ethanol yield [32][33] in S. cerevisiae. However, QTL mapping only allows the allocation of the genetic determinants to intervals in the genome instead of identifying the determinants themselves. Therefore, once the QTLs have been identified, they must be down-scaled to the gene and/or nucleotide level. In the present work, we have used the unbiased approach named reciprocal hemizygosity analysis (RHA) [34]. This functional analysis is based on the construction of two isogenic strains in the hybrid diploid background from both parent strains that genetically differ only in the alleles of one copy of a specific candidate gene. By comparing the phenotypes of the two strains, it will be revealed whether an allele from one genetic background is advantageous over that from the other [29]. In order to speed up the identification of causatives genes when studying all genes in one QTL an extension of the RHA method can be used. This approach is called ‘bulk’ RHA (bRHA) and instead of comparing alleles for each single gene, a fragment with multiple genes is deleted in the hybrid strain [27].

2. Background

The work developed in the present thesis is a continuation of the work developed by the doctoral student Vaskar Mukherjee in 2016 [6]. In his work, high-throughput phenotypic evaluation of 279 S. cerevisiae strains allowed identification of highly osmotolerant strains that managed to grow on plates containing 50 and 55% (w/v) glucose. X6003 (CAT1) displayed the highest rate of fermentation and produced the second highest ethanol yield under VHG conditions, being therefore selected as superior parental strain. On the other hand, Ethanol Red had the highest ethanol yield, but it took a long time to reach the stationary phase. Therefore, it was considered that this strain still had room for improvement when it came to VHG fermentation [6]. Ethanol Red is a widely employed industrial bioethanol yeast strain used in first generation bioethanol production and is well known for its robustness and stress tolerance [7]. Ethanol Red was then selected as the inferior parental strain in order to identify superior alleles from X6003 that can specifically improve VHG fermentation efficiency of Ethanol Red [6]. Both parental strains were sporulated and 24 haploid segregants from each were subjected to VHG fermentation (YP+25% glucose +25% sorbitol). For X6003, three segregants were selected, that showed a fermentation profile even better than the parental strain. For Ethanol Red, four segregants were selected based on the VHG fermentation profiles which are similar to that of Ethanol Red. The diploid strain 4DX42A (X6003-4DXER-42A) was selected as the parental hybrid for QTL study. QTL mapping was achieved by Pooled-segregant whole-genome sequencing. This allowed the identification of 10 QTLs involved in superior fermentation performance under VHG conditions (Figure 2.1) [6]. In the present work, two of the 10 QTLs (QTL 7 and QTL 8) were studied in detail. RHA strains for groups of genes were constructed that were used in bRHA, in which their phenotype was evaluated by subjecting them to VHG fermentation and comparing their fermentation profile.

3. Materials and Methods

3.1. Strains and Growth Conditions

All control strains used (X6003-4D (JT 23795.16), ER-42A (JT 22931.1) and 4DX42A (JT 28237)) were obtained from Mukherjee [6] and all single deletion strains were obtained from a collection of gene-deletion mutants constructed by Giaever [35]. All yeast cells were cultured in liquid and solid YPD medium. YPD medium consists of deionized water with 1% (w/v) yeast extract (Merck KGaA, Germany), 2% (w/v) bacterial peptone (OXOID Ltd, England) and 2% (w/v) glucose (Sigma-Aldrich, USA). Solid YPD medium also contains 1.5% (w/v) granulated Difco™ agar. For selective medium, geneticin (Labconsult, Belgium) was added after the medium was autoclaved to reach a final concentration of 100 μg/mL.
Figure 2.1. SNP variant frequency mapping against the SNP position on all sixteen chromosomes of *S. cerevisiae*. The upper panel shows the genetic mapping of the SNP frequency of the superior and the unselected pools. The red and the black line represent the predicted SNP frequency of the selected and the unselected pool, respectively. Medium panel shows the confidence interval between the superior and the unselected pool. A significant deviation of the selected pool from the unselected pool indicates candidate QTLs linked to osmotolerance. Bottom panel shows the significance of the linkage by calculating the p value (represented by the blue line). The identified significantly linked regions are marked by red dashed vertical lines [6].

3.2. Bulk Reciprocal Hemizygosity Analysis (bRHA)
Reciprocal-hemizygosity analysis is a tool developed by Steinmetz and coworkers and can be applied to delete one ORF or larger sections of the genome (bulk RHA) [30][34]. Two different types of diploid mutants were constructed using the parental hybrid 4Dx42A (JT 28237). In one mutant the deletion was made in the chromosome derived from the inferior parent and in the other the deletion was made in the chromosome derived from the superior parent (Figure 3.1).

Figure 3.2. Strategy used for the construction of the strains used in Bulk reciprocal-hemizygosity analysis (bRHA). Two types of strains can be obtained when creating the hybrids: in one mutant the targeted allele from the inferior parent chromosome was deleted (Hybrid 1) and in the other, the targeted allele from the superior parent chromosome was deleted (Hybrid 2).

The aforementioned mutants were constructed using the “split-marker” recombination method (Figure 3.2) [36]. Transforming molecules that contain a homologous region with the flanking regions of the block in study and a part of the selection marker *kanMX*, which gives resistance to geneticin, were amplified from a deletion strain which has the target gene replaced by the selection marker [35]. Amplification of the transforming molecules was accomplished by performing a polymerase chain reaction (PCR) on a Thermo Cycler using 50 µL reactions. PCR products were detected on 1.1% (w/v) agarose gel by electrophoresis.

Figure 3.3. Schematic representation of the “split-marker” recombination. The two transformation cassettes have a flanking region at the end (LHR and RHR), homologous to the sequence upstream and downstream the block to be deleted, and have an overlapping sequence region within the *kanMX* marker, which allows the homologous recombination when both of the cassettes are co-transformed in the yeast hybrid X6003-4DxER-42A.

3.3. Yeast Transformation
The transformation protocol used is based on the LiAc/SS-DNA/PEG procedure described by Gietz and coworkers [37]. Overnight cultures of the parental hybrid 4Dx42A were inoculated to an OD_{600} of 0.4 and grown for 3-4 hours until OD_{600} 1.5 in 50 mL of YPD medium. Cells were washed with 25 mL of water and then washed with 1 mL of 0.1M Lithium Acetate (LiAC)
(Sigma-Aldrich, USA). Afterwards, cells were re-
suspended in 0.1M LiAC according to the initial OD$_{600}$
(OD$_{600}=0.9$: 300 µL 0.1M LiAC). For each
transformation, 300 µL of PLI solution (solution
containing 0.1M LiAC (Sigma-Aldrich, USA) and 40%
(v/v) PEG 3350 (Polyethelene glycol) (Sigma-Aldrich,
USA)), 5 µL of SS-DNA (Sigma-Aldrich, USA) and 25
µL of each of both “split-marker” PCR products were
added to 50 µL of cell suspension. The whole
procedure was performed under sterile conditions. The
transformation mix was incubated at 42°C for 30
minutes. Afterwards, the transformation mix was
centrifuge and the cell pellet was re-suspended in 1 mL
of YPD medium and incubated for 3 to 4 hours at 30°C.
Transformations were plated in selective YPD plate
containing geneticin.

3.4. Genotypic Evaluation of the Transformation
Genotypic evaluation of the transformants was
accomplished by SNP PCR. Therefore, two sets of
primers were used in which one set amplified the
sequence present in the chromosome derived from the
superior parent and the other amplified the sequence
present in the chromosome derived from the inferior
parent. SNP PCR was performed on a Thermo Cycler
using 20 µL reactions. PCR products were detected on
1.1% (w/v) agarose gel by electrophoresis.

3.5. Phenotypic Evaluation: Very-High-Gravity
(VHG) Fermentation
The procedure used mimics the procedure used by
Mukherjee [6]. Strains were pre-cultured overnight in 3
mL YPD medium at 30 °C and continuously shaken at
200 rpm. Subsequently, 50 mL YPD medium was
inoculated with the strains at a starting OD$_{600}$ of 0.75
and incubated at 30 °C, 200 rpm for two days until
stationary phase. The pre-cultures’ OD$_{600}$ were
measured and fermentation tubes were inoculated at a
final OD$_{600}$ of 4 in a total volume of 80 mL YP medium
containing 25%(w/v) glucose and 30%(w/v) sorbitol.
Semi-anerobic batch fermentations were performed in
cylindrical glass tubes with a rubber stopper containing
cotton plugged glass pipe to release CO$_2$. The
fermentations were performed at 30 °C and
continuously stirred at 120 rpm. To follow the course of
the fermentation the weight loss of the tubes due to
CO2 release was used. All fermentations were
performed in duplicate. This system has been used
frequently to mimic the industrial fermentation
conditions [7][32].

4. Results and Discussion
4.1. Dissection of QTL 8
QTL8 is located in Chromosome XIII between locations
377831 and 442005 (64174 bp). SNP variant frequency
analysis identified this region to be highly significant (p
value 1E-06) and the region showed 71% association
with the superior parent X6003-4D [6]. In order to study
QTL 8 the genes that belong to this QTL were divided
into five groups of genes (blocks) (Figure 4.1). For each
block, RHA strains were constructed and genotypic
evaluation of the transformants was accomplished by
SNP PCR. The primers used were located within one of
the ends of the block. For all RHA strains subjected to
VHG fermentation, a second genotypic evaluation was
performed by SNP PCR within the other end of the
block in order to control if the whole block was deleted
and to reconfirm the results obtained in the first SNP
PCR. For each block, at least three transformants with
the superior type present and three transformants with
the inferior type present were studied under VHG
conditions and their fermentation profile compared with
the fermentation profile of three control strains: the
haploid superior parent 4D, the haploid inferior parent
42A and the parental hybrid 4Dx42A.

![Figure 4.1. Overview of all ORFs that belong to QTL 8. ORFs above the straight line are ORFs on the Watson strand and ORFs below the straight line are ORFs on the Crick strand. The grey arrows below each line represent the different Blocks outlining the ORFs that belong to each Block.](image)

Block 18. RHA strains for Block 18 showed no
difference in the fermentation performance under high
gravity conditions. These results suggest that there is
no gene of interest in this block with an allele that
confers a superior fermentation performance. However,
a small difference between the fermentation profiles
from the RHA strains and that of 4Dx42A can be observed (Figure 4.1). These results might suggest that one or more genes within Block 18 is associated with hyperosmotic resistance, since deletion of those genes in one of the chromosomes results in a slightly worse performance than when the cells present both copies, indicating a dosage effect. To confirm the results obtained a second fermentation was performed using different RHA strains than the ones used in the first VHG fermentation (Results not shown). The results obtained in the second fermentation were not conclusive regarding the possible association of a gene from Block 18 with hyperosmotic resistance. However, no difference in the fermentation performance of the RHA strains was observed. Therefore, the study with Block 18 was finished with the conclusion that there is no gene of interest in this block.

**Discussion QTL 8.** After the study of the five blocks that compose QTL 8 no block was pin-pointed that could contain a causative gene that confers a better fermentation performance under VHG conditions. Identification of the QTL is based on statistics and probabilities, which means that there is no guarantee that a causative gene conferring a better performance exists in all identified QTLs. Hence, the most straightforward explanation is that, although QTL 8 is a significant region with a high association with the superior parent, there is no superior allele responsible for the better fitness of the superior segregant X6003-4D under VHG conditions in QTL 8. It is also possible that the statistical method used by Mukherjee is too sensitive. If the threshold value that defines a region as significant is too narrow several regions can be marked as significant even if they are not. In his work, Mukherjee identified 10 QTLs responsible for superior fermentation performance under VHG conditions. This number is considerably higher than the number of QTLs identified in other studies where the average was 5 QTLs [29][30][31][32][33]. Thus, it can be argued that some of the regions marked as QTLs are actually not significant and that QTL 8 is one of those regions, explaining why no causative gene was found.

More complex possibilities can also explain the events in discussion. Gene-gene interactions or epistasis between one or more causative genes of QTL 8 might also explain why no phenotypic effect was observed even if QTL 8 has one or more causative genes. It can be hypothesized that QTL 8 has a causative gene that confers a better performance to yeast under VHG conditions, but that this gene interacts with another gene that masks its allelic effects. If, in the dominant gene, one of the alleles is dominant and the other one is recessive, it might be necessary to study double deletion strains of the epistatic genes in order to see the expected phenotype. Therefore, with the approach used in this thesis, if the epistatic genes are located in different QTLs or if they are in the same QTL but divided in different blocks, the phenotype might not be observed and the genes will not be identified. It is also possible that, within one block, there are two alleles with opposite affects that will neutralize each other. Therefore, it would be necessary to employ other approaches to identify these genes.
4.2. Dissection of QTL 7

QTL 7 is located in chromosome XII between chromosomal locations 908788-971173. This region was significantly (p value 0.0075) linked with superior fermentation under hyperosmotic stress. This region showed 78% association with the superior parent X6003-4D. Fine mapping did not reduce the length of the center of the QTL, but increased the maximum SNP variant frequency to 81% and reduced the p value to 0.0002 [6]. In order to study QTL 7 the genes that belong to this QTL were divided into six groups of genes (Figure 4.3). To study in detail QTL 7 the same approach used to study QTL 8 was applied. Due to time constraints it was not possible to studied Block 12 and Block 14.

Block 13. Block 13 consists only of the gene DUS3. This gene was previously associated with hyperosmotic stress tolerance [38] and presented one non-synonymous mutation and mutations in the promoter as well as in the terminator. Thus, this gene was considered a candidate gene by Mukherjee and consequently studied using RHA analysis [6]. A difference in fermentation performance was observed for the RHA strains of DUS3. However, the difference observed suggests that the DUS3 allele from the inferior strain is the one conferring a better fermentation performance under high-gravity conditions instead of the DUS3 allele from the X6003-4D strain as expected [6]. In the present work, RHA strains for Block 13 showed no difference in the fermentation performance under VHG conditions (Figure 4.4). To confirm these results, a second fermentation was performed (Results not shown). The results obtained in the second fermentation were in agreement with the results obtained in the first fermentation. Although the results obtained are not in agreement with the results from Mukherjee, they were not completely unexpected since some further studies with this gene had already presented results that were not concordant with the results shown by Mukherjee. The study with Block 13 was finalized concluding that DUS3 is not a causative gene.

**Figure 4.3.** Overview of all ORFs that belong to QTL 7. Figure indications are described in Figure 4.1.

**Figure 4.4.** VHG fermentation curves of RHA strains from Block 13 (DUS3). Graph indications are as described in Figure 4.2.

Block 15. RHA strains for Block 15 showed no difference in the fermentation performance under high gravity conditions. These results suggest that there is no gene of interest in this block with an allele that confers a superior fermentation performance (Results not shown). Therefore, the studies with this block were finalized.

Block 16. A difference in fermentation performance was observed for the RHA strains of Block 16. The RHA results for Block 16 were not very conclusive, although they suggest that a difference between the fermentation performances of the two types of RHA strains might exist (Figure 4.5). All strains with the superior type show fermentation profiles similar to that of the one from the
hybrid 4Dx42A and all strains with the inferior type, but one showed a lower fermentation profile than the RHA strains with the superior type. To confirm if the low fermentation performances of the RHA strains with the inferior type prevails, a second fermentation should be performed with different RHA strains. Due to time constraints the second fermentation was not performed.

Two main hypotheses can explain the difference between the RHA strains’ performances: one of the genes within Block 16 is a causative gene which has a superior allele that confers a better fermentation performance to the RHA strains or DCK1 is the causative gene. DCK1 belongs to the adjacent block 17, but part of its promoter is inside Block 16. Partial deletion of this promoter could affect its expression although the gene was not deleted. It should be noted that both hypotheses are not mutually exclusive, since a QTL can hold more than one causative gene. To study in detail Block 16, RHA strains for all genes should be investigated.

**Block 17.** Block 17 consists only of the gene DCK1. A difference in fermentation performance was observed for the RHA strains of DCK1 (Figure 4.6). All strains with the superior type showed fermentation profiles almost identical to that of the hybrid 4Dx42A and all strains with the inferior type show a lower fermentation profile than the RHA strains with the superior type. These results clearly suggest that the DCK1 allele from the X6003-4D strain (superior parent) confers a better fermentation performance under VHG conditions than the allele from the ER-42A strain (inferior parent). To confirm these results a second fermentation with different RHA strains was performed (Results not shown). The results from the second fermentation were in agreement with the results from the first fermentation.

**Figure 4.5.** VHG fermentation curves of RHA strains from Block 16. Graph indications are as described in Figure 4.2.

**Figure 4.6.** VHG fermentation curves of RHA strains from Block 17 (DCK1). Graph indications are as described in Figure 4.2.

Only a handful of studies about DCK1 have been released until the present day. This gene was only characterized in 2015 by Schmitz and coworkers where it was identified as an upstream regulator of Rho5. It was also proposed that Dck1 may form a complex with Lmo1 that acts as a GEF (Guanine nucleotide exchange factor) for Rho5 [39]. Single deletion strains of DCK1 and LMO1 were hyper-resistant against typical cell wall integrity (CWI) stress agents, a feature reminiscent of a rho5 deletion strain [40], providing evidence that DCK1 and LMO1 are epistatic to RHO5 [39]. It can therefore be argued that the results observed are an epistatic effect of Rho5, meaning that the lower VHG fermentation performance of the
transformants without the superior allele from *DCK1* is a result of an improper activation of Rho5.

Rho5 is a small GTPase from the Rho-type family and acts as a molecular switch in signal transduction pathways [41]. Although Rho5 is not a well characterized protein, different studies have already designated Rho5 to participate in quite diverse processes and influence different signaling pathways introducing several hypotheses of how Rho5 is involved in the osmotic stress response and consequently how Dck1 could be involved. The most direct explanation is the interaction between Rho5 and Ste50 indicated by Annan and coworkers [42]. Ste50p is involved in the activation of the HOG pathway in response to hyperosmotic shock [16]. Expression of an active allele from *RHO5* in a ste50 deletion strain resulted in a growth defect and was osmotically lethal, suggesting that Rho5 plays a role in the cellular response to hyperosmotic stress in a Ste50p-dependent manner [42]. Rho5 was also indicated as a negative regulator in CWI signaling [40]. Responses mediated by the HOG pathway lead to increased turgor pressure, which is interpreted by the CWI pathway to coordinate cell wall strength and cell expansion and thereby diminish turgor pressure [14]. Thus, activation of Rho5 under VHG conditions may be related to these adjustments in the cell wall and its role in CWI signaling. Another function of Rho5 was proposed in oxidant-induced apoptosis, when rho5 mutants were hyper-resistant to hydrogen peroxide treatment [45]. An overlap of the transcriptional response to osmotic shock and to oxidative stress has been reported in several studies [14]. Therefore, VHG conditions may activate Rho5 in the same way that it is activated under oxidative stress triggering the functions of Rho5 under this particular stress.

Although the interaction of Dck1 with Rho5 presents itself as the most probable explanation for the connection of *DCK1* with the fitness of yeast under hyperosmotic stress, other possible explanations should be explored as well. Schmitz and coworkers note in their work that, although their genetic data define Rho5 as the major target of the dimeric GEF, the possibility that other small GTPases may also be affected by it cannot be excluded [39]. Another protein that belongs to the Rho-family is Cdc42 [44]. The current hypothesis for the activation mechanism of the Sho1 branch in the HOG pathway identifies Cdc42 as one of the proteins that play a role in its activation [16]. Therefore, an interaction between Cdc42 and Dck1 could also explain the results obtained. Rho5p sequence analysis revealed that Rho5 contains a Ras-like effector region, which is identical to that of Cdc42. These results strengthen the idea that Dck1 or the complex Dck1/Lmo1 can regulate both Rho5 and Cdc42.

**Discussion**

QTL 7. Although the study of the six blocks that compose QTL 7 was not complete, the results obtained identified at least one causative gene that confers a better fermentation performance to the superior parent X6003-4D under VHG conditions in this QTL. RHA strains of *DCK1* (Block 17) and RHA strains for Block 16 showed a difference in fermentation performance, suggesting that in those blocks the background from X6003-4D confers a better fitness to yeast under VHG conditions than the background from ER-42A. These results can be unrelated, meaning that QTL7 holds two candidate genes: *DCK1* and one of the genes within Block 16. Other possibility is that these results are related and there is only one candidate gene that affects the fermentation performance of RHA strains of both blocks. Therefore, there are two possibilities: *DCK1* is the causative gene and a part of its promoter was deleted in Block 16 resulting in the appearance of the phenotype when studying RHA strains of Block 16 or *RPN13* (last gene in Block 16) is the causative gene and part of its promoter was deleted in Block 17 resulting in the appearance of the phenotype when studying RHA strains of Block 17. To clarify the results further studies should be performed with both blocks.

5. Conclusion

In this thesis, two QTLs were studied in detail. The first QTL was QTL 8 that was divided into five groups of genes. The studies with this QTL were finalized concluding that most probably there is no causative gene in this QTL that can be identified with the approach used.

QTL 7 was the second QTL studied in this work. The genes in this QTL were divided into six groups. Due to time constraints it was not possible to study the whole QTL in detail. Therefore, VHG fermentations of the RHA strains for Block 12 and Block 14 should be performed to investigate whether there is a causative gene in one of these blocks. Also, Block 16 should be down-scaled to the gene level in order to understand why RHA strains for this block present a difference in fermentation performance. Nevertheless, the gene
DCK1 was identified as most probably being a causative gene, since the RHA strains with the DCK1 allele from X603-4D presented a better fermentation performance under VHG conditions than the RHA strains with the DCK1 allele from ER-42A. DCK1 encodes a subunit of a GEF complex alongside with LMO1 that is believed to regulate primarily Rho5, a small GTPase [39]. DCK1 was never associated with hyperosmotic stress resistance; however null mutants of RHO5 were identified as osmo-sensitive strains [38] which might suggest that the role of DCK1 in osmotolerance is indeed related with the interaction between Dck1 and Rho5. Further studies with this gene should be done in order to elucidate its role in the hyperosmotic stress resistance of yeast. Furthermore, studies that explain why the allele from X6003-4D confers a better fitness than the allele from ER-42A under VHG conditions and what its impact is should also be explored.

This work also demonstrated the advantages of using an unbiased approach when identifying causative genes within a QTL. QTL 7 had been previously studied by Mukherjee using a biased approach where only genes that were previously associated with hyperosmotic stress resistance were studied. With this approach it was not possible to explain the high association of QTL 7 with X6003 [6]. With the approach used in the present thesis it was possible to identify at least one causative gene in this QTL. Therefore, all QTLs identified by Mukherjee should be studied in detail using “bulk” RHA analysis. An interesting QTL to study next is QTL 5 since it has the highest association with the superior parent X6003-4D (73%) after QTL 7, no work has been done yet in this QTL and, according to Saccharomyces Genome Database (SGD), three of its genes were previously associated with hyperosmotic resistance (MDS3, GCN1 and CDC55).

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