

**Gerald Richter**

# **Membrane Engineering in Yeast**

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Univ.-Prof. Dipl.-Ing. Dr.techn. Helmut Schwab

Ass.-Prof. Dipl.-Ing. Dr.techn. Harald Pichler

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Von dem, was du erkennen und wissen willst, musst du Abschied nehmen, wenigstens auf eine Zeit. Erst, wenn du die Stadt verlassen hast, siehst du, wie hoch sich ihre Türme über die Häuser erheben.

(Friedrich Nietzsche 1833-1900)

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## **Kurzfassung**

*Pichia pastoris*, Ergosterolbiosynthese, Membran - Engineering

Sterole sind ein wichtiger Bestandteil der Membranen in Eukaryoten, wobei Ergosterol die mengenmäßig verbreitetste Form in Hefen darstellt. Die Ergosterolbiosynthese aus Acetyl-CoA ist einer der komplexesten biochemischen Wege. Durch genau jene Vielfalt ist es möglich, an unterschiedlichen Punkten im Biosyntheseweg einzugreifen und den Sterolhaushalt der Zelle zu verändern. In ihrem Sterolhaushalt veränderte Hefezellen sind sowohl in grundlegenden Studien der Zell- und Molekularbiologie als auch in biotechnologischen Prozessen von hohem Interesse. Die Konstruktion von *Pichia pastoris* Stämmen mit veränderter Sterolzusammensetzung und ihre Analyse ist das Thema dieser Arbeit.

## **Abstract**

*Pichia pastoris*, ergosterol biosynthesis pathway, membrane engineering

Sterols are a main compound of eukaryotic membranes, and ergosterol is the most abundant form in yeast. The formation of ergosterol from acetyl-CoA is one of the most complex biochemical pathways. This variety of enzymes enables us to intervene at different points of the ergosterol biosynthesis pathway and shift the sterol composition of the cell. Yeasts with altered sterol composition are not only of high importance in cell as well as molecular biology but are highly relevant in industrial biotechnology. In this work, *Pichia pastoris* strains with modified sterol patterns have been conducted and analysed.

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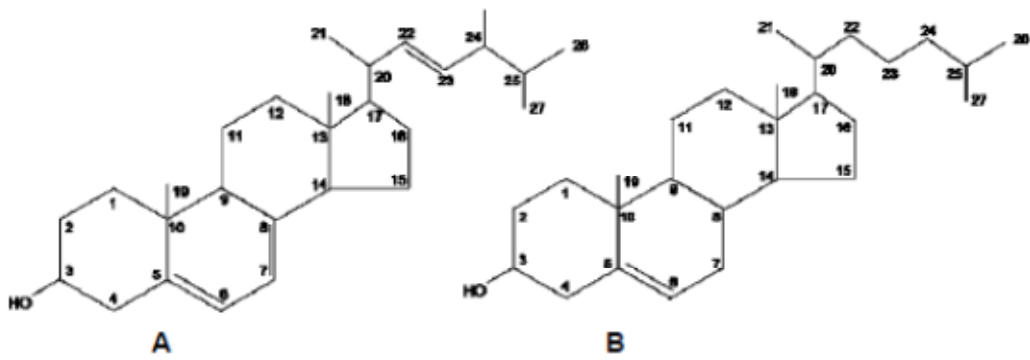
## List of Abbreviations

|         |                                       |
|---------|---------------------------------------|
| aa      | amino acid                            |
| bidest  | double distilled                      |
| bp      | base pairs                            |
| DC      | disruption cassette                   |
| DG      | diglycerides                          |
| DHCR    | dehydrocholesterol reductase          |
| DMSO    | dimethyl sulfoxid                     |
| dNTP    | deoxynucleotid triphosphate           |
| DTT     | dithiothreitol                        |
| ER      | endoplasmic reticulum                 |
| ERG     | ergosterol related gene               |
| EtOH    | ethanol                               |
| Fig     | figure                                |
| FS      | free sterols                          |
| G418    | geneticin 418 sulfate                 |
| GC/MS   | gas chromatography/ mass spectroscopy |
| his     | histidin                              |
| kan     | kanamycin                             |
| LB      | Luria-Bertani Broth (Lennox)          |
| MeOH    | methanol                              |
| min     | minute(s)                             |
| Mut     | mutant                                |
| MCS     | multiple cloning site                 |
| OD      | optical density                       |
| O/N     | over night                            |
| ONC     | over night culture                    |
| PBS     | phosphate buffered saline             |
| PCR     | polymerase chain reaction             |
| PEG     | polyethylenglycol                     |
| PL      | phospholipids                         |
| Prepgel | preparative agarose gel               |
| rpm     | revolutions per minute                |
| RT      | room temperature                      |
| s       | second(s)                             |
| T       | temperature                           |
| TG      | triglycerides                         |
| TLC     | thin layer chromatography             |
| WT      | wild-type                             |
| YPD     | yeast extract peptone dextrose        |

# 1 Introduction

## 1.1 Sterols in eukaryotic membranes

Sterols are a subcategory of steroids and they consist of a characteristic tetracyclic ring system (Figure 1). Largely, sterols are only present in eukaryotic membranes and apparently they confer properties or functions to membranes that are not required in prokaryotes (Matyash et al., 2004; Guan et al., 2009).



**Figure 1: Chemical structure of ergosterol (A) and cholesterol (B), with correct labelling of the C - atoms (Maczek, 2009)**

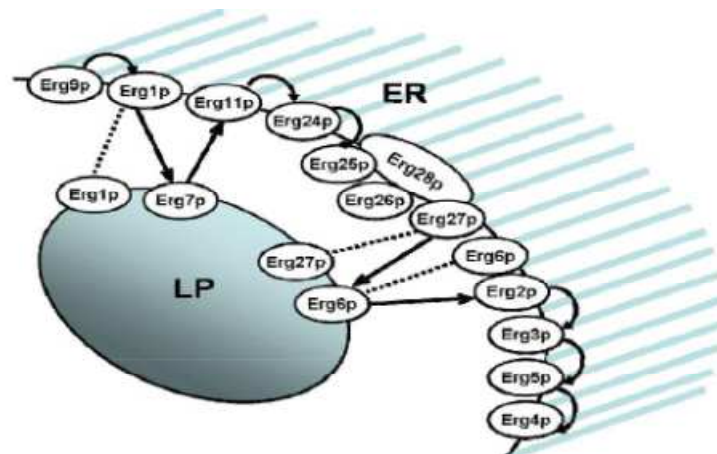
The structural distinctions between ergosterol, the main sterol of fungi, and cholesterol, which is the predominant sterol in mammalian membranes, are rather small. The difference between both sterols plays a critical role in vivo and essentially lead to altered cellular functions (Gaber et al., 1989).

It has been reported, that ergosterol is required for proper tryptophane uptake in yeast by affecting the transport of the tryptophan permease (Tat2p) to the plasma membrane. *Erg6* mutants show an increased deficiency in the transport mechanism (Umebayashi and Nakano, 2003). Many precursors of ergosterol in the biosynthesis pathway are involved in various cellular operations, such as mating (Aguilar et al., 2010).

Sterol homeostasis in mammalian cells is regulated by various feedback control mechanisms, transcriptional as well as posttranscriptional. The membrane bound transcription factor SREBP (sterol regulatory element binding protein) controls sterol uptake and synthesis (Hughes and Espenshade, 2007). In fission yeast, SREBPs are relevant in both oxygen sensing and sterol metabolism (Bien and Espenshade, 2010).

## 1.2 The ergosterol biosynthesis pathway

The ergosterol biosynthesis pathway can be divided into the pre- and the post-squalene pathway, which altogether consist of nearly thirty biochemical reactions. Most of the enzymes are located in the ER membrane, except Erg1p, Erg6p and Erg7p, which are pre-dominantly present in lipid particles.



**Figure 2: Overview of the enzymes involved in the ergosterol biosynthesis pathway, which are either localized in the endoplasmic reticulum (ER) or in lipid particles (LP) (Maczek, 2009)**

Newly formed lipid particles (droplets) appear to originate from the ER. Also, both compartments are functionally connected and efficient exchange of membrane proteins is possible (Jacquier et al., 2011; Adeyo et al., 2011).

### **1.2.1 The pre – squalene pathway**

Initially, ergosterol synthesis starts with the condensation of acetyl-CoA molecules. Two acetyl-CoA molecules are converted into acetoacetyl-CoA by Erg10p (acetoacetyl-CoA thiolase). Erg13p, the HMG-CoA synthase, catalyses the formation of HMG-CoA, which is subsequently reduced to form mevalonate by Hmg1p and Hmg2p (HMG-CoA reductases 1 and 2). These two isoenzymes show feedback inhibition by ergosterol (Bard and Downing, 1981).

Mevalonate is phosphorylated by Erg12p (mevalonate kinase) to form phosphomevalonate, which is further phosphorylated by Erg8p, leading to the formation of mevalonate 5-pyrophosphate. The latter is decarboxylated by Erg19p (mevalonate pyrophosphate decarboxylase) to isopentenyl pyrophosphate (IPP). Consequently, IPP is converted to dimethylallyl pyrophosphate (DMAPP) by the enzyme IDI1 (isopentenyl diphosphate isomerase). Erg20p (geranyl/farnesyl pyrophosphate decarboxylase) is essential for the formation of both geranylpyrophosphate (GPP) and farnesylpyrophosphate (FPP). Finally, two FPP molecules are fused by Erg9p (squalene synthase) to form squalene.

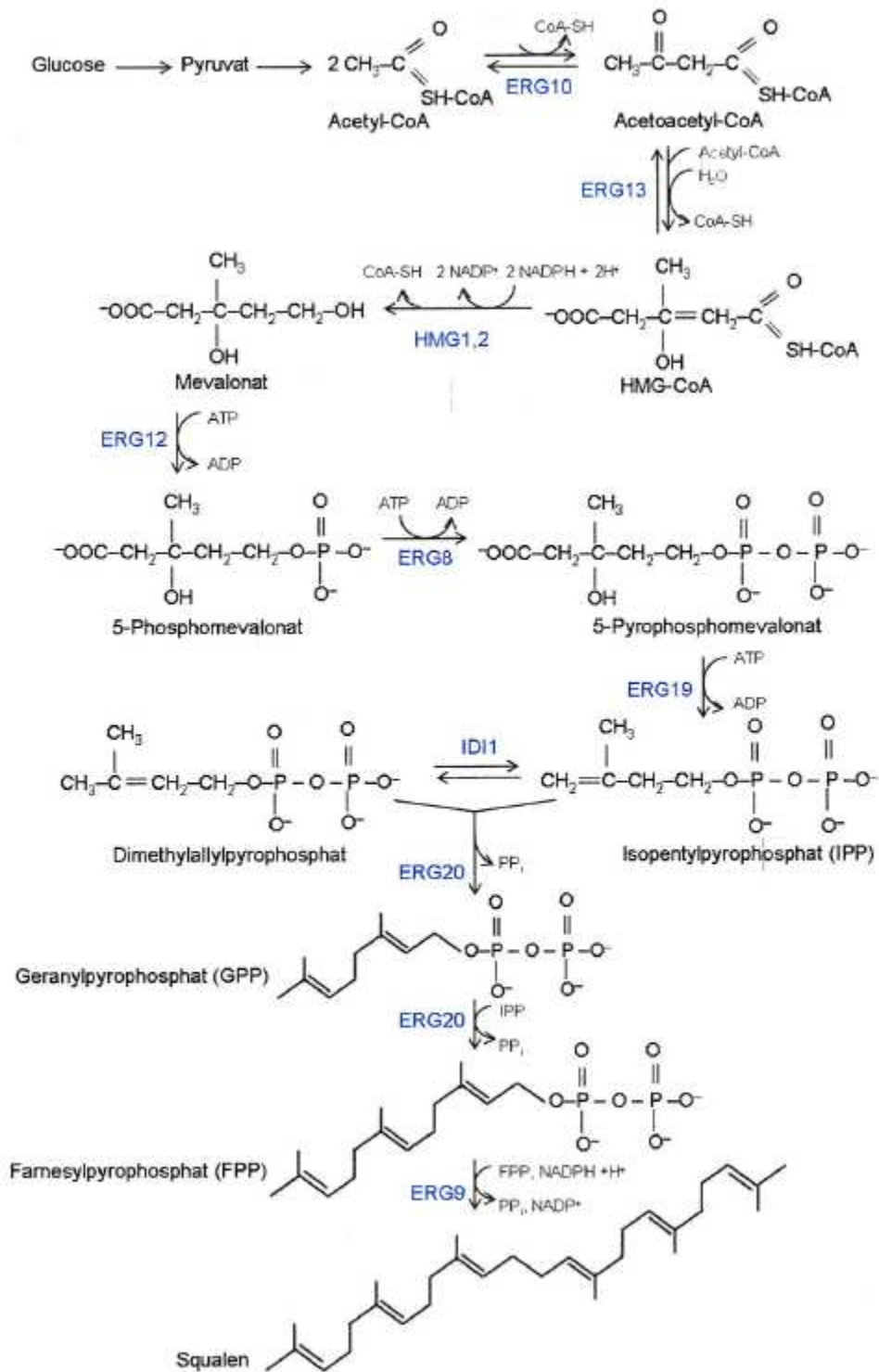


Figure 3: Overview of the pre - squalene pathway of ergosterol biosynthesis (Maczek, 2009)

### 1.2.2 The post – squalene pathway

Firstly, squalene epoxide is formed by Erg1p (squalene epoxidase) from squalene. The reaction itself requires oxygen and makes ergosterol synthesis an aerobic process. Lanosterol synthase (Erg7p) facilitates the formation of lanosterol from squalene epoxide. The methyl group at the C14 position of lanosterol is removed by Erg11p (sterol C14 demethylase) yielding 4,4-dimethylcholesta-8,14,24-trienol, a double bond of which is saturated by Erg24p (C14 sterol reductase). Four proteins (Erg25 – 28p) operate as a complex in the formation of zymosterol.

Zymosterol is methylated at the C24 position by Erg6p (sterol C24 methyltransferase), a critical step which distinguishes fungal and mammalian sterols. After the formation of fecosterol, Erg2p (C8 sterol isomerase) shifts the double bond at the C8 to the C7 position present in episterol. Erg3p (C5 sterol desaturase) introduces a double bond at the C5 position, which is highly conserved in all eukaryotic cells. In the two final steps of the pathway, double bonds in the sterol side chain are introduced by Erg5p (C22 sterol desaturase), and eliminated by Erg4p (C24(28) sterol reductase) yielding the final product ergosterol.

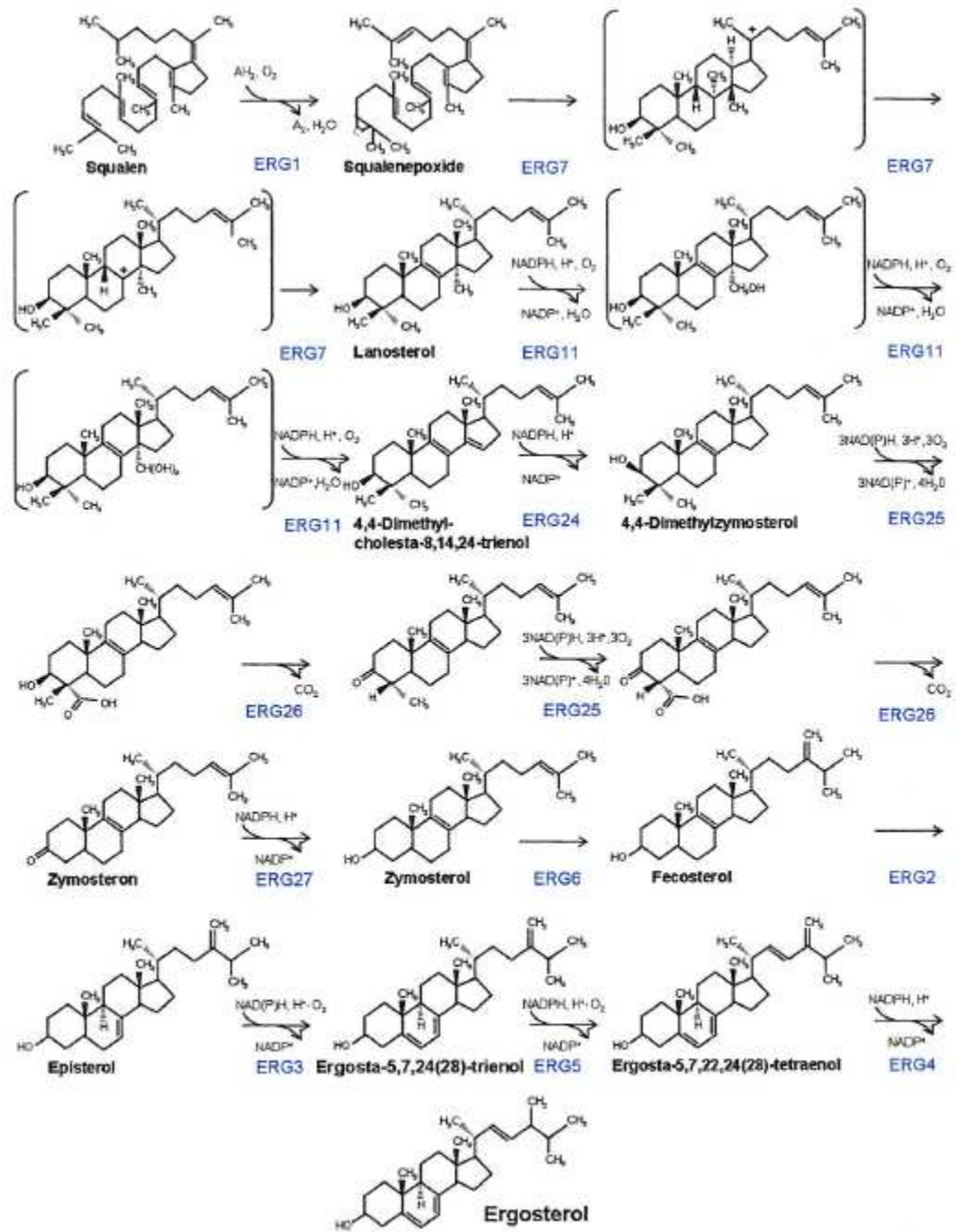


Figure 4: Overview of the post - squalene pathway of ergosterol biosynthesis (Maczek, 2009)

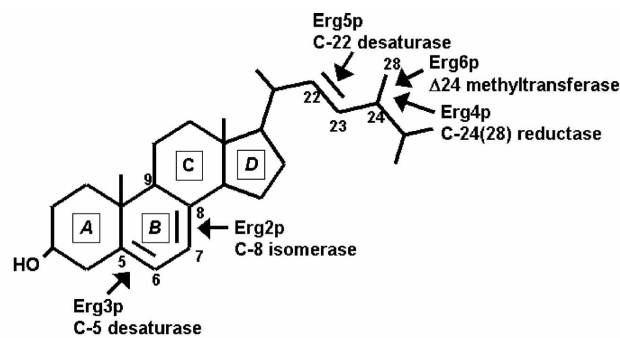
## 1.3 Genes involved in this work

### 1.3.1 The role of the *ERG5* and *ERG6* genes in the ergosterol biosynthesis pathway

In order to create a cholesterol producing *Pichia pastoris* strain, two genes of the ergosterol biosynthesis pathway had to be knocked out – the *ERG5* and *ERG6* genes.

The gene product of the *ERG5* gene, the C22 sterol desaturase, is localized in the endoplasmic reticulum. It catalyzes the conversion of its substrate ergosta-5,7,24(28)-trienol to ergosta-5,7,22,24(28)-tetraenol, the ultimate precursor of ergosterol biosynthesis pathway. As its name implies, it introduces a double bond at the C22 position of the sterol side chain (Pichler, 2005)

The C24 methyltransferase, encoded by the *ERG6* gene, is localized in lipid particles and to a lesser extent in the ER (Zinser et al., 1993). The addition of a methyl group at the C24 position is needed for the conversion of zymosterol to fecosterol.



**Figure 5: Enzymes involved in the formation of ergosterol, including Erg5p and Erg6p (Heese-Peck et al., 2002)**



### 1.3.2 Dehydrocholesterol reductases of *Danio rerio*

Both dehydrocholesterol reductases used in this work, *DHCR7* and *DHCR24* genes, of *Danio rerio* (zebrafish) had been codon optimized for the expression in the yeast *Saccharomyces cerevisiae* (Souza et al., 2011). Both gene products specifically remove double bonds of the sterol structure, *DHCR7* at position 7 and *DHCR24* at position 24, respectively.

### 1.4 Strategy of creating a cholesterol producing cell line

The first step in order to create a cholesterol producing cell line is to knock out the *ERG5* and *ERG6* genes. In addition to that, *DHCR7* and *DHCR24* had to be integrated into the *Pichia pastoris* genome. This was achieved by the assembly of knockin cassettes, bearing the dehydrocholesterol genes under the control of the GAP promoter and a resistance marker. These knockin cassettes were determined to selectively knock out both genes of the ergosterol biosynthesis pathway and introducing the *DHCR7* and *DHCR24* genes.

Both knockin cassettes also featured flanking upstream and downstream regions of the *ERG* genes, so the knockout could be achieved by homologous recombination. The flanking regions were chosen to be longer than 500 bp, to make sure that the integration would not likely occur elsewhere in the genome.

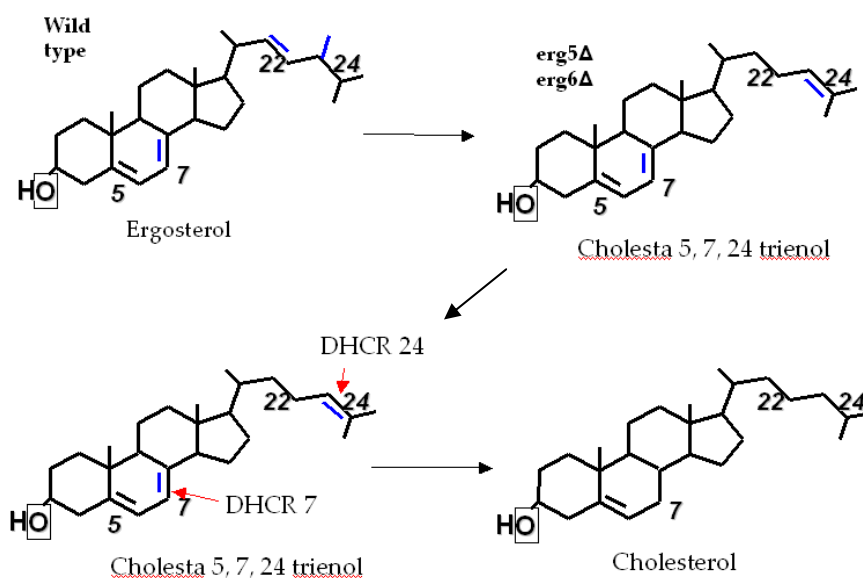


Figure 6: Strategy of creating a cholesterol producing cell line (Cleiton Martins de Souza, personal communication)

This work features basically three variants of genetically modified *Pichia pastoris* CBS7435  $\Delta ku70his4$  wild type strain. First, the single knockout strains  $\Delta erg5 - DHCR7$  and  $\Delta erg6 - DHCR24$ , which were created via single transformation experiments of the knockin cassettes. For the creation of the double mutant ( $\Delta erg5/6 - DHCR7/24$ ), the cholesterol producing strain, two transformation events were conducted. We will see, that a significant shift of the sterol patterns occurred.

## 1.5 Protein - Lipid interactions

Biological membranes confine cellular compartments and ensure the exchange of energy, biological molecules and information between the inside and the environment of the cell (García-Sáez and Schwille, 2008). Both lipid and protein composition of the cell play an essential role in this process. The large diversity of the lipid structures allows a diversity of chemical and physical properties for the membrane bilayer that affects protein function and their organization (Dowhan et al., 2004).

It has been shown that activity of membrane associated proteins is highly dependent on the lipid environment. In order to characterize the strains created during my work, I tried to investigate the correlation of the altered sterol composition and a possible change in protein function. Therefore, Pdr12p was selected as a target membrane protein for this investigation.

Pdr12p, which is a member of the ABC transporter family of proteins, is one of several anion efflux transporters in yeast. Its main function is the effective prevention of anion accumulation inside the cell. Yeasts, which are lacking Pdr12p, have been shown to be hypersensitive against weak organic acids. Pdr12p is under the control of the War1p transcription factor, named for weak acid resistance (Kren et al., 2003).

Pdr12p is also responsible for the ATP dependent efflux of fluorescein from the cell. Fluorescein is a fluorescent dye that had already been used for the characterisation of Pdr12p activity in yeast (Holyoak et al., 1999; Lushchak et al., 2008).

## **1.6 Targets of this Master thesis**

The aim of my Master thesis was the generation of a *Pichia pastoris* strain, which shows a significant shift in the overall sterol composition from ergosterol to cholesterol. This was achieved by sequentially knocking out the *ERG5* and *ERG6* genes and introducing functional *DHCR7* and *DHCR24* genes of zebrafish. Furthermore, I succeeded in characterizing the created strain variants in terms of sterol distribution and started to investigate sterol – protein interactions in the case of Pdr12p.

## 2 Materials

### 2.1 Strains

**Table 1: Yeast strains used in this work**

| Strain   | Genotype  | Source                 |
|--|---|------------------------|
| <i>P. p.</i> CBS7435 wild type                                 | CBS7435 $\Delta$ ku70his4   | IMBT strain collection |
| <i>P. p.</i> CBS7435 $\Delta$ ku70his4                         | $\Delta$ erg5-DHCR7   | This work              |
| <i>P. p.</i> CBS7435 $\Delta$ ku70his4                         | $\Delta$ erg6-DHCR24  | This work              |
| <i>P. p.</i> CBS7435 $\Delta$ ku70his4<br>“Cholesterol strain” | $\Delta$ erg5-DHCR7 $\Delta$ erg6-DHCR24  | This work              |
| <i>S. c.</i> CEN.PK2   | MATa/ MAT $\alpha$ ; ura3-52/ ura3-52;<br>trp1-289/ trp1-289; leu2-3_112/<br>leu2-3_112; his3 $\Delta$ 1/ his3 $\Delta$ 1; MAL2-<br>8C/ MAL2-8C; SUC2/ SUC2 | EUROSCARF              |

**Table 2: *E. coli* strains used in this work**

| Strain    | Genotype  | Source     |
|-----------|---|------------|
| Top10F'   | F' {lacIq Tn10 (TetR)} mcrA $\Delta$ (mrr-hsdRMS-mcrBC)<br>$\Phi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 recA1 araD139 $\Delta$ (ara-leu)7697 galU<br>galK rpsL endA1 nupG | Invitrogen |
| BL21 Gold | <i>E. coli</i> B F <sup>-</sup> ompT hsdS(rB <sup>-</sup> mB <sup>-</sup> ) dcm <sup>+</sup> Tetr gal endA Hte  | Stratagene |

### 2.2 Plasmids

**Table 3: Plasmids used in this work**

| Plasmid                | Features                          | Source    |
|------------------------|-----------------------------------|-----------|
| pGAPZ A                |                                   | IMBT      |
| pPpKanSynPDI           |                                   | IMBT      |
| pGAPZA - DHCR7c        | 5' ERG5 - GAP - DHCR7 -<br>Zeocin | This work |
| pPpKanSynPDI - DHCR24c | 5' ERG6 - GAP - DHCR24 -<br>KanMX | This work |
| pJet1.2 - DHCR7c       | DHCR7 knockin cassette            | This work |
| pJet1.2 - DHCR24c      | DHCR24 knockin cassette           | This work |

## 2.3 Primers

**Table 4: Primers for the amplification of the *DHCR7* and *DHCR24* genes**

| <b>Primername</b> | <b>Sequence 5' – 3'</b>                    |
|-------------------|--|
| Fw_DHCR7          | CCGGAATTCATGATGGCTTCAGATAGAGTT             |
| Rv_DHCR7          | ATAGTTTAGCGGCCGCTCATCAGAAAATATTTGGCAATAATC |
| Fw_DHCR24         | CCGGAATTCATGGACCCATTGTTGTATTTGG            |
| Rv_DHCR24         | ATAGTTTAGCGGCCGCTTATTAATGTCTAGCAGATTTAC    |

**Table 5: Primers for the amplification of the flanking regions of the *ERG5* and *ERG6* genes**

| <b>Primername</b> | <b>Sequence 5' – 3'</b>  |
|-------------------|--|
| Fw_Erg5upSt       | GGAAGATCTCTCGAGTCTAGAGGTTGAAGAGAGAAGATCG<br>TG                   |
| Rv_Erg5upGAP      | GACACCAAGACATTTCTACAAAAAAGATCTATTTAGCAAA<br>GAGATTAAGTTGGAGGAGGG |
| Fw_Erg5downStuI   | AAAAGGCCTTAGAGTATCGGATATTTATTTAGTTTATTA                          |
| Rv_Erg5downStuI   | AAATCCGGATTGTGGTATCGTTTCTGGTGG                                   |
| Fw_Erg6upSt       | GGAAGATCTCTCGAGGAGCTCGAGTAGAGCAGAGAGCAAG<br>C                    |
| Rv_Erg6upGAP      | GACACCAAGACATTTCTACAAAAAAGATCT<br>TTTCGGTAATAAACGAGTGTTGAGTAG    |
| Fw_KanErg6down3   | ATAGACGAAGATAAGTTTTGTAGATGC                                      |
| Rv_Erg6SacIneu    | GGAGCTCATTATTGGGCTTTCAGACGG                                      |

**Table 6: PCR amplification of the intermediate *DHCR7* and *DHCR24* constructs**

| <b>Primername</b> | <b>Sequence 5' – 3'</b>             |
|-------------------|-------------------------------------|
| Fw_GAPup          | CGTTAAGGGATTTTGGTCATG               |
| Rv_ZeoXhoIneu     | GGCCTCGAGTCAGTCCTGCTCCTCGGC         |
| Fw_AOX1down       | GACGCAGATCGGGAACACTG                |
| Rv_KMXXhoIneu     | GGCCTCGAGTTAGAAAACTCATCGAGCATCAAATG |

**Table 7: Primers for the PCR amplification of the *DHCR7* and *DHCR24* constructs**

| <b>Primername</b> | <b>Sequence 5' – 3'</b> |
|-------------------|-------------------------|
| Fw_Erg5up         | GGTTGAAGAGAGAAGATGGTG   |
| Rv_Erg5down       | TTGTGGTATCGTTTCTGGTTG   |
| Fw_Erg6up         | GAGTAGAGCAGAGAGCAAGC    |
| Rv_Erg6down       | ATTATTGGGCTTTCAGACGG    |

**Table 8: Primers for sequencing of the *DHCR24* construct**

| <b>Primername</b> | <b>Sequence 5' – 3'</b> |
|-------------------|-------------------------|
| FwErg6Seq         | CCCATCGTTTAGATAGTGAAA   |
| Fw GAPseq1        | CTCTGAAATATCTGGCTCC     |
| Fw DHCR24seq1     | GGACCCATTGTTGTATTTGG    |
| Fw DHCR24seq2     | GCTAATATGGGTCAAGTTAC    |
| Fw DHCR24seq3     | CCATTGAGACATTATTATC     |
| Fw DHCR24seq4     | CATTTTGAAGCTACATCTTC    |
| Fw DHCR24downseq  | GATTAAGTGAGACCTTCG      |
| Fw Kanseq1        | GACTTCAAACGCCAATATG     |
| Fw Kanseq2        | CCTCTTCCGACCATCAAGC     |
| Fw Kanseq3        | CCTTCATTACAGAAACGG      |
| Fw Erg6downseq1   | CGTGTCTAATAATGAAATC     |

**Table 9: Primers for colony PCR of the  $\Delta$ *erg5* *DHCR7* transformants**

| <b>Primername</b>       | <b>Sequence 5' – 3'</b>     |
|-------------------------|-----------------------------|
| Rv_DHCR7ctr (Fw primer) | ATTGGGAAAGATATACTGCTGCTG    |
| Rv_ZeoXhoI              | GGCCTCGAGTCAGTCCTGCTCCTCGGC |
| Fw_Erg5Ric              | ATGTCGCAAGTCATTTCAAG        |
| Rv_Erg5Ric              | CCTGCTCGTCTCTATGTTTGAAC     |
| Fw_Erg5up2              | AGACATAAGTGGAAGAAGCC        |
| Rv_DHCR7                | CTAACTCTATCTGAAGCCATC       |

**Table 10: Primers for colony PCR of the  $\Delta$ *erg6* *DHCR24* transformants**

| <b>Primername</b> | <b>Sequence 5' – 3'</b>         |
|-------------------|---------------------------------|
| Fw_DHCR24         | CCGGAATTCATGGACCCATTGTTGTATTTGG |
| Rv_DHCR24         | CTAAGCCACCCAAATACAAC            |
| Fw_Erg6Ric        | ATGACTACCTCTACAACACTGAAC        |
| Rv_Erg6Ric        | TTCTGGCAACGTATAACATC            |
| Fw_Erg6up2        | GAGATGTGGAAATGGTGTAAAG          |
| Rv_DHCR24         | CTAAGCCACCCAAATACAAC            |

## 2.4 Instruments and Devices

**Table 11: Instruments and devices used in this work**

| <b>Instrument/Devices</b>                       | <b>Supplier</b>                                  |
|---|--|
| Automatic TLC sampler                           | CAMAG  |
| Centrifuges                                     | Centrifuge 5810R, Eppendorf, Germany             |
|   | Centrifuge 5415R, Eppendorf, Germany             |
| Electrophoresis gel chambers                    | PowerPac Basic + Sub - Cell GT, Biorad, USA      |
| Electrotransformators                           | MicroPulser, BIO - RAD, USA                      |
| Electroporation Cuvettes<br>(2 mm gap)          | Molecular BioProducts Inc., USA                  |
| Eppendorf tubes                                 | Greiner bio - one International AG               |
| Falcon Tubes                                    | Greiner bio - one International AG               |
| GC caps   | VWR International, GmbH                          |
| GC vials  | VWR International, GmbH                          |
| GC clamp  | VWR International, GmbH                          |
| Glass bottles                                   | Schott/ Duran                                    |
| Glass beads                                     | Carl Roth GmbH & Co KG                           |
| Incubator (30°C and 37°C)                       | Binder GmbH                                      |
| Flasks  | Simax  |
| Laminar flow chamber                            | Gelaire Flow Laboratories                        |
| Microscope DM LB2                               | Leica Microsystems GmbH                          |
| Vortexer  | Vortex - Genie2, Scientific Industries Inc, USA  |
| PCR machines                                    | GeneAmp PCR System 2700, Applied Biosystems, USA |
| PCR tubes                                       | Greiner bio - one International AG               |
| Petridishes                                     | Greiner bio - one International AG               |
| Photometer                                      | BioPhotometer, Eppendorf, Germany                |
| Pipette tips                                    | Greiner bio - one International AG               |
| Pipettes  | Pipetman P20N Gilson Inc, USA                    |
|   | Pipetman P200N Gilson Inc, USA                   |
|   | Pipetman P1000N Gilson Inc, USA                  |
| Pyrex tubes                                     | Pyrex, Incorp.                                   |
| Scanner   | HP scanjet 4370                                  |
| Shaker  | HT MiltronII, Infors AG, Switzerland             |
| Thermomixer                                     | Thermomixer comfort, Eppendorf, Germany          |
| TLC chamber                                     | CAMAG  |
| TLC scanner                                     | CAMAG  |
| TLC silica plates                               | Merck GmbH                                       |
| Transferpipettes (200 – 1000<br>µL; 10 – 50 µL) | Brand GmbH, Germany                              |
| UV cuvettes                                     | Greiner bio - one International AG               |
| Vibrax  | Vibrax VXR basic, IKA GmbH & Co KG, Germany      |

## 2.5 Reagents

**Table 12: Reagents used in this work**

| <b>Reagent</b>                               | <b>Supplier</b>                            |
|--|--|
| Acetic acid                                  | Carl Roth GmbH & Co KG                     |
| Agar   | BD Bacto- Becton, Dickinson and Company    |
| Agarose                                      | Biozym Scientific GmbH                     |
| Bicine - NaOH                                | Carl Roth GmbH & Co KG                     |
| Chloroform                                   | Carl Roth GmbH & Co KG                     |
| Cholesterol                                  | Sigma- Aldrich Corp.                       |
| CloneJET™ PCR Cloning Kit                    | Fermentas- Thermo Fisher Scientific Inc.   |
| dATP   | Fermentas- Thermo Fisher Scientific Inc.   |
| dCTP   | Fermentas- Thermo Fisher Scientific Inc.   |
| Deionised water                              | Fresenius Kabi Austria GmbH                |
| Deoxy-2-glucose                              | Sigma Aldrich Corp.                        |
| dGTP   | Fermentas- Thermo Fisher Scientific Inc.   |
| Diethylether                                 | Carl Roth GmbH & Co KG                     |
| DMSO   | Carl Roth GmbH & Co KG                     |
| DreamTaq buffer                              | Fermentas- Thermo Fisher Scientific Inc.   |
| DreamTaq DNA polymerase                      | Fermentas- Thermo Fisher Scientific Inc.   |
| DreamTaq™ Buffer                             | Fermentas- Thermo Fisher Scientific Inc.   |
| DreamTaq™ Polymerase                         | Fermentas- Thermo Fisher Scientific Inc.   |
| DTT  | Carl Roth GmbH & Co KG                     |
| dTTP   | Fermentas- Thermo Fisher Scientific Inc.   |
| Easy DNA Kit                                 | Invitrogen Corp.                           |
| EDTA   | Carl Roth GmbH & Co KG                     |
| Ethanol                                      | Australco Handels GmbH                     |
| Ethyl acetate                                | Carl Roth GmbH & Co KG                     |
| Ethylene glycol                              | Sigma Aldrich Corp.                        |
| FastAP™ Buffer                               | Fermentas- Thermo Fisher Scientific Inc.   |
| FastAP™ thermosensitive alkaline phosphatase | Fermentas- Thermo Fisher Scientific Inc.   |
| Fluorescein diacetate                        | Sigma Aldrich Corp.                        |
| GeneJET™ Plasmid Miniprep Kit                | Fermentas- Thermo Fisher Scientific Inc.   |
| GeneRuler™ 1kb Plus DNA Ladder               | Fermentas- Thermo Fisher Scientific Inc.   |
| GeneRuler™ DNA Ladder Mix                    | Fermentas- Thermo Fisher Scientific Inc.   |
| Geneticin sulfate                            | Gibco/ Invitrogen- Life Technologies Corp. |
| Glucose Monohydrate                          | Carl Roth GmbH & Co KG                     |
| Glycerol                                     | Carl Roth GmbH & Co KG                     |
| HCl  | Carl Roth GmbH & Co KG                     |
| HEPES Buffer                                 | Merck GmbH                                 |
| Magnesium chloride                           | Carl Roth GmbH & Co KG                     |
| Mass Ruler DNA Ladder Mix                    | Fermentas- Thermo Fisher Scientific Inc.   |
| MassRuler™ DNA Ladder                        | Fermentas- Thermo Fisher Scientific Inc.   |
| Methanol                                     | Carl Roth GmbH & Co KG                     |



|   |  |
|---|--|
| N'O'-bis(trimethylsilyl)-trifluoroacetamid          | Sigma Aldrich Corp.                      |
| n-heptane   | Carl Roth GmbH & Co KG                   |
| PCR DNA and Gel band purification kit               | GE Healthcare                            |
| PEG 4000  | Sigma Aldrich Corp.                      |
| Peptone   | BD Biosciences                           |
| Petrol ether  | Carl Roth GmbH & Co KG                   |
| Phusion Buffer HF                                   | Fermentas- Thermo Fisher Scientific Inc. |
| Phusion DNA Polymerase                              | Fermentas- Thermo Fisher Scientific Inc. |
| Pyridine  | Carl Roth GmbH & Co KG                   |
| Pyrogallol  | Carl Roth GmbH & Co KG                   |
| Roti Prep - Plasmid Kit                             | Carl Roth GmbH & Co KG                   |
| Sodium hydroxide                                    | Carl Roth GmbH & Co KG                   |
| T4 DNA Ligase                                       | Fermentas- Thermo Fisher Scientific Inc. |
| T4 DNA Ligase Buffer                                | Fermentas- Thermo Fisher Scientific Inc. |
| Tween20   | Carl Roth GmbH & Co KG                   |
| Wizard SV Gel Slice and PCR Product Preparation Kit | Promega                                  |
| Yeast extract                                       | Carl Roth GmbH & Co KG                   |
| $\beta$ -Mercaptoethanol                            | Carl Roth GmbH & Co KG                   |

## 2.6 Media and Buffers

**Table 13: Media and buffers used in this work**

| <b>Medium</b>   | <b>Composition</b>  |
|---|---|
| LB  | 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 20 g/L agar   |
| LB - Kan  | LB medium + 1 mL kanamycin stock solution (1000x)/1 L   |
| LB - Zeo  | LB medium + 1 mL zeocin stock solution (1000x)/1 L  |
| 2 Deoxy - D - Glucose 100x stock ( 500 mM)            | 0.08208 g in 1 mL bidest. H <sub>2</sub> O  |
| BEDS (10 mL)  | 1 mL 0.1 M bicine NaOH (10x), 300 µL ethylene glycol (3%), 500 µL DMSO (5%), 2 mL 5 M sorbitol, 6.2 mL bidest H <sub>2</sub> O  |
| Bicine NaOH stock pH 8,3 (0,1 M)                      | 1.63g N,N - Bis - (2 - hydroxyethyl) - glycine in 100 mL bidest H <sub>2</sub> O  |
| DTT stock solution (1 M)                              | 1.54g dithiotreitol dissolved in 10 mL bidest H <sub>2</sub> O  |
| EDTA stock solution (0,5 M)                           | 93.05 g EDTA disodium salt, dissolved in 400 mL bidest H <sub>2</sub> O ; pH adjusted to about 8, solution topped up to a final volume of 500 mL                        |
| Fluorescein Diacetate 100x stock (5 mM)               | 0.01041 g in 5 mL DMSO  |
| HEPES NaOH pH 7 (50 mM)                               | 2.383 g/ 200 mL bidest H <sub>2</sub> O   |
| SOC   | 20 g/L bacto tryptone, 0.58 g/L NaCl, 5 g/L bacto yeast extract, 2 g/L MgCl <sub>2</sub> , 0.16 g/L KCl, 2.46 g/L MgSO <sub>4</sub> , 3.46 g/L dextrose                 |
| Sorbitol (5 M)  | 91.1 g sorbitol in 100 mL bidest H <sub>2</sub> O   |
| TAE buffer (1x) for electrophoresis                   | 242 g Tris base dissolved in 750 mL deionized water; addition of 57.1 mL glacial acid acid and 100 mL of 0.5 M EDTA (pH 8.0) solution adjusted to a final volume of 1 L |
| TAE buffer (50x) - stock solution for electrophoresis | 200 mL of TAE (50x), 9.8 L bidest H <sub>2</sub> O  |
| YPD   | 10 g/L yeast extract (1%), 20 g/L peptone (2%) , 20 g/L glucose (2%), 20 g/L agar (2%)  |
| YPD - G418  | YPD medium + 300 mg/L G418 sulfate  |
| YPD - Zeo   | YPD medium + 100 mg/L zeocin  |

## 3 Methods

### 3.1 Construction of the *DHCR7* and *DHCR24* knockin cassettes

The construction of the knockin cassettes involved several cloning steps. Both dehydrocholesterol reductase genes were placed under the control of the glyceraldehyde 3-phosphate (GAP) promoter for constitutive expression.

The pGAPZ A vector was the starting point for the construction of the *DHCR7* knockin cassette. The vector already contained the GAP promoter and a Zeocin resistance marker for selection of positive transformants in both *E. coli* and *Pichia pastoris*. The 5' *ERG5* fragment for homologous recombination in *Pichia* was cloned upstream of the GAP promoter. Due to the fact, that the pGAPZ A vector was lacking a restriction site for the integration of the 3' *ERG5* fragment shortly after the zeocin marker cassette, the 3' *ERG5* was cloned via blunt end ligation into another vector system, the pJET1.2/blunt. This new vector offered several restriction sites for the integration of the unfinished knockin cassette of pGAPZ A. Finally, the whole cassette was amplified via PCR and used for transformation into *Pichia pastoris*.

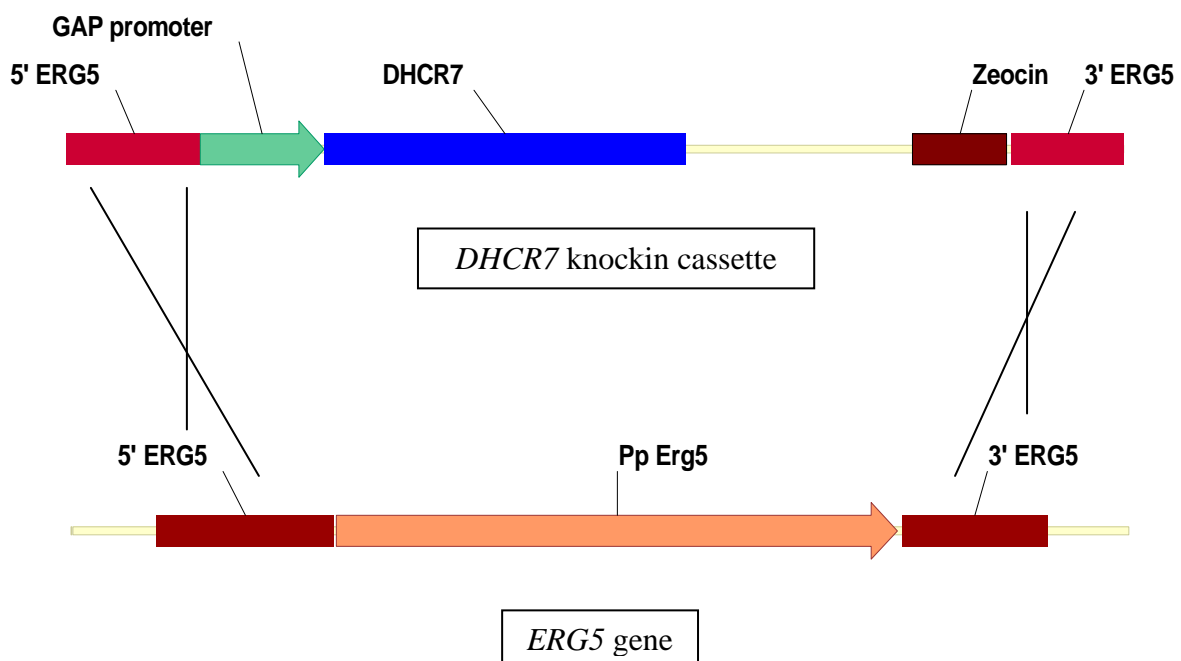
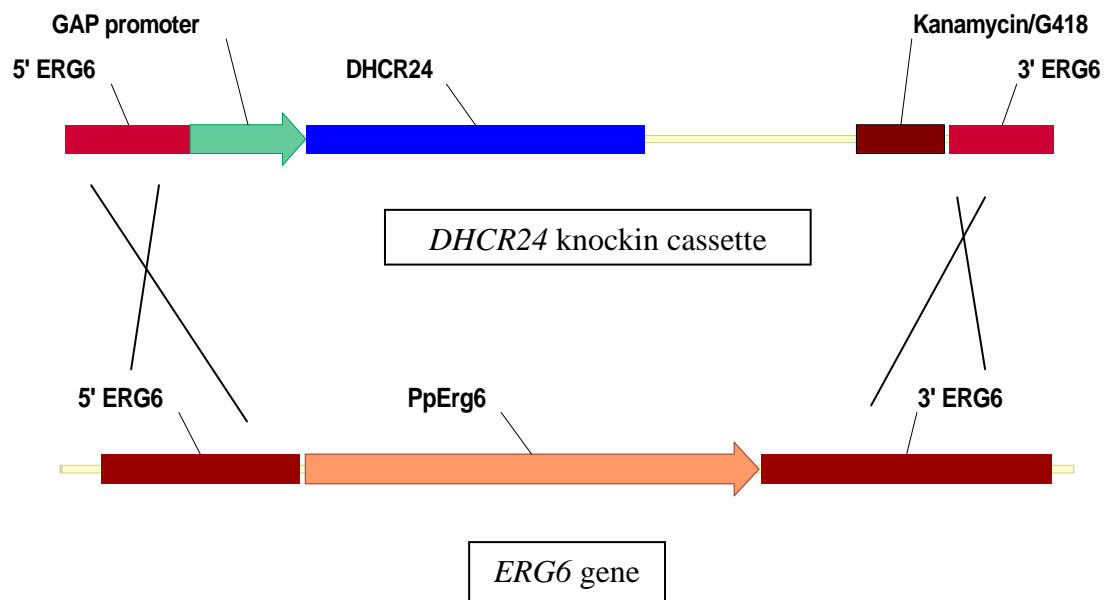


Figure 7: Overview of the *DHCR7* knockin cassette and its integration into the *Pichia pastoris* genome

The strategy for the *DHCR24* knockin cassette was different. I started out with the pPpKanSynPDI vector, which carries a kanamycin resistance cassette. The kanamycin marker confers resistance to the aminoglycoside G418 in *Pichia pastoris* (Webster and Dickson, 1983). The *AOX1* promoter of the pPpKanSynPDI vector was replaced with the GAP promoter. Subsequently, the 5' *ERG6* fragment was inserted upstream the GAP promoter and the SynPDI cassette was replaced by the *DHCR24* gene. The final steps were analogous to the construction of the *DHCR7* construct, involving the pJET1.2/blunt - 3' *ERG6* intermediate product.



**Figure 8: Overview of the *DHCR24* knockin cassette and its integration into the *Pichia pastoris* genome**

The individual steps of creating the *DHCR 7* and *DHCR24* knockin cassettes can be found in the results section.

## **3.2 General Methods**

All of the following methods have been carried out several times during my Master thesis. Nevertheless, the individual reaction setup will be described in detail in each section of the corresponding experiment.

### **3.2.1 Preparation of plasmids, PCR products and DNA Fragments**

All plasmids were isolated with “Roti Prep - Plasmid Kit” from Roth according to the supplier’s manual and finally eluted with 50  $\mu$ L ddH<sub>2</sub>O. DNA fragments and PCR products were purified over standard DNA agarose gels and extracted with “Wizard SV Gel Slice and PCR Product Preparation” by Promega or the “PCR DNA and Gel band purification kit” by GE Healthcare, as described in the product manuals.

### **3.2.2 Ligation experiments**

All ligations were done with the T4 DNA ligase by Fermentas and the corresponding ligation buffer according to the supplier’s manual. For sticky end ligations, I generally used an insert to vector ratio of 3:1. The exact amount of DNA for the ligation reactions was determined using the “Ligation calculator”, an online – tool offered by Invitrogen. The reaction itself was either incubated for 2 h at room temperature or O/N at 16°C. All ligation experiments were coupled with a control ligation, which served as an indicator of vector re - ligation.

Blunt end ligations were also carried out, generally using a 5:1 insert to vector ratio. During the final stages of constructing the knockin cassettes, I used the CloneJET™ PCR Cloning Kit by Fermentas.

### **3.2.3 Dephosphorylation of DNA fragments**

FastAP™ thermosensitive alkaline phosphatase by Fermentas was used for dephosphorylation of vectors before blunt end ligations were carried out. The incubation times were modified in order to yield the best results for each experiment.

### **3.2.4 Electrocompetent *E. coli* cells**

30 mL of LB media were inoculated with the chosen *E. coli* strain and incubated over night at 37°C and 220 rpm. The following day, the main culture of 500 mL of LB media was inoculated with 5 mL of the ONC and incubated at 37°C until reaching an OD between 0.7 and 0.9, which usually took between two and four h. After transferring the culture to chilled 500 mL centrifuge bottles, they were cooled on ice for 30 min before harvesting the cells at 2,000 x g and 4°C for 15 min. The supernatant was discarded and the pellet was carefully resuspended in pre-chilled 500 mL ddH<sub>2</sub>O. The suspension was centrifuged as before and the supernatant was discarded. Then, the cell pellet was resuspended in 35 mL of pre-chilled, sterile 10% glycerol and centrifuged at 4,000 x g and 4°C for 15 min. After discarding the supernatant, the pellet was resuspended in 1 mL ice-cold, sterile 10% glycerol before aliquoting the electrocompetent cells to 80 µL into sterile Eppendorf tubes. The cells were frozen in liquid N<sub>2</sub> and stored at -80°C until needed (Ploier, 2010).

### **3.2.5 Electroporation of *E. coli***

The transformation of plasmids into electrocompetent *E. coli* cells TOP10F' followed a standard procedure. Eighty µL of electrocompetent cells, prepared as described above, were thawed on ice. After adding 2 µL of a plasmid preparation and transferring the mixture to chilled transformation cuvettes, they were incubated on ice for ten minutes before pulsing them in the electroporator. Immediately after the electro-pulse, 1 mL of SOC medium was added and the cells were regenerated at 37°C for one h at 650 rpm before plating them onto selective media (Ploier, 2010).

### **3.2.6 Preparation of electrocompetent *Pichia pastoris* cells**

All transformations in this work are based on the condensed protocol for competent cell preparation and transformation of *Pichia pastoris* (Lin – Cereghino et al., 2005), which worked very well for the purpose of my thesis.

For the preparation of competent *Pichia pastoris* cells, an ONC of 5 mL YPD media was inoculated with a single colony and incubated at 30°C and 180 rpm until the following day. An aliquot of the ONC was then used for the inoculation of the main culture (50 ml total volume) to an OD<sub>600</sub> of 0.2. The cells were grown to an OD<sub>600</sub> of 0.8 to 1.0 at 30°C and 120 rpm.

Harvesting of the cells was performed at 3000 rpm (Eppendorf 5810R) for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in a mixture of 9 mL BEDS and 1 mL DTT. After resuspension, the harvested cells were incubated for 5 min at 30°C and 100 rpm. Another centrifugation step was performed at 3000 rpm for 5 min at 4°C and the supernatant was again discarded.

The cell pellet was resuspended in 0.5 mL BEDS and aliquoted in 80 µL portions into sterile 1.5 mL Eppendorf tubes. For better transformation efficiency the cells were directly used for electrotransformation.

### **3.2.7 Electrotransformation of *Pichia pastoris***

The best transformation results were achieved when 1 to 1.2 µg linearized DNA was mixed with 80 µL electrocompetent cells. The cells were incubated for 2 min on ice and transferred into a sterile electroporation cuvette for electroporation. The electroporator was set to 1500 V, 200 Ω, 25 µF. Immediately after the electro pulse, 0.5 mL of 1 M sorbitol and 0.5 ml of YPD medium were added. The transformed cells were recovered at 30°C for 3 h before plating them on selective media.

### 3.2.8 Colony PCR

The transformants were tested for the correct integration of the *DHCR 7* and *DHCR24* genes into the *ERG5* and *ERG6* loci according to the standard colony PCR protocol. Therefore, a single colony of about 1 mm diameter was resuspended in 25  $\mu$ L of sterile H<sub>2</sub>O bidest. The mixture was heated for 5 min at 95°C and then immediately cooled on ice for 5 min. After centrifugation for 5 min at maximum speed, the cell debris was pelleted and the supernatant containing the genomic DNA could immediately be used as template for Colony PCR.

Three different primer pairs were used to make sure that the knockin cassettes had integrated into the *ERG5* and *ERG6* loci, respectively.

### 3.2.9 GC/MS Sterol analysis

Sterol analysis was performed as previously described (Quail and Kelly, 1996; Tuller et al., 1999). GC/MS samples were prepared by harvesting 15 OD<sub>600</sub> units of ONC in Pyrex tubes. The culture medium was discarded after centrifugation at 1600 rpm (Eppendorf 5810R) for 5 min and the pellets were resuspended in 0.6 mL methanol, 0.4 mL pyrogallol (0.5% dissolved in methanol) and 0.4 mL 60% aqueous KOH each. After addition of 5  $\mu$ L of 2 mg/mL cholesterol standard, the pellets were vigorously vortexed and heated for 2 h on a sandbath at 90°C. After cooling to room temperature, saponified lipids were extracted three times with 1 mL of n-heptane. For good extraction efficiency, the samples were vortexed for one minute. The phases were separated by centrifugation at 1500 rpm for 3 min. The upper phases were collected in a new Pyrex tube, while the lower phases were re-extracted. The combined upper phases of each sample were dried under a stream of nitrogen. The extracted sterols were dissolved in 10  $\mu$ L of pyridine and derivatized with 10  $\mu$ L of N'O'-bis(trimethylsilyl)-trifluoroacetamid to improve volatility. Then, the samples were incubated for 10 min at room temperature and diluted in 50  $\mu$ L ethylacetate (Ploier, 2010). The sterols were analysed by Prof. Dr. Erich Leitner at the Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology. The technical data of GC-MS analysis are described as follows:



**Table 14: Technical data of the GC – MS**

|                  |  |
|------------------|--|
| GLC              | HP 5890 Series II Plus with Electronic Pressure Control and 6890 automated liquid sampler (ALS)  |
| Injector         | Split/splitless 270°C, mode: splitless, purge on: 2 min  |
| Injection volume | 1 µL   |
| Column           | HP 5-MS (Crosslinked 5% Phenyl Methyl Siloxane),<br>30 m x 0.25 mm i.d. x 0.25 µm film thickness |
| Carrier          | Helium, 5.0  |
| Flow             | 0.9 mL, linear velocity 35.4 cm/s, constant flow   |
| Oven             | 100°C (1 min), ramp of 10°C/min to 250°C (0 min) and ramp of<br>3°C/min to 300°C (0 min)         |
| Detector         | selective Detector HP 5972 MSD   |
| Ionization       | EI, 70 eV  |
| Mode             | Scan, scan range: 100-550 amu, 2.58 scans/s  |
| EM Voltage       | Tune Voltage   |
| Tune             | Auto Tune  |

### 3.2.10 Gel electrophoresis

The separation and purification of DNA fragments by agarose gel electrophoresis was performed according to standard protocols. One % agarose gels in TAE buffer were run at 80 V for about 80 min for preparative gels and 120 V for 45 min for analytical gels. The sizes of DNA fragments were assessed by comparison to the standard “GeneRuler 1kb Plus DNA Ladder”, “GeneRuler DNA Ladder” and “MassRuler DNA Ladder” by Fermentas (Ploier, 2010).

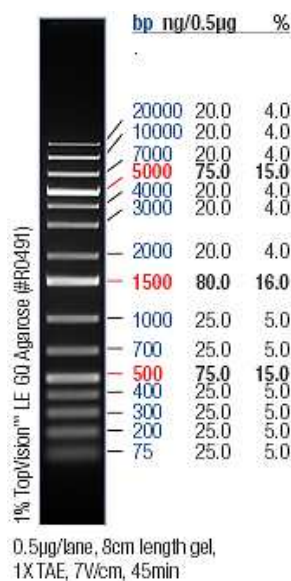


Figure 9: GeneRuler™ 1kb Plus DNA Ladder

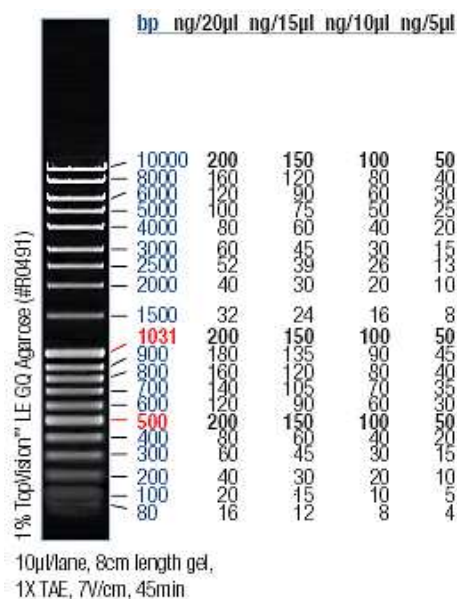
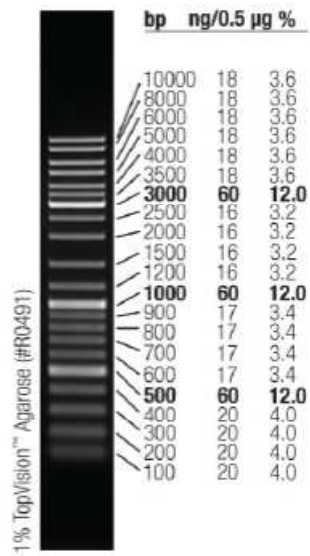


Figure 10: MassRuler™ DNA Ladder



0.5 µg/lane, 8 cm length gel,  
1X TAE, 7 V/cm, 45 min

**Figure 11: GeneRuler™ DNA Ladder**

### 3.2.11 Lipid extraction

Lipids were extracted by the method of Folch (1957). To get equal amounts of each strain, 250 OD<sub>600</sub> units of early stationary cultures were harvested in Pyrex glass tubes. The cell pellets were resuspended in 4 mL CHCl<sub>3</sub>:MeOH (2:1, v/v) and agitated in the presence of glass beads for one hour at room temperature on a Vibrax set to full speed to extract the total lipids. After sedimenting the glass beads at 1500 rpm for 3 min the crude lipid extract was transferred to a new Pyrex tube. Proteins and non-polar substances were removed by sequentially washing the organic phase with 2 mL of 0.034% MgCl<sub>2</sub>, 2 mL of 2 N KCl:MeOH (4:1, v/v) and 1.5 mL of CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (3:48:47, per vol.). Each washing step consisted of 2 min of vortexing and subsequent centrifugation to separate the phases. The upper, aqueous phases were always discarded. The lipid extracts were dried under a stream of nitrogen and finally dissolved in 50 µL of CHCl<sub>3</sub>:MeOH (2:1, v/v). These lipid extracts were used for TLC analysis immediately or stored at -20°C (Ploier, 2010).

### 3.2.12 Thin layer chromatography

In order to analyze the distribution of free sterols and steryl esters, a separation on silica TLC plates was carried out. Spotting of the prepared lipid extracts was either done manually or automatically. The separation of free and esterified sterols was achieved by the use of two main solvent mixtures. The first solvent mixture, petrol ether/diethyl ether/acetic acid (49:49:2, v/v), was prepared and after 30 min of equilibration in the TLC chamber, the TLC plates were developed until reaching about two thirds of the plate's height. The plate was dried completely and then transferred to the second TLC chamber for the final developing step, which was achieved by the pre – equilibrated solvent mixture petrol ether/diethyl ether (49:1, v/v). This time, the second TLC plate was developed until the solvent mixture reached the top of the plate.

Free sterols and steryl esters were quantified relatively using a TLC scanner at 275 nm. Then, bands of free sterols, steryl esters, triglycerides and squalene were visualized by charring. TLC plates were dipped into a solution of 0.4 g MnCl<sub>2</sub>, 60 mL H<sub>2</sub>O, 60 mL methanol and 4 mL H<sub>2</sub>SO<sub>4</sub> conc. for 10 sec and then heated at 105°C for 40 min (Ploier, 2010).

### 3.2.13 FDA Analysis:

**Table 15: Overview of the strains used in the FDA assay**

| Number | Strain   |
|--------|--|
| 1      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 (WT)  |
| 2      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 $\Delta$ erg5 <i>DHCR7</i>  |
| 3      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 $\Delta$ erg6 <i>DHCR24</i>   |
| 4      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 $\Delta$ erg5- <i>DHCR7</i> $\Delta$ erg6- <i>DHCR24</i> “Cholesterol strain” |
| 5      | - <i>S.c.</i> CEN.PK2 (generously provided by Birgit Ploier)   |

Cells were grown in YPD to early exponential phase and acetic acid was added to a final concentration of 0.25 mM for the induction of Pdr12p. Cells were grown further until they reached the early stationary phase.  $1 \cdot 10^8$  cells were used for the assay and washed 3 times with 1 mL 50 mM HEPES – NaOH, pH 7. A counting chamber (Neubauer) was used to determine the actual cells/ml. The cells were incubated in 5 mM 2 – deoxyglucose and 50  $\mu$ M FDA for 2 h at 30°C. The incubation served for the accumulation of FDA in the cells. Again, the cells were washed 3 times with 1 mL 50 mM HEPES – NaOH, pH 7 to remove excess FDA, which had not accumulated in the cells. Finally, the pellet was resuspended in 1 mL 50 mM HEPES – NaOH, pH 7, and incubated for another 5 min at 30°C before the start of the assay.

The assay was started by the addition of glucose (2 mM final concentration).

At specific time points, i.e. 0, 5, 10, 20, 30 min, samples were taken, centrifuged and the supernatants were used for fluorescence measurement. FDA fluorescence measurements were conducted using a spectrofluorimeter with an excitation wavelength of 435 nm and an emission wavelength of 525 nm.

### **3.2.13.1 Glucose dependent/independent efflux of fluorescein**

In order to investigate the glucose dependent efflux of fluorescein, cells were prepared according to the standard procedure. After the final washing steps, glucose was added to a final concentration of 2 mM and incubated for 2 h.

For the glucose independent efflux, the cells were prepared according to the standard procedure, but no glucose was added after the final washing steps. After 2 h of incubation the totally accumulated fluorescein in the supernatants was determined.

This experiment also featured the *Saccharomyces cerevisiae* CEN.PK2 wild type strain, to check if there is a difference in the efflux of fluorescein between the yeast wild type strains and the strains I generated.

## 4 Results

### 4.1 Construction of the knockin cassettes

#### 4.1.1 PCR Amplification of the *DHCR7* and *DHCR24* genes

The dehydrocholesterol reductase genes were amplified following the standard protocol of Finnzymes Phusion polymerase. The primers, which were used in the PCR reaction, featured *EcoRI* (Fw\_primer) and *NotI* (Rv\_primer) restriction sites for molecular cloning into pGAPZ A.

**Table 16: PCR amplification of the *DHCR7* gene**

| Reagent                          | Amount ( $\mu\text{L}$ ) |
|----------------------------------|--------------------------|
| H <sub>2</sub> O                 | 33                       |
| Phusion buffer (5x)              | 10                       |
| dNTP mix (10 mM)                 | 1                        |
| Primer Fw_ <i>DHCR7</i>          | 2                        |
| Primer Rv_ <i>DHCR7</i>          | 2                        |
| pPCR-Script <i>D._erio_DHCR7</i> | 1.5                      |
| Phusion polymerase               | 0.5                      |
| Total volume                     | 50                       |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 60°C/30 s - 72°C/50 s) x 30 - 72°C/10 min - 4°C/ $\infty$

**Table 17: PCR amplification of the *DHCR24* gene**

| Reagent                           | Amount ( $\mu\text{L}$ ) |
|-----------------------------------|--------------------------|
| H <sub>2</sub> O                  | 33.5                     |
| Phusion buffer (5x)               | 10                       |
| dNTP mix (10 mM)                  | 1                        |
| Primer Fw_ <i>DHCR24</i>          | 2                        |
| Primer Rv_ <i>DHCR24</i>          | 2                        |
| pPCR-Script <i>D._erio_DHCR24</i> | 1                        |
| Phusion polymerase                | 0.5                      |
| Total volume                      | 50                       |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 55°C/30 s - 72°C/50 s) x 30 - 72°C/10 min - 4°C/ $\infty$

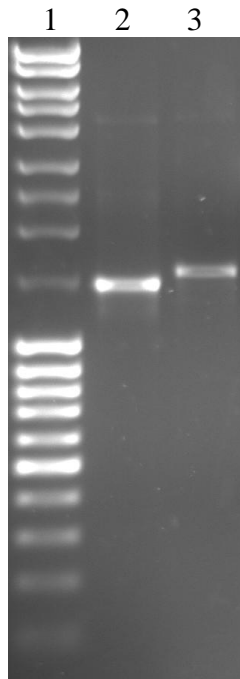


Figure 12: Agarose gel after the PCR amplification of the *DHCR7* and *DHCR24* genes

Table 18: Agarose gel after the PCR amplification of the *DHCR7* and *DHCR24* genes

| Sample ID | Description               | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|---------------------------|--------------------------|--------------------|
| 1         | MassRuler® DNA ladder mix | 5                        | -                  |
| 2         | <i>DHCR7</i>              | 5                        | 1447               |
| 3         | <i>DHCR24</i>             | 5                        | 1561               |



### 4.1.2 PCR amplification of the flanking regions of the *ERG5* and *ERG6* genes

The upstream and downstream regions of the *ERG5* and *ERG6* genes were amplified to pursue the homologous integration of the artificial *DHCR7* and *DHCR24* cassettes into the *Pichia pastoris* genome. The fragments were amplified using primers, introducing restriction sites for the purpose of molecular construction of the knockin cassettes.

The genomic DNA of the *Pichia pastoris* CBS7435  $\Delta$ ku70his4 strain served as template for the PCR reaction and was provided by Tamara Wriessnegger.

**Table 19: PCR amplification of the 5' *ERG5* region**

| Reagent   | Amount ( $\mu$ L) |
|---|-------------------|
| H <sub>2</sub> O                                  | 33                |
| Phusion buffer (5x)                               | 10                |
| dNTP mix (10 mM)                                  | 2                 |
| Primer Fw_Erg5upSt                                | 2                 |
| Primer Rv_Erg5upGAP                               | 2                 |
| Genomic DNA <i>P.p.</i> CBS7435 $\Delta$ ku70his4 | 0.5               |
| Phusion polymerase                                | 0.5               |
| Total volume                                      | 50                |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 64°C/50 s -72°C/20 s) x 30 - 72°C/10 min - 4°C/ $\infty$

**Table 20: PCR amplification of the 3' *ERG5* region**

| Reagent   | Amount ( $\mu$ L) |
|---|-------------------|
| H <sub>2</sub> O                                  | 33                |
| Phusion buffer (5x)                               | 10                |
| dNTP mix (10 mM)                                  | 2                 |
| Primer Fw_Erg5downStuI                            | 2                 |
| Primer Rv_Erg5downStuI                            | 2                 |
| Genomic DNA <i>P.p.</i> CBS7435 $\Delta$ ku70his4 | 0.5               |
| Phusion polymerase                                | 0.5               |
| Total volume                                      | 50                |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 60°C/50 s -72°C/20 s) x 30 - 72°C/10 min - 4°C/ $\infty$

**Table 21: PCR amplification of the 5' *ERG6* region**

| Reagent   | Amount ( $\mu\text{L}$ ) |
|---|--------------------------|
| H <sub>2</sub> O  | 33                       |
| Phusion buffer (5x)                                     | 10                       |
| dNTP mix (10 mM)  | 2                        |
| Primer Fw_Erg6upSt                                      | 2                        |
| Primer Rv_Erg6upGAP                                     | 2                        |
| Genomic DNA <i>P.p.</i> CBS7435 $\Delta\text{ku70his4}$ | 0.5                      |
| Phusion polymerase                                      | 0.5                      |
| Total volume  | 50                       |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 63°C/50 s -72°C/20 s) x 30 - 72°C/10 min - 4°C/ $\infty$

**Table 22: PCR amplification of the 3' *ERG6* region**

| Reagent   | amount ( $\mu\text{L}$ ) |
|---|--------------------------|
| H <sub>2</sub> O  | 33                       |
| Phusion buffer (5x)                                     | 10                       |
| dNTP mix (10 mM)  | 2                        |
| Primer Fw_KanErg6down3                                  | 2                        |
| Primer Rv_Erg6SacIneu                                   | 2                        |
| Genomic DNA <i>P.p.</i> CBS7435 $\Delta\text{ku70his4}$ | 0.5                      |
| Phusion polymerase                                      | 0.5                      |
| Total volume  | 50                       |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 64°C/50 s -72°C/20 s) x 30 - 72°C/10 min - 4°C/ $\infty$

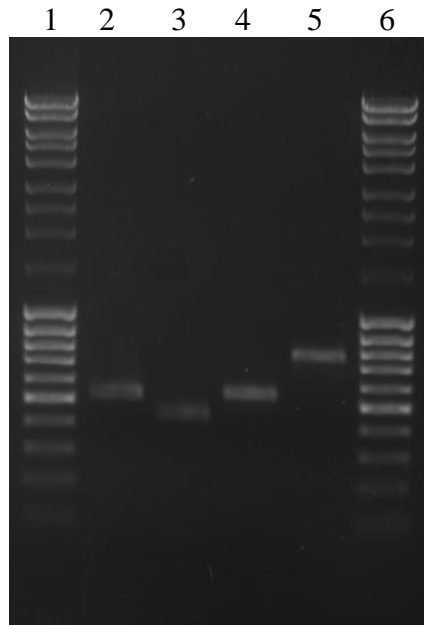


Figure 13: Agarose gel after the amplification of the *ERG5* and *ERG6* fragments

Table 23: Lane description of Figure 13

| Sample ID | Description               | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|---------------------------|--------------------------|--------------------|
| 1         | MassRuler® DNA ladder mix | 5                        | -                  |
| 2         | 5' <i>ERG5</i>            | 2                        | 544                |
| 3         | 3' <i>ERG5</i>            | 2                        | 449                |
| 4         | 5' <i>ERG6</i>            | 2                        | 531                |
| 5         | 3' <i>ERG6</i>            | 2                        | 763                |
| 6         | MassRuler® DNA ladder mix | 5                        | -                  |

### 4.1.3 Integration of the *DHCR7* gene into the pGAPZ A vector

The starting point for this knockin cassette was the pGAPZ A vector.

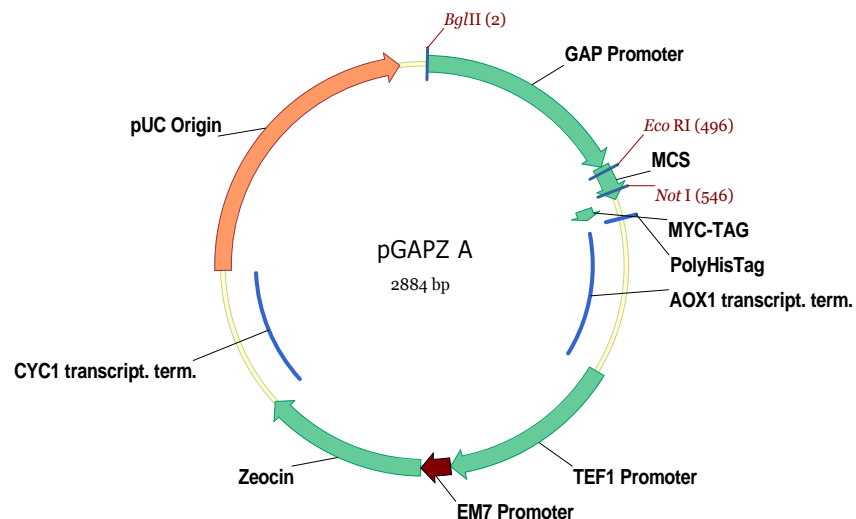


Figure 14: VectorNTi map of pGAPZ A

The vector features the GAP promoter and a Zeocin resistance marker, the *DHCR7* gene was inserted into the MCS of the vector at *EcoRI* and *NotI* restriction sites. In order to integrate the *DHCR7* gene into the pGAPZ A vector, both DNA fragments had to be cut with the enzymes *EcoRI* and *NotI*.

Table 24: Restriction cut of the cleaned up *DHCR7* PCR product

| Reagent                             | Amount ( $\mu\text{L}$ ) |
|-------------------------------------|--------------------------|
| cleaned up <i>DHCR7</i> PCR product | 50                       |
| Buffer O                            | 7                        |
| <i>EcoRI</i>                        | 1                        |
| <i>NotI</i>                         | 1                        |
| H <sub>2</sub> O                    | 11                       |
| Total volume                        | 70                       |

**Table 25: Restriction cut of the pGAPZ A vector**

| Reagent          | Amount ( $\mu\text{L}$ ) |
|------------------|--------------------------|
| pGAPZ - A        | 50                       |
| Buffer O         | 7                        |
| <i>Eco</i> RI    | 1                        |
| <i>Not</i> I     | 1                        |
| H <sub>2</sub> O | 11                       |
| Total volume     | 70                       |

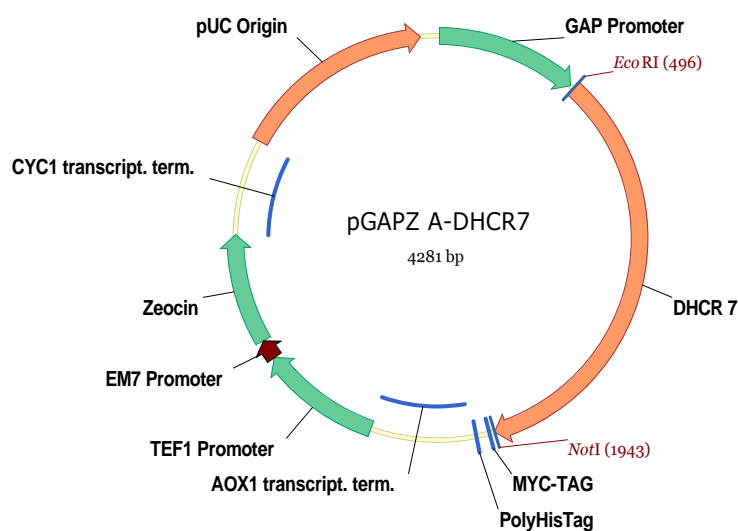
The linearized pGAPZ A was eluted on a preparative gel and cleaned up using the “Wizard SV Gel Slice and PCR Product Preparation” kit by Promega.

#### 4.1.4 Ligation of pGAPZ A vector and *DHCR7*

**Table 26: Ligation of pGAPZ A and *DHCR7***

| Reagent                                    | Amount ( $\mu\text{L}$ ) |
|--|--------------------------|
| pGAPZ A vector (20 ng/ $\mu\text{l}$ )     | 2                        |
| <i>DHCR7</i> insert (6 ng/ $\mu\text{l}$ ) | 13                       |
| Ligase buffer (10x)                        | 3                        |
| T4 DNA ligase                              | 1                        |
| H <sub>2</sub> O                           | 11                       |
| Total volume                               | 30                       |

Ligation was done O/N at 16°C.



**Figure 15: Vector NTI map of the pGAPZ A – *DHCR7* vector**

To check whether the *DHCR7* gene had been inserted into the pGAPZ A vector, an aliquot of the plasmid isolation was cut with *EcoRI* and *NotI*. If the *DHCR7* gene had been successfully integrated, an extra band at 1447 bp was expected.

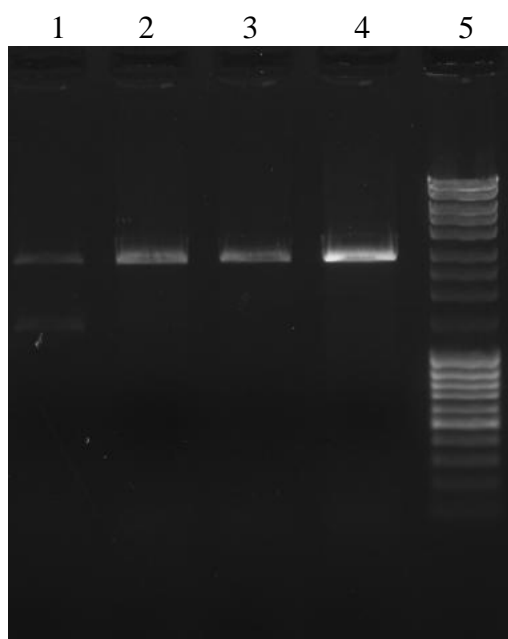


Figure 16: Gel after plasmid isolation and restriction cut with *EcoRI* and *NotI*

Table 27: Lane description of Figure 16

| Sample ID | Description               | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|---------------------------|--------------------------|--------------------|
| 1         | pGAPZ A – <i>DHCR7</i>    | 5                        | 2884, 1447         |
| 2         | pGAPZA only*              | 5                        | 2884               |
| 3         | pGAPZA only*              | 5                        | 2884               |
| 4         | Control pGAPZ A           | 5                        | 2884               |
| 5         | MassRuler® DNA ladder mix | 5                        | -                  |

\* negative transformants

The plasmid shown in lane 1 was used further.

#### 4.1.5 Preparation of the 5' *ERG5* fragment and the pGAPZ A – *DHCR7* vector for integration thereof

**Table 28: Restriction cut of the pGAPZA - *DHCR7* PCR product**

| Reagent                       | Amount ( $\mu\text{L}$ ) |
|-------------------------------|--------------------------|
| pGAPZ A – <i>DHCR7</i> vector | 25 (500ng)               |
| Buffer O                      | 4                        |
| <i>Bg</i> III                 | 3                        |
| H <sub>2</sub> O              | 8                        |
| Total volume                  | 40                       |

**Table 29: Restriction cut of the 5' *ERG5* fragment**

| Reagent                 | Amount ( $\mu\text{L}$ ) |
|-------------------------|--------------------------|
| 5' <i>ERG5</i> fragment | 12.5 (500 ng)            |
| Buffer O                | 2                        |
| <i>Bg</i> III           | 3                        |
| H <sub>2</sub> O        | 2.5                      |
| Total volume            | 20                       |

Following the restriction cuts, the fragments were cleaned up using the “Wizard SV Gel Slice and PCR Product Preparation Kit” by Promega.

#### 4.1.6 Dephosphorylation of the *Bgl*III cut pGAPZA – *DHCR7* vector

FastAP™ thermosensitive alkaline phosphatase by Fermentas was used for removal of 5' and 3' phosphates of DNA fragments. Many times, the dephosphorylation reaction was not very effective, so the incubation time and the amount of enzyme used for the reaction were changed from case to case.

**Table 30: Dephosphorylation of pGAPZ A – *DHCR7***

| Reagent                                   | Amount (μL) |
|---|-------------|
| pGAPZ A – <i>DHCR7</i> <i>Bgl</i> III cut | 50          |
| FastAP™ Buffer (10x)                      | 6           |
| FastAP™                                   | 1           |
| H <sub>2</sub> O                          | 3           |
| Total volume                              | 60          |

Incubation of the dephosphorylation reaction lasted for 10 min at 37°C and after adding an additional μL of FastAP™, the first step was repeated. Alkaline phosphatase was heat inactivated at 75°C for 5 min.

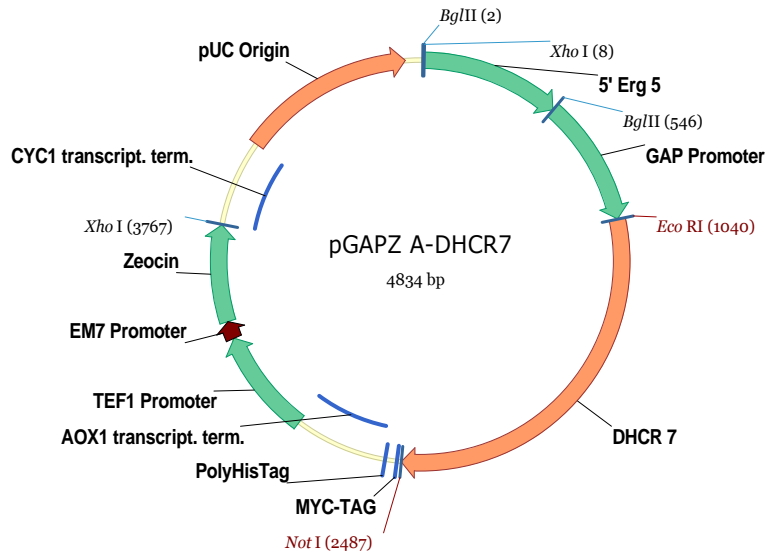
#### 4.1.7 Ligation of the 5' *ERG5* fragment into the pGAPZ A – *DHCR7* vector construct

**Table 31: Ligation of the 5' *ERG5* fragment into the pGAPZ A – *DHCR7* vector**

| Reagent                                   | Amount (μL) |
|---|-------------|
| pGAPZ A – <i>DHCR7</i> vector (100 ng/μl) | 5           |
| 5' <i>ERG5</i> insert (37 ng/μl)          | 1.2         |
| Ligase buffer (10x)                       | 2           |
| T4 DNA ligase                             | 2           |
| H <sub>2</sub> O                          | 9.8         |
| Total volume                              | 20          |

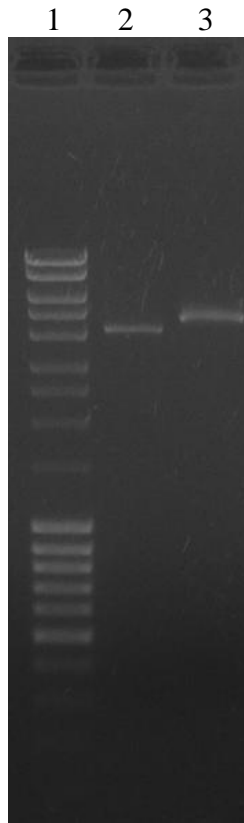
Ligation was done O/N at 16°C.





**Figure 17: Vector NTI map of pGAPZ A including the *DHCR7* gene and the 5' *ERG5* region**

After ligation and transformation into *E. coli* TOP10F', positive colonies were picked and the plasmids isolated. Plasmids were linearized with *Eco*RI in order to determine the correct size and the integration of the 5' *ERG5* fragment. The size of the 5' *ERG5* fragment inserted into the pGAPZ A – *DHCR7* vector is 544 bp.



**Figure 18: Agarose gel to confirm the integration of the 5' *ERG5* fragment**

**Table 32: Lane description of Figure 18**

| <b>Sample ID</b> | <b>Description</b>                      | <b>Volume (μL)</b> | <b>Visible bands (bp)</b> |
|------------------|---|--------------------|---------------------------|
| 1                | MassRuler® DNA ladder mix               | 5                  | -                         |
| 2                | pGAPZ A – <i>DHCR7</i>                  | 3                  | 4290                      |
| 3                | 5' <i>ERG5</i> – pGAPZ A – <i>DHCR7</i> | 3                  | 4834                      |

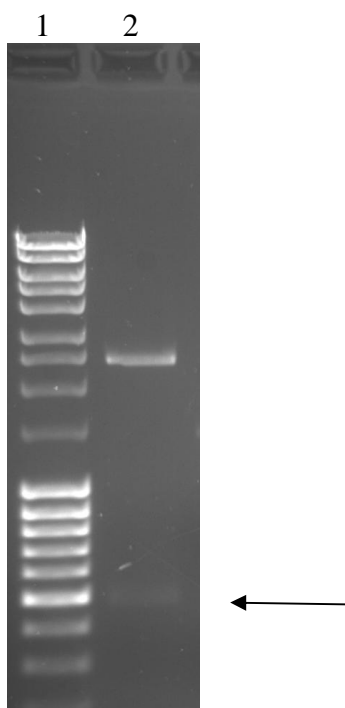
### 4.1.8 Preparation of the GAP promoter from the pGAPZ A vector

The GAP promoter was isolated from the pGAPZ A vector via *Eco*RI and *Bgl*III restriction cuts for integration into the pPpKanSynPDI vector.

**Table 33: Restriction cut of pGAPZ A to isolate the GAP promoter**

| Reagent          | Amount ( $\mu$ L) |
|------------------|-------------------|
| pGAPZ A          | 50 (1 $\mu$ g)    |
| Buffer O         | 7                 |
| <i>Eco</i> RI    | 4                 |
| <i>Bgl</i> III   | 4                 |
| H <sub>2</sub> O | 5                 |
| Total volume     | 70                |

The reaction was incubated for 2 h at 37°C and the GAP promoter fragment and the vector backbone were separated on a preparative gel.



**Figure 19: Agarose gel after restriction cut of pGAPZ A vector**

**Table 34: Lane description of Figure 19**

| Sample ID | Description               | Volume ( $\mu$ L) | Visible bands (bp) |
|-----------|---------------------------|-------------------|--------------------|
| 1         | MassRuler® DNA ladder mix | 5                 | -                  |
| 2         | pGAPZ A; GAP promoter     | 3                 | 2390, 494          |

The slight band of 494 bp in lane 2 corresponds to the successfully cut out GAP promoter.

#### 4.1.9 Removal of the *AOXI* promoter of pPpKanSynPDI vector

In order to replace the *AOXI* promoter of the pPpKanSynPDI vector by the GAP promoter, the *AOXI* promoter was cut from the vector backbone.

**Table 35: Reaction setup for the removal of the *AOXI* promoter**

| Reagent          | Amount ( $\mu\text{L}$ ) |
|------------------|--------------------------|
| pGAPZ A          | 8.4 (1 $\mu\text{g}$ )   |
| Buffer O         | 2                        |
| <i>Eco</i> RI    | 4                        |
| <i>Bgl</i> II    | 4                        |
| H <sub>2</sub> O | 1.6                      |
| Total volume     | 20                       |

The reaction was incubated for 2 h at 37°C and separated on a prepel. The vector backbone was cut out of the gel and purified for ligation.



Figure 20: Preparative agarose gel after excision of the *AOX1* promoter

Table 36: Lane description of Figure 20

| Sample ID | Description               | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|---------------------------|--------------------------|--------------------|
| 1         | MassRuler® DNA ladder mix | 5                        | -                  |
| 2         | pPpKanSynPDI; <i>AOX1</i> | 3                        | 4598, 931          |

The *AOX1* promoter fragment of 931 bp was successfully removed from the pPpKanSynPDI vector.

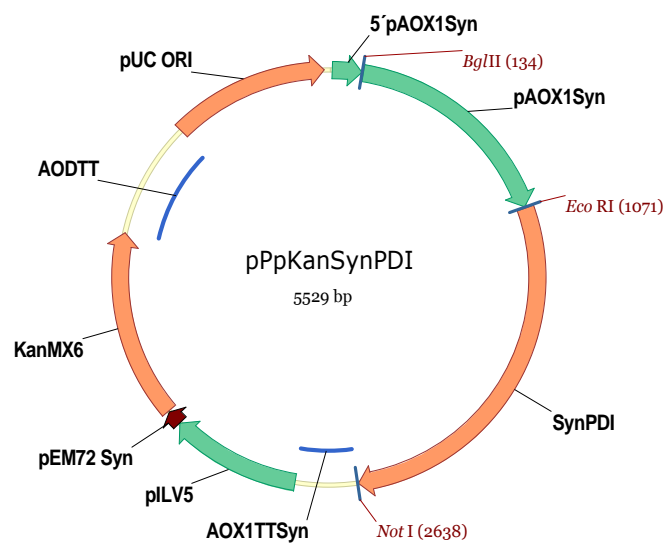


Figure 21: Vector NTI map of the pPpKanSynPDI vector

#### 4.1.10 Ligation of the GAP promoter into the pPpKanSynPDI (minus AOX1) vector

Table 37: Inserting the GAP promoter into the pPpKanSynPDI vector

| Reagent                      | Amount ( $\mu\text{L}$ ) |
|------------------------------|--------------------------|
| pPp KanSynPDI vector (50 ng) | 3.1                      |
| GAP promoter (16 ng)         | 16                       |
| Ligase buffer                | 4                        |
| T4 DNA ligase                | 1                        |
| H <sub>2</sub> O             | 15.9                     |
| Total volume                 | 40                       |

Ligation was performed O/N at 16°C. After inactivation and desalting of the ligation reaction, the construct was transformed into *E. coli* TOP10F'.

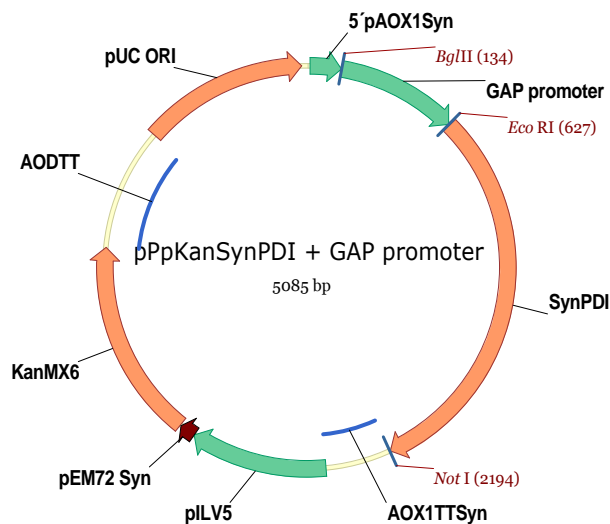
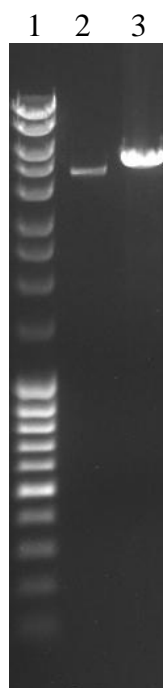


Figure 22: Vector NTI map of the pPpKanSynPDI vector with the GAP promoter

Transformants were checked for the integration of the GAP promoter by linearization with *NotI*.



**Figure 23: Agarose gel to confirm the integration of the GAP promoter into the pPpKanSynPDI vector backbone**

**Table 38: Lane description of Figure 23**

| Sample ID | Description                                      | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|--|--------------------------|--------------------|
| 1         | MassRuler® DNA ladder mix                        | 5                        | -                  |
| 2         | pPpKanSynPDI (minus <i>AOXI</i> )                | 3                        | 4598               |
| 3         | pPpKanSynPDI (minus <i>AOXI</i> ) + GAP promoter | 3                        | 5081               |

Lane 3 shows the linearized vector construct with the integrated GAP promoter fragment.

#### 4.1.11 Preparation of the 5' *ERG6* fragment and the pPpKanSynPDI – GAP vector for ligation

The 5' *ERG6* fragment was *Bgl*III cut and cloned upstream of the newly introduced GAP promoter after attaching *Bgl*III sites by PCR.

**Table 39: Restriction cut of the 5' *ERG6* fragment**

| Reagent                          | Amount (μL) |
|----------------------------------|-------------|
| 5' <i>ERG6</i> fragment (500 ng) | 10          |
| Buffer O                         | 2           |
| <i>Bgl</i> III                   | 3           |
| H <sub>2</sub> O                 | 5           |
| Total volume                     | 20          |

**Table 40: Restriction cut of the pPpKanSynPDI - GAP**

| Reagent                     | Amount (μL) |
|-----------------------------|-------------|
| pPpKanSynPDI – GAP (500 ng) | 20          |
| Buffer O                    | 3           |
| <i>Bgl</i> III              | 3           |
| H <sub>2</sub> O            | 4           |
| Total volume                | 30          |

Incubation of both restriction cuts O/N at 37°C. Further dephosphorylation of the vector was performed to avoid re-ligation of the vector.



#### 4.1.12 Dephosphorylation of the *Bgl*III cut pPpKanSynPDI – GAP vector

Table 41: Dephosphorylation of the pPpKanSynPDI – GAP vector

| Reagent                                      | Amount (μL) |
|--|-------------|
| pPpKanSynPDI – GAP vector <i>Bgl</i> III cut | 50          |
| Fast AP Buffer (10x)                         | 6           |
| Fast AP                                      | 1           |
| H <sub>2</sub> O                             | 3           |
| Total volume                                 | 60          |

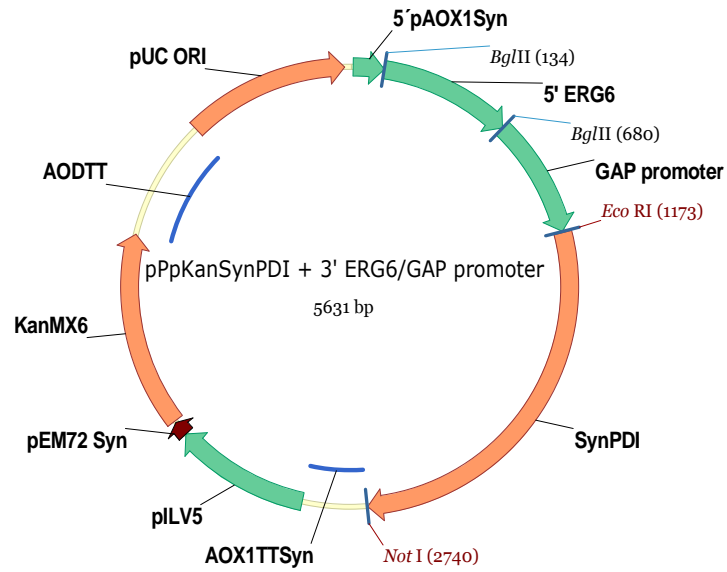
The dephosphorylation reaction was incubated for 10 min at 37°C, which was repeated after adding an additional μL of FastAP™. Alkaline phosphatase was heat inactivated at 75°C for 5 min.

#### 4.1.13 Ligation of the 5' *ERG6* fragment and the pPpKanSynPDI – GAP vector

Table 42: Ligation of 5' *ERG6* fragment and the pPpKanSynPDI – GAP vector

| Reagent                            | Amount (μL) |
|------------------------------------|-------------|
| pPpKanSynPDI - GAP vector (100 ng) | 4.5         |
| 5' <i>ERG6</i> fragment (29 ng)    | 1.1         |
| Ligase buffer                      | 2           |
| T4 DNA ligase                      | 2           |
| H <sub>2</sub> O                   | 10.4        |
| Total volume                       | 20          |

Ligation was performed for 2 h at room temperature. After inactivation and desalting of the ligation reaction, the construct was transformed into *E. coli* TOP10F'.



**Figure 24: Vector NTI map of the pPpKanSynPDI vector with integrated GAP promoter and 5' *ERG5* fragment.**

After ligation and transformation, plasmids were purified and linearized (with *NotI*) to check for the integration of the 5' *ERG6* fragment (546 bp).

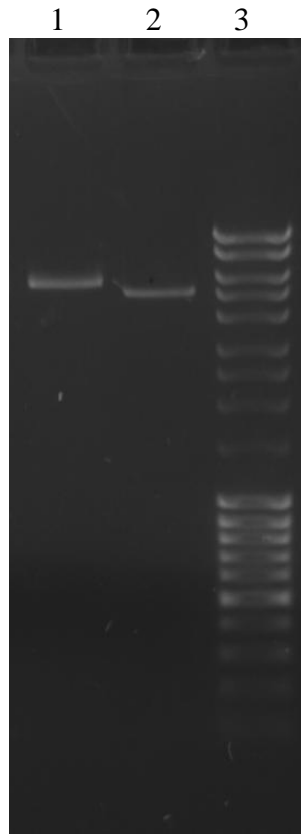


Figure 25: Agarose gel after correct integration of the 5' *ERG6* fragment

Table 43: Lane description of Figure 25

| Sample ID | Description                         | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|-------------------------------------|--------------------------|--------------------|
| 1         | pPpKanSynPDI + 5' <i>ERG6</i> + GAP | 5                        | 5641               |
| 2         | pPpKanSynPDI + GAP                  | 3                        | 5095               |
| 3         | MassRuler® DNA ladder mix           | 5                        | -                  |

The 5' *ERG6* fragment of 546 bp was successfully integrated into the pPpKanSynPDI - GAP construct, as shown in lane 1.

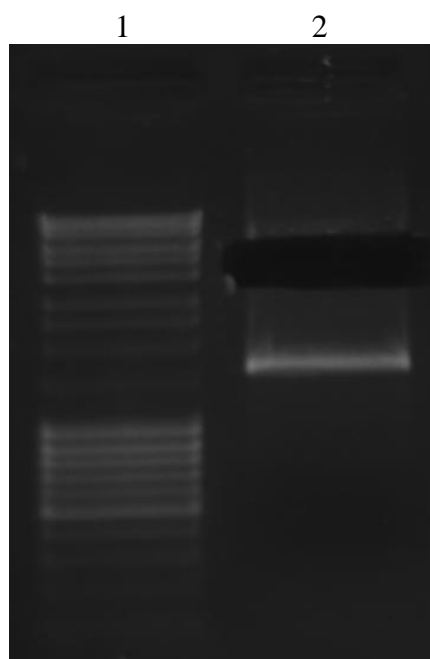
#### 4.1.14 Removal of the SynPDI fragment from the pPpKanSynPDI – 5' *ERG6* – GAP vector

The SynPDI gene was replaced by *DHCR24* using *EcoRI* and *NotI*.

**Table 44: Restriction cut to remove the SynPDI fragment**

| Reagent   | Amount (μL) |
|---|-------------|
| pPpKanSynPDI – 3' <i>ERG6</i> GAP vector (500 ng) | 20          |
| Buffer O (10x)                                    | 3           |
| <i>EcoRI</i>                                      | 3           |
| <i>NotI</i>                                       | 3           |
| H <sub>2</sub> O                                  | 1           |
| Total volume                                      | 30          |

The double digest was done O/N at 37°C and the vector backbone was purified from a prep gel.



**Figure 26: Preparative agarose gel showing the removal of the SynPDI fragment.**

**Table 45: Lane description of Figure 26**

| Sample ID | Description  | Volume (μL) | Visible bands (bp) |
|-----------|--|-------------|--------------------|
| 1         | MassRuler® DNA ladder mix                            | 5           | -                  |
| 2         | pPpKanSynPDI + 5' <i>ERG6</i> + GAP; SynPDI fragment | 3           | 4075, 1560         |

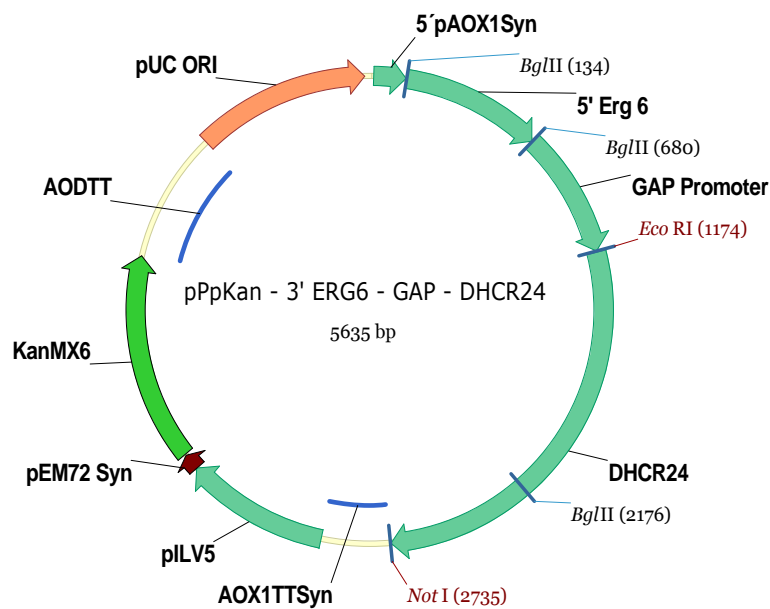
After removal of the SynPDI fragment, the *DHCR24* gene was cloned into the vector. The vector backbone was eluted on a preparative agarose gel and purified for ligation.

#### 4.1.15 Integration of the *DHCR24* gene into the pPpKan – 5' *ERG6* – GAP vector

**Table 46: Ligation of the *DHCR24* gene and the pPpKan – 5' *ERG6* – GAP vector**

| Reagent                                       | Amount (μL) |
|---|-------------|
| pPpKan – 5' <i>ERG6</i> – GAP vector (100 ng) | 5           |
| <i>DHCR24</i> gene (120 ng)                   | 2           |
| Ligase buffer                                 | 2           |
| T4 DNA ligase                                 | 1           |
| H <sub>2</sub> O                              | 10          |
| Total volume                                  | 20          |

Ligation was performed for 2 h at room temperature. After inactivation and desalting of the ligation reaction, the construct was transformed into *E. coli* TOP10F'.



**Figure 27: Vector NTI map showing the integration of the *DHCR24* gene into the pPpKan – 5' *ERG6* – GAP vector**

Isolated plasmids were cut with *Bgl*III, which should lead to three distinctive fragments in case of successful integration of the *DHCR24* gene. The *DHCR24* gene also bears a restriction site for *Bgl*III, which was responsible for the fragmentation.

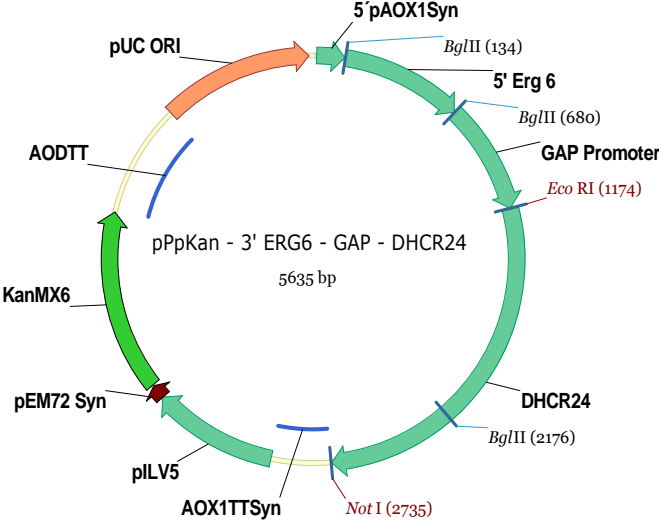
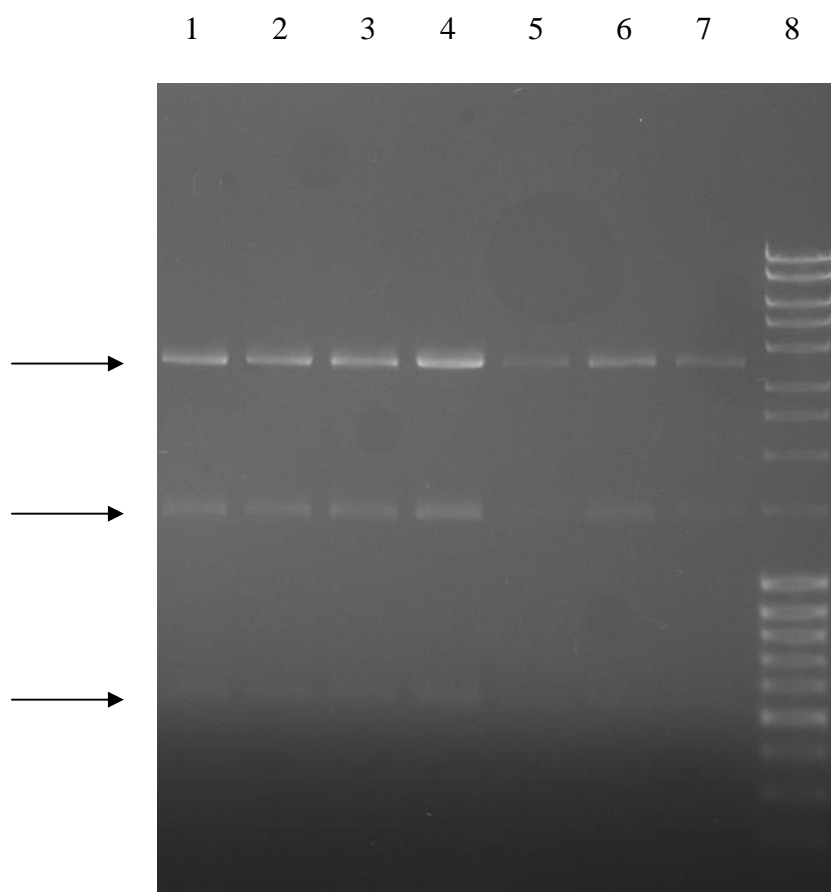


Figure 28: Vector NTI map of the final construct including the distinctive *Bgl*III restriction sites



**Figure 29:** Agarose gel after integration of the *DHCR24* gene, showing the fragmentation pattern

**Table 47: Lane description of Figure 29**

| Sample ID | Description               | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|---------------------------|--------------------------|--------------------|
| 1 - 7     | positive transformants    | 5                        | 3593, 1496, 546    |
| 1         | MassRuler® DNA ladder mix | 5                        | -                  |

Transformants were identified by the significant fragmentation pattern.

#### 4.1.16 The pJET1.2/blunt vector

In order to construct the whole *DHCR7* and *DHCR24* knockin cassettes, the pJet1.2/blunt vector turned out to be an excellent tool for the final cloning steps. The 3' *ERG5* and 3' *ERG6* fragments were integrated into the blunt-end PCR product cloning site. The MCS containing an *XhoI* restriction site served for the integration of the already constructed intermediate knockin cassettes.

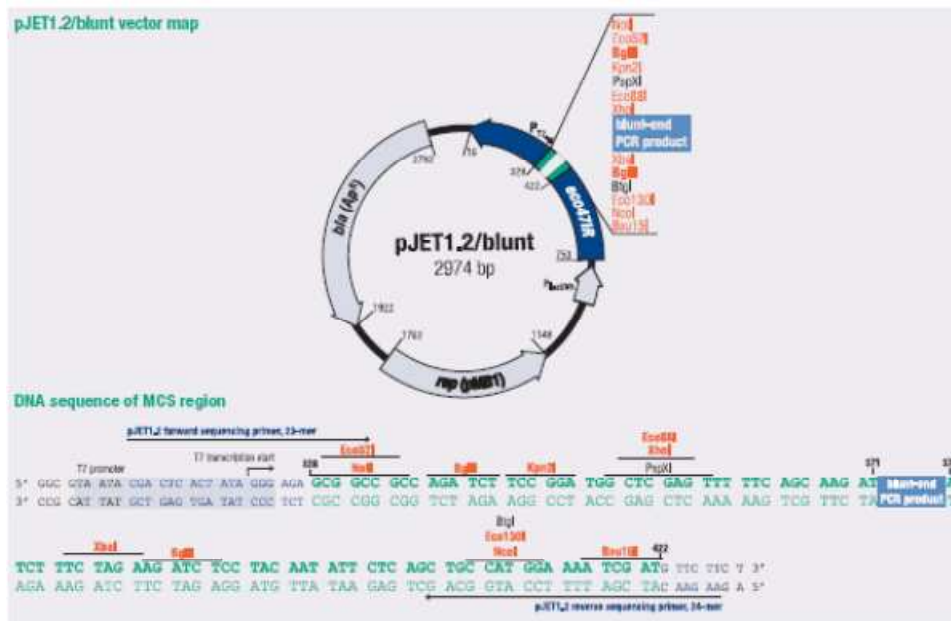


Figure 30: Overview of the pJET1.2/blunt vector from Fermentas



#### 4.1.17 Blunt end ligation of the 3' *ERG5* and 3' *ERG6* regions into the pJET1.2/blunt vector

Table 48: Reaction setup for blunt-end ligation of 3' *ERG5* and 3' *ERG6* regions in the pJET1.2/blunt vector system

| Reagent                         | Amount ( $\mu\text{L}$ ) |
|---------------------------------|--------------------------|
| Reaction buffer (2x)            | 10                       |
| pJet1.2 blunt                   | 0.5                      |
| 3' <i>ERG5</i> / 3' <i>ERG6</i> | 1                        |
| T4 DNA ligase                   | 1                        |
| H <sub>2</sub> O                | 7.5                      |
| Total volume                    | 20                       |

The reaction was incubated for 30 min at 22°C and the ligation was inactivated at 65°C for 10 min as described in the “CloneJET PCR Cloning Kit” by Fermentas.

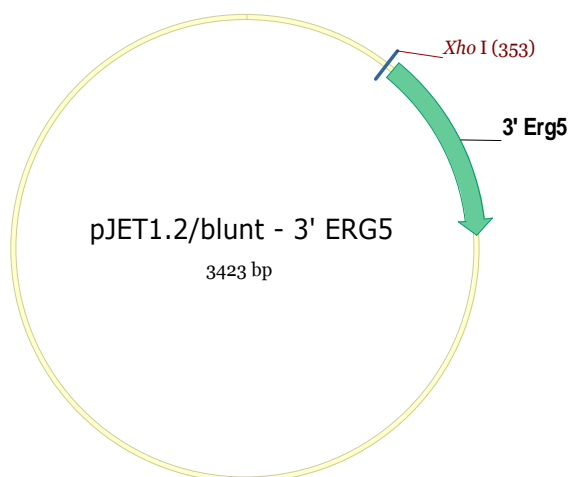


Figure 31: Vector NTI map of pJET1.2/blunt - 3' *ERG 5*

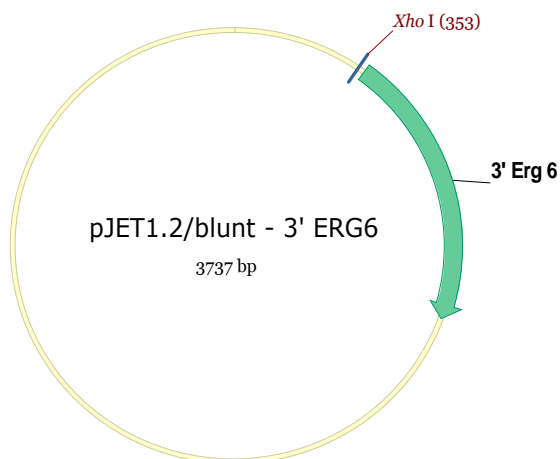
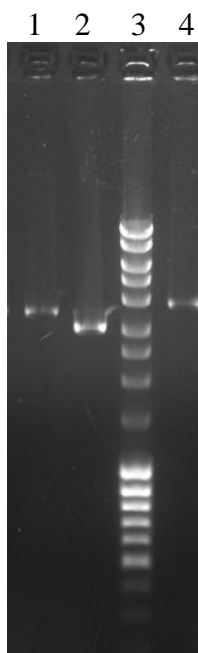


Figure 32: Vector NTI map of pJET1.2/blunt - 3' *ERG 6*

Isolated plasmids were cut with *NotI* in order to linearize the plasmid.



**Figure 33: Agarose gel after linearization of the isolated pJET1.2/blunt – 3' *ERG* 5 and 6 vectors**

**Table 49: Lane description of Figure 33**

| Sample ID | Description                    | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|--------------------------------|--------------------------|--------------------|
| 1         | pJET1.2/blunt + 3' <i>ERG5</i> | 3                        | 3423               |
| 2         | pJET1.2/blunt                  | 3                        | 2947               |
| 3         | MassRuler® DNA ladder mix      | 5                        | -                  |
| 4         | pJET1.2/blunt + 3' <i>ERG6</i> | 3                        | 3737               |

The orientation of the 3' *ERG5* and 3' *ERG6* fragments was confirmed by sequencing of the pJET1.2/blunt – 3' *ERG* 5 and 3' *ERG6*, respectively.

#### 4.1.18 PCR amplification of “5’ *ERG5* – GAP – *DHCR7* – ZeoR” and “5’ *ERG6* – GAP – *DHCR24* – KanMX” fragments

In order to incorporate an *XhoI* restriction site directly after the ZeoR/KanMX markers for cloning the fragments into the pJET1.2/blunt – 3’ *ERG5* and *ERG6* vectors, respectively.

**Table 50: PCR amplification of the “5’ *ERG5* – GAP – *DHCR7* – ZeoR” fragment**

| Reagent   | Amount (µL) |
|---|-------------|
| H <sub>2</sub> O  | 35          |
| Phusion buffer (10x)  | 5           |
| dNTP mix (10 mM)  | 5           |
| Primer Fw_GAPup   | 2           |
| Primer Rv_ZeoXhoIneu  | 2           |
| „pPpKan -5’ <i>ERG5</i> – GAP – <i>DHCR7</i> – ZeoR“ vector (20 ng) | 0.5         |
| Phusion polymerase  | 0.5         |
| Total volume  | 50          |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 66°C/30 s -72°C/1:45 min) x 30 - 72°C/10 min - 4°C/∞

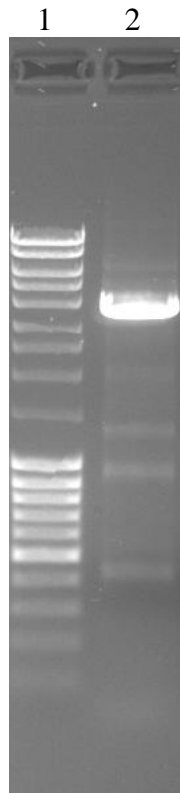
**Table 51: PCR amplification of the “5’ *ERG6* – GAP – *DHCR24* – KanMX” fragment**

| Reagent   | Amount (µL) |
|---|-------------|
| H <sub>2</sub> O  | 34.5        |
| Phusion buffer (10x)  | 5           |
| dNTP mix (10 mM)  | 5           |
| Primer Fw_AOX1down  | 2           |
| Primer Rv_RvKMXXhoIneu  | 2           |
| „pPpKan -5’ <i>ERG6</i> – GAP – <i>DHCR24</i> – KanMX“ vector (20 ng) | 1           |
| Phusion polymerase  | 0.5         |
| Total volume  | 50          |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 69°C/30 s -72°C/2 min) x 30 - 72°C/10 min - 4°C/∞

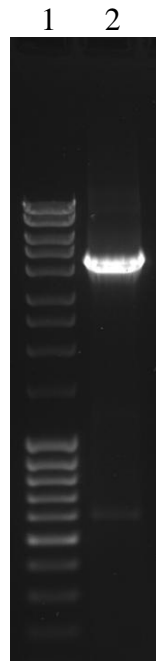
PCR fragments were gel purified.



**Figure 34: PCR amplification of “5’ *ERG 5* – GAP – *DHCR7* – ZeoR” fragment**

**Table 52: Lane description of Figure 34**

| <b>Sample ID</b> | <b>Description</b>                          | <b>Volume (μL)</b> | <b>Visible bands (bp)</b> |
|------------------|---|--------------------|---------------------------|
| 1                | MassRuler® DNA ladder mix                   | 5                  | -                         |
| 2                | amplified fragment with<br>unspecific bands | 5                  | 3764                      |



**Figure 35: PCR amplification of “5’ *ERG6* – GAP – *DHCR24* – KanMX” fragment**

**Table 53: Lane description of Figure 35**

| <b>Sample ID</b> | <b>Description</b>                          | <b>Volume (μL)</b> | <b>Visible bands (bp)</b> |
|------------------|---|--------------------|---------------------------|
| 1                | MassRuler® DNA ladder mix                   | 5                  | -                         |
| 2                | amplified fragment with<br>unspecific bands | 5                  | 4286                      |

#### 4.1.19 Excision of the “5’ *ERG5* – GAP – *DHCR7* – ZeoR” and “5’ *ERG6* – GAP – *DHCR24* – KanMX” fragments

The two fragments were acquired by the restriction cut with *XhoI*.

**Table 54: Restriction cut of the cleaned up “5’ *ERG5* – GAP – *DHCR7* – ZeoR” fragment**

| Reagent                      | Amount (μL) |
|------------------------------|-------------|
| Cleaned up PCR product (1μg) | 14          |
| Buffer R                     | 2           |
| <i>XhoI</i>                  | 3           |
| H <sub>2</sub> O             | 1           |
| Total volume                 | 20          |

**Table 55: Restriction cut of the cleaned up “5’ *ERG6* – GAP – *DCHR24* – KanMX” fragment**

| Reagent                      | Amount (μL) |
|------------------------------|-------------|
| Cleaned up PCR product (1μg) | 8           |
| Buffer R                     | 2           |
| <i>XhoI</i>                  | 3           |
| H <sub>2</sub> O             | 7           |
| Total volume                 | 20          |

Both reactions were incubated O/N at 37°C and cleaned up for ligation with the linearized pJET1.2/blunt – 3’ *ERG* 5 and 6 vectors.

#### 4.1.20 Dephosphorylation of the “5’ *ERG5* – GAP – *DHCR7* – ZeoR” and “5’ *ERG6* – GAP – *DHCR24* – KanMX” fragments

**Table 56: Desphosphorylation of the “5’ *ERG5* – GAP – *DHCR7* – ZeoR” and “5’ *ERG6* – GAP – *DHCR24* – KanMX” fragments**

| Reagent              | Amount (μL) |
|----------------------|-------------|
| PCR fragments        | 50          |
| Fast AP Buffer (10x) | 6           |
| Fast AP              | 1           |
| H <sub>2</sub> O     | 3           |
| Total volume         | 60          |

The dephosphorylation reaction was incubated for 10 min at 37°C which was repeated after adding an additional μL of FastAP™. Alkaline phosphatase was heat inactivated at 75°C for 5 min.

#### 4.1.21 Ligation to acquire the final *DHCR7* and *DHCR24* constructs

In order to yield the whole *DHCR 7* and *DHCR24* constructs, the intermediate fragments were ligated into the corresponding pJET1.2/blunt – 3' *ERG 5* and 3' *ERG6* vectors, respectively.

**Table 57: Ligation of the *DHCR7* construct**

| Reagent  | Amount ( $\mu\text{L}$ ) |
|--|--------------------------|
| Reaction buffer (10x)                                | 2                        |
| pJET1.2/blunt – 3' <i>ERG5</i> (136 ng)              | 4.5                      |
| “5' <i>ERG5</i> – GAP – <i>DHCR7</i> – ZeoR” (50 ng) | 1                        |
| T4 DNA ligase  | 2                        |
| H <sub>2</sub> O                                     | 10.5                     |
| Total volume   | 20                       |

**Table 58: Ligation of the *DHCR24* construct**

| Reagent  | Amount ( $\mu\text{L}$ ) |
|--|--------------------------|
| Reaction buffer (10x)                                  | 2                        |
| pJET1.2/blunt – 3' <i>ERG6</i> (130 ng)                | 4                        |
| “5' <i>ERG6</i> – GAP – <i>DHCR24</i> – KanMX” (50 ng) | 1.5                      |
| T4 DNA ligase  | 2                        |
| H <sub>2</sub> O                                       | 10.5                     |
| Total volume   | 20                       |

Ligation was accomplished for 2 h at RT, inactivation for 10 min at 65°C.

After ligation and transformation, positive transformants were screened and their plasmids isolated. Aliquots of the isolated plasmids were linearized. The linearized pJET1.2/blunt – *DHCR7* construct should have a size of 7182 bp.

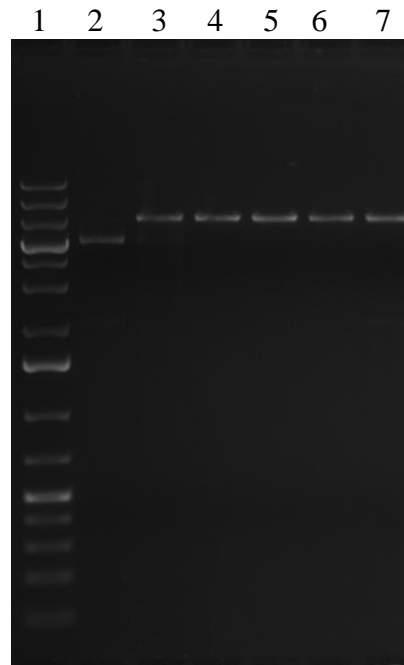


Figure 36: Agarose gel showing the linearized pJET1.2/blunt – *DHCR7* construct

Table 59: Lane description of Figure 36

| Sample ID | Description                              | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|--|--------------------------|--------------------|
| 1         | GeneRuler™ 1kb Plus DNA Ladder           | 5                        | -                  |
| 2         | uncut pJET1.2/blunt – <i>DHCR7c</i>      | 2                        | 6000               |
| 3-7       | linearized pJET1.2/blunt – <i>DHCR7c</i> | 2                        | 7182               |

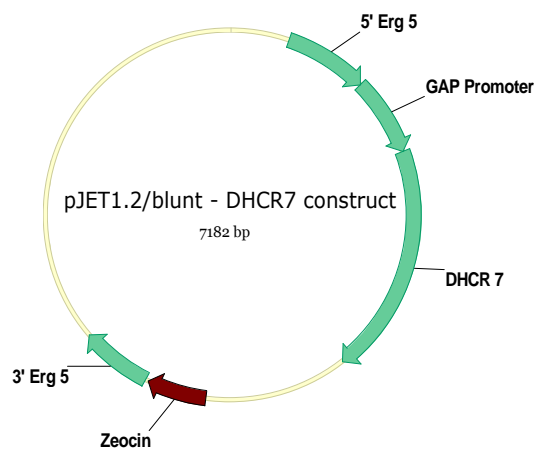


Figure 37: Overview of the pJET1.2/blunt – *DHCR7* construct



The pJET1.2/blunt – *DHCR24* construct should have a size of 8012 bp.

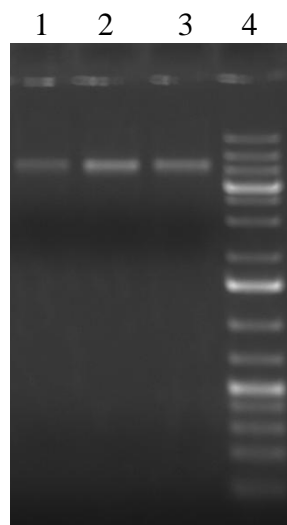
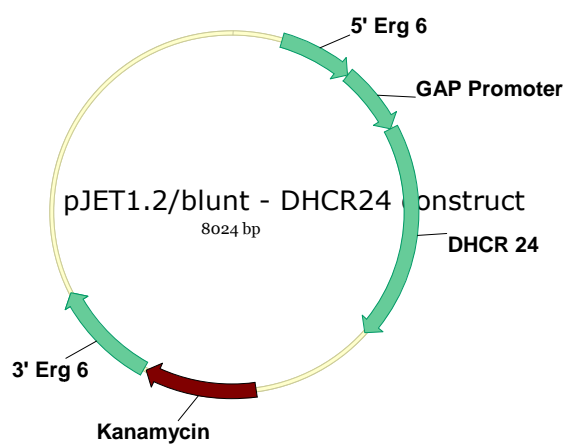


Figure 38: Agarose gel showing the linearized pJET1.2/blunt – *DHCR24* construct

Table 60: Lane description of Figure 39

| Sample ID | Description                               | Volume (μL) | Visible bands (bp) |
|-----------|---|-------------|--------------------|
| 1-3       | linearized pJET1.2/blunt – <i>DHCR24c</i> | 2           | 8012               |
| 4         | GeneRuler™ 1kb Plus DNA Ladder            | 5           | -                  |

Through the integration of the *DHCR7* and *DHCR24* intermediate constructs into the pJET1.2/blunt – 3' *ERG 5* and *ERG6* vectors, the knockin cassettes were completed and ready for amplification and transformation into *Pichia pastoris*.



ct

#### 4.1.22 PCR amplification of the *DHCR7* and *DHCR24* knockin cassettes

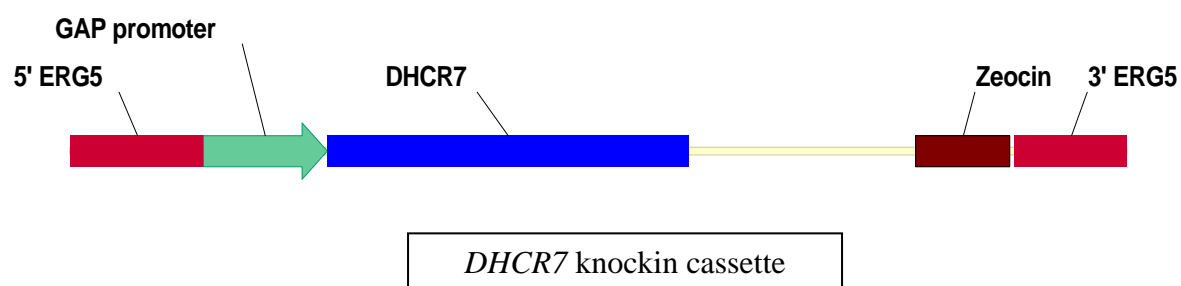
Both knockin cassettes had been finished with the integration of the “5’ *ERG5* – GAP – *DHCR7* – ZeoR” and “5’ *ERG6* – GAP – *DCHR24* – KanMX” fragments into the pJET1.2/blunt vector system, which already carried the 3’ *ERG5* and 3’ *ERG6* regions. The PCR amplification of the whole knockin cassette was performed for the transformation into *Pichia pastoris*.

**Table 61: Amplification of the *DHCR7* construct**

| Reagent                                | Amount (µL) |
|--|-------------|
| H <sub>2</sub> O                       | 29.5        |
| Phusion buffer (5x)                    | 10          |
| dNTP mix (10 mM)                       | 5           |
| Primer Fw_Erg5up                       | 2           |
| Primer Rv_Erg5down                     | 2           |
| Template pJET1.2 blunt – <i>DHCR7c</i> | 1           |
| Phusion polymerase                     | 0.5         |
| Total volume                           | 50          |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 64°C/50 s - 72°C/2:10 min) x 35 - 72°C/10 min - 4°C/∞



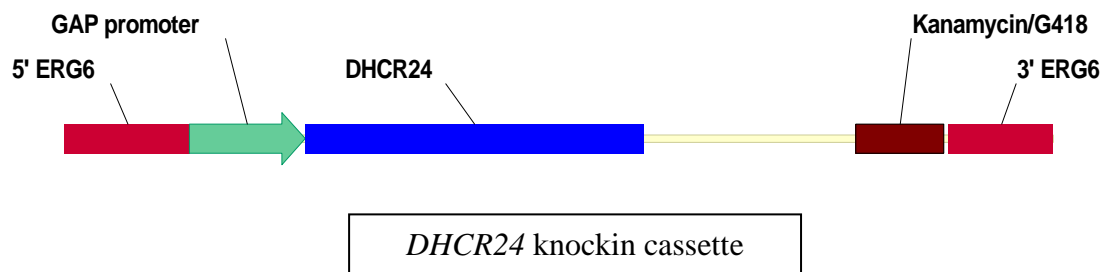
**Figure 40: Overview of the final *DHCR7* knockin cassette**

**Table 62: Amplification of the *DHCR24* construct**

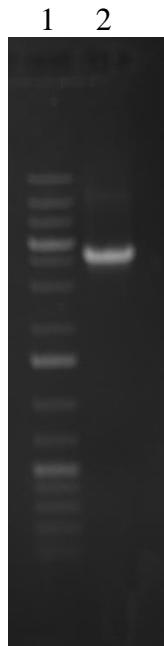
| Reagent                                 | Amount ( $\mu\text{L}$ ) |
|---|--------------------------|
| H <sub>2</sub> O                        | 28                       |
| Phusion buffer (5x)                     | 10                       |
| dNTP mix (10 mM)                        | 5                        |
| Primer Fw_Erg6up                        | 2                        |
| Primer Rv_Erg6down                      | 2                        |
| Template pJET1.2 blunt – <i>DHCR24c</i> | 2.5                      |
| Phusion polymerase                      | 0.5                      |
| Total volume                            | 50                       |

PCR cycling conditions:

98°C/30 s - (98°C/10 s - 59°C/30 s - 72°C/2:30 min) x 35 - 72°C/10 min - 4°C/ $\infty$



**Figure 41: Overview of the final *DHCR24* knockin cassette**



**Figure 42: Agarose gel after the amplification of the *DHCR7* knockin cassette**

**Table 63: Lane description of Figure 42**

| <b>Sample ID</b> | <b>Description</b>             | <b>Volume (<math>\mu</math>L)</b> | <b>Visible bands (bp)</b> |
|------------------|--------------------------------|-----------------------------------|---------------------------|
| 1                | GeneRuler™ 1kb Plus DNA ladder | 5                                 | -                         |
| 2                | <i>DHCR7</i> knockin cassette  | 5                                 | 4215                      |

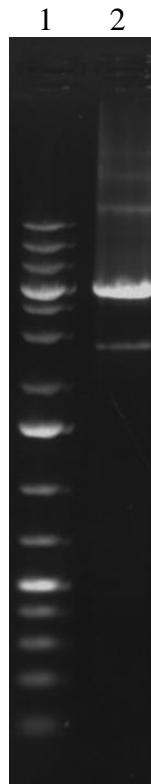


Figure 43: Agarose gel after the amplification of the *DHCR24* knockin cassette

Table 64: Lane description of Figure 43

| Sample ID | Description  | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|--|--------------------------|--------------------|
| 1         | GeneRuler™ 1kb Plus DNA ladder                       | 5                        | -                  |
| 2         | <i>DHCR24</i> knockin cassette with unspecific bands | 5                        | 5068               |

## 4.2 Colony PCR results

### 4.2.1 Colony PCR results to confirm the $\Delta erg5$ - *DHCR7* strain

Three different primer pairs were used to make sure that the knockin cassettes had integrated into the *ERG5* and *ERG6* loci, respectively.

**Table 65: Primer pairs to check on the correct integration of the *DHCR7* construct**

| PCR number | Primer pair  | Amplified fragments   |
|------------|--|---|
| 1          | Rv_DHCR7ctr<br>(actually forward primer)<br>Rv_ZeoXhoI | Amplification of a part of the integrated <i>DHCR7</i> knockin cassette (1322 bp size)  |
| 2          | Fw_Erg5Ric<br>Rv_Erg5Ric                               | Amplification of the <i>ERG5</i> gene, should give no product in the case of positive intergration of the knockout cassette (1561 bp size)                            |
| 3          | Fw_Erg5up2<br>Rv_DHCR7                                 | Amplification of a fragment upstream the <i>ERG5</i> locus to the 3' end of the <i>DHCR7</i> ; proves the correct integration in the <i>ERG5</i> locus (1249 bp size) |

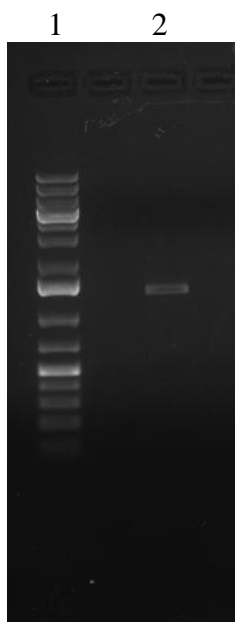
**Table 66: Colony PCR setup to check for the integrated *DHCR7* construct**

| Reagent                | Amount ( $\mu$ L) |
|------------------------|-------------------|
| H <sub>2</sub> O       | 28.5              |
| Phusion buffer HF (5x) | 10                |
| dNTP mix (2,5 mM each) | 2                 |
| Primer Fw              | 2                 |
| Primer Rv              | 2                 |
| Template; genomic DNA  | 5                 |
| Phusion polymerase     | 0.5               |
| Total volume           | 50                |

PCR cycling conditions:

1. 98°C/30 s - (98°C/10 s - 64°C/50 s - 72°C/40 s) x 30 - 72°C/10 min - 4°C/ $\infty$
2. 98°C/30 s - (98°C/10 s - 64°C/50 s - 72°C/45 s) x 30 - 72°C/10 min - 4°C/ $\infty$
3. 98°C/30 s - (98°C/10 s - 58°C/50 s - 72°C/40 s) x 30 - 72°C/10 min - 4°C/ $\infty$

The first colony PCR reaction was done with a primer pair, i.e. Rv\_DHCR7ctr, actually a forward primer and Rv\_ZeoXhoI2, amplifying a fragment ranging from the *DHCR7* gene to the end of the zeocin resistance cassette. In case of a positive transformant, the fragment should have a size of 1322 bp.



**Figure 44: Agarose Gel of colony PCR 1 product**

**Table 67: Lane description of Figure 44**

| Sample ID | Description                    | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|--------------------------------|--------------------------|--------------------|
| 1         | GeneRuler™ 1kb Plus DNA ladder | 5                        | -                  |
| 2         | product of PCR1                | 5                        | 1322               |

Out of the three possible positive transformants from PCR 1, two other colony PCRs were done.

The lanes 2 – 4 represent the colony PCR amplification of the *ERG5* gene in the *Pichia pastoris* genome, which should give no product. A distinctive band with the size of 1561 bp would mean that the *ERG5* gene was not knocked out. Lanes 5 – 7 represent the amplification of a fragment upstream the *ERG5* locus to the 3' end of the *DHCR7* – proving the correct integration in the *ERG5* locus (1249 bp size).

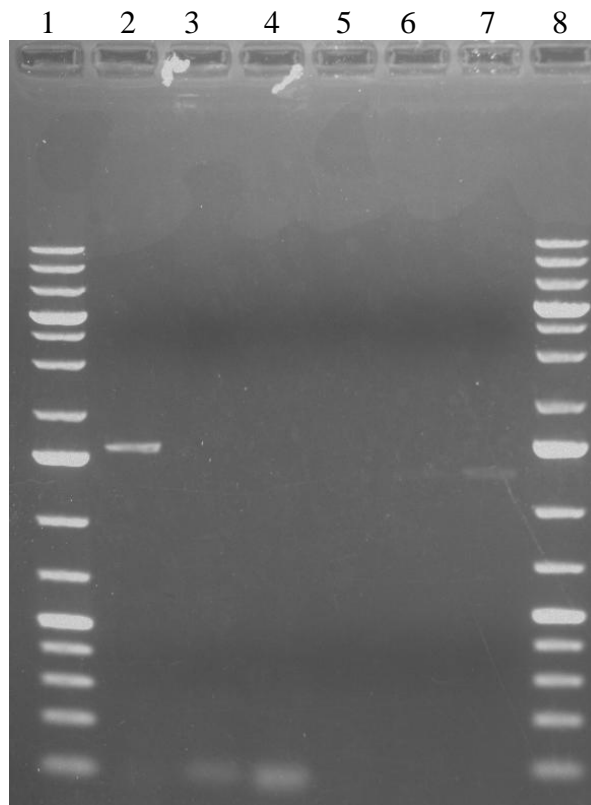


Figure 45: Agarose gel of colony PCR products 2 and 3

Table 68: Lane description of Figure 45

| Sample ID | Description  | Volume (μL) | Visible bands (bp) |
|-----------|--|-------------|--------------------|
| 1         | GeneRuler™ 1kb Plus DNA ladder   | 5           | -                  |
| 2         | product of PCR 2 – specific for the <i>ERG5</i> gene                         | 5           | 1561               |
| 3 -4      | no product for PCR 2   | 5           | -                  |
| 5         | no product for PCR 3   | 5           | -                  |
| 6 - 7     | product of PCR 3 - positive integration of the <i>DHCR7</i> knockin cassette | 5           | 1249               |
| 8         | GeneRuler™ 1kb Plus DNA ladder   | 5           | -                  |

Lane 7 indicates that  $\Delta erg5$  - *DHCR7* strain was successfully constructed. The *ERG5* gene had successfully been knocked out and *DHCR7* integrated into the genome.



## 4.2.2 Colony PCR results to confirm the $\Delta erg6$ - *DHCR24* strain

**Table 69: Primer pairs to check for the correct integration of the *DHCR24* construct**

| PCR number | Primer pair              | Amplified fragments  |
|------------|--------------------------|--|
| 1          | Fw_DHCR24<br>Rv_DHCR24   | Amplification of the integrated <i>DHCR24</i> gene (1568 bp size)  |
| 2          | Fw_Erg6Ric<br>Rv_Erg6Ric | Amplification of the <i>ERG6</i> gene, should give no product in the case of positive integration of the knockin cassette (1132 bp size)                               |
| 3          | Fw_Erg6up2<br>Rv_DHCR24  | Amplification of a fragment upstream the <i>ERG6</i> locus to the 3' end of the <i>DHCR24</i> ; proves the correct integration in the <i>ERG6</i> locus (1236 bp size) |

**Table 70: Colony PCR setup to check for the integrated *DHCR24* construct**

| Reagent                | Amount ( $\mu\text{L}$ ) |
|------------------------|--------------------------|
| H <sub>2</sub> O       | 28.5                     |
| Phusion buffer HF (5x) | 10                       |
| dNTP mix (2,5 mM each) | 2                        |
| Primer Fw              | 2                        |
| Primer Rv              | 2                        |
| Template; genomic DNA  | 5                        |
| Phusion polymerase     | 0.5                      |
| Total volume           | 50                       |

PCR cycling conditions:

1. 98°C/30 s - (98°C/10 s - 74°C/50 s - 72°C/45 s) x 30 - 72°C/10 min - 4°C/ $\infty$
2. 98°C/30 s - (98°C/10 s - 58°C/50 s - 72°C/30 s) x 30 - 72°C/10 min - 4°C/ $\infty$
3. 98°C/30 s - (98°C/10 s - 62°C/50 s - 72°C/35 s) x 30 - 72°C/10 min - 4°C/ $\infty$

The first colony PCR reaction amplified the integrated *DHCR24* gene of 1568 bp in size. The agarose gel shows that the *DHCR24* cassette had been integrated into the *Pichia* genome.

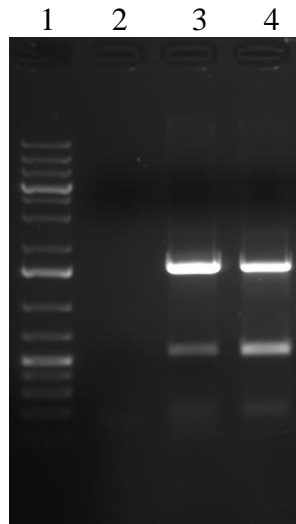


Figure 46: Agarose gel of colony PCR 1 to confirm the integration of the *DHCR24* knockin cassette

Table 71: Lane description of Figure 46

| Sample ID | Description                             | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|---|--------------------------|--------------------|
| 1         | GeneRuler™ 1kb Plus DNA ladder          | 5                        | -                  |
| 2         | no PCR product                          | 5                        | -                  |
| 3 -4      | product for PCR 1 with unspecific bands | 5                        | 1568               |

The second colony PCR reaction, which is specific for the *ERG6* gene, should yield no product. Lane number 4 shows a band with the size of 1132 bp, indicating that the *ERG6* gene had not been knocked out in this particular strain.

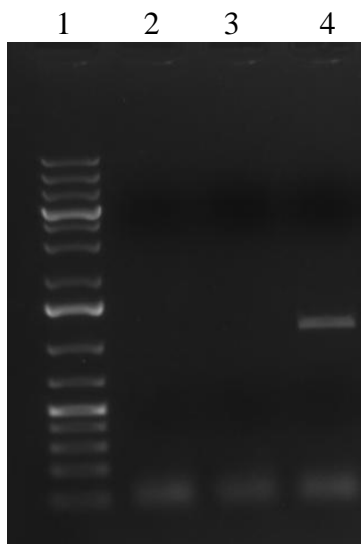


Figure 47: Agarose gel of colony PCR 2, specific for the *ERG6* gene

Table 72: Lane description of Figure 47

| Sample ID | Description                         | Volume ( $\mu$ L) | Visible bands (bp) |
|-----------|-------------------------------------|-------------------|--------------------|
| 1         | GeneRuler™ 1kb Plus DNA ladder      | 5                 | -                  |
| 2-3       | no PCR product                      | 5                 | -                  |
| 4         | PCR product of the <i>ERG6</i> gene | 5                 | 1132               |

To finally prove the correct integration of the *DHCR24* knockin cassette, a colony PCR was conducted to amplify a fragment ranging from upstream the *ERG6* gene in the *Pichia pastoris* genome to the *DHCR24* gene (1120 bp).

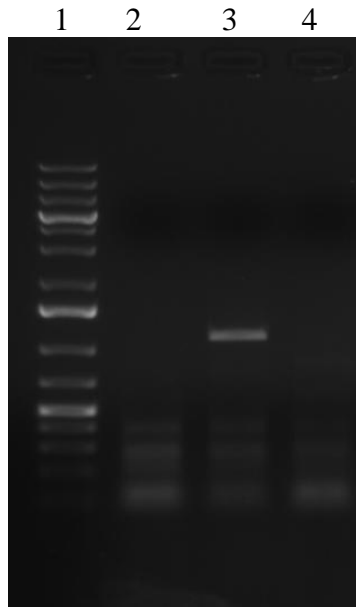


Figure 48: Agarose gel of colony PCR 3 to confirm the correct integration of the *DHCR24* knockin cassette

Table 73: Lane description of Figure 48

| Sample ID | Description                    | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|--------------------------------|--------------------------|--------------------|
| 1         | GeneRuler™ 1kb Plus DNA ladder | 5                        | -                  |
| 2 + 4     | no PCR product                 | 5                        | -                  |
| 3         | Colony PCR                     | 5                        | 1236               |

Lane 3 represents a positive transformant – the *DHCR24* knockin cassette had been successfully integrated into the *ERG6* locus.

### 4.2.3 Colony PCR to confirm the “cholesterol strain” ( $\Delta erg5 - DHCR7 \Delta erg6 - DHCR24$ )

Two different primer pairs were chosen to investigate the correct integration of the *DHCR7* and *DHCR24* knockin cassettes into the *ERG5* and *ERG6* loci, respectively.

**Table 74: Primer pairs to check for the correct integration of the *DHCR7/24* constructs**

| PCR number | Primer pair             | Amplified fragments   |
|------------|-------------------------|---|
| 1          | Fw_Erg5up2<br>Rv_DHCR7  | Amplification of a fragment upstream the <i>ERG5</i> loci to the 3' end of the <i>DHCR7</i> ; proves the correct integration in the <i>ERG5</i> locus (1249 bp size)  |
| 2          | Fw_Erg6up2<br>Rv_DHCR24 | Amplification of a fragment upstream the <i>ERG6</i> loci to the 3' end of the <i>DHCR24</i> ; proves the correct integration in the <i>ERG6</i> locus (1236 bp size) |

The PCR was done with the DreamTaq™ Polymerase by Fermentas.

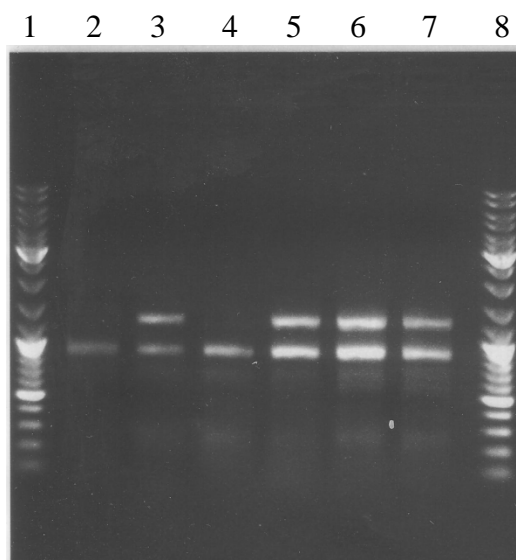
**Table 75: Colony PCR setup for identification of the “cholesterol” strain**

| Reagent                | Amount (μL) |
|------------------------|-------------|
| H <sub>2</sub> O       | 15.5        |
| DreamTaq™ buffer (10x) | 3           |
| dNTP mix (2.5 mM each) | 2           |
| Primer Fw              | 2           |
| Primer Rv              | 2           |
| Template; genomic DNA  | 5           |
| DreamTaq™ Polymerase   | 0.5         |
| Total volume           | 30          |

PCR cycling conditions

1. 95°C/1 min - (95°C/30 s - 55°C/50 s - 72°C/1:30 min) x 30 - 72°C/10 min - 4°C/∞
2. 95°C/1 min - (95°C/30 s - 55°C/50 s - 72°C/1:30 min) x 30 - 72°C/10 min - 4°C/∞

In colony PCR 1 a fragment of the size of 1249 bp was expected, if the integration of the *DHCR7* gene into the *ERG5* locus had worked out.

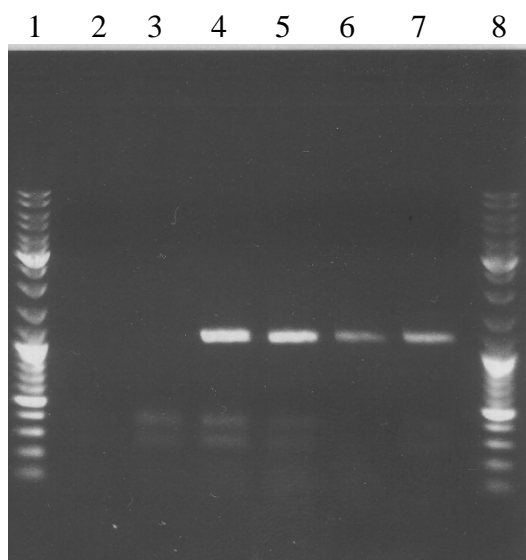


**Figure 49: Agarose gel to confirm the integration of the *DHCR7* construct in the *Pichia pastoris* genome**

**Table 76: Lane description of Figure 49**

| Sample ID | Description                                | Volume ( $\mu\text{L}$ ) | Visible bands (bp) |
|-----------|--|--------------------------|--------------------|
| 1         | GeneRuler™ DNA ladder                      | 5                        | -                  |
| 2 + 4     | no product of PCR 1, only unspecific bands | 5                        | -                  |
| 3,5,6,7   | PCR product with unspecific bands          | 5                        | 1249               |
| 8         | GeneRuler™ DNA ladder                      | 5                        | -                  |

Lanes 3, 5, 6, and 7 represent positive transformants regarding the correct integration of the *DHCR7* construct. To yield the cholesterol strain, also the *DHCR24* knockin cassette had to be inserted in the *ERG6* locus, which was confirmed in the following colony PCR.



**Figure 50:** Agarose gel to confirm the integration of the *DHCR24* construct in the *Pichia pastoris* genome

**Table 77:** Lane description of Figure 50

| Sample ID | Description                                | Volume ( $\mu$ L) | Visible bands (bp) |
|-----------|--|-------------------|--------------------|
| 1         | GeneRuler™ DNA ladder                      | 5                 | -                  |
| 2 + 3     | no product of PCR 2, only unspecific bands | 5                 | -                  |
| 4,5,6,7   | PCR product with unspecific bands          | 5                 | 1236               |
| 8         | GeneRuler™ DNA ladder                      | 5                 | -                  |

In lane 4, there is a clear PCR product, which confirms the integration of the *DHCR24* cassette in the *ERG6* locus. However, PCR 1 of this transformant had been negative, meaning that this mutant does not contain the *DHCR7* knockin cassette in the *ERG5* locus, but maybe somewhere else. Lanes 5 to 7 represent positive double mutants because they show both significant PCR products of the correct size.

Finally, the strain construction was accomplished.

### 4.3 GC/MS analysis

The analysis of the sterols present in the wild type and the different mutant strains were essential for confirming the integration of the knockin cassettes. The relative amounts of the sterol species were determined.

#### 4.3.1 GC/MS chromatogram of *Pichia pastoris* $\Delta ku70his4$ (wild type)

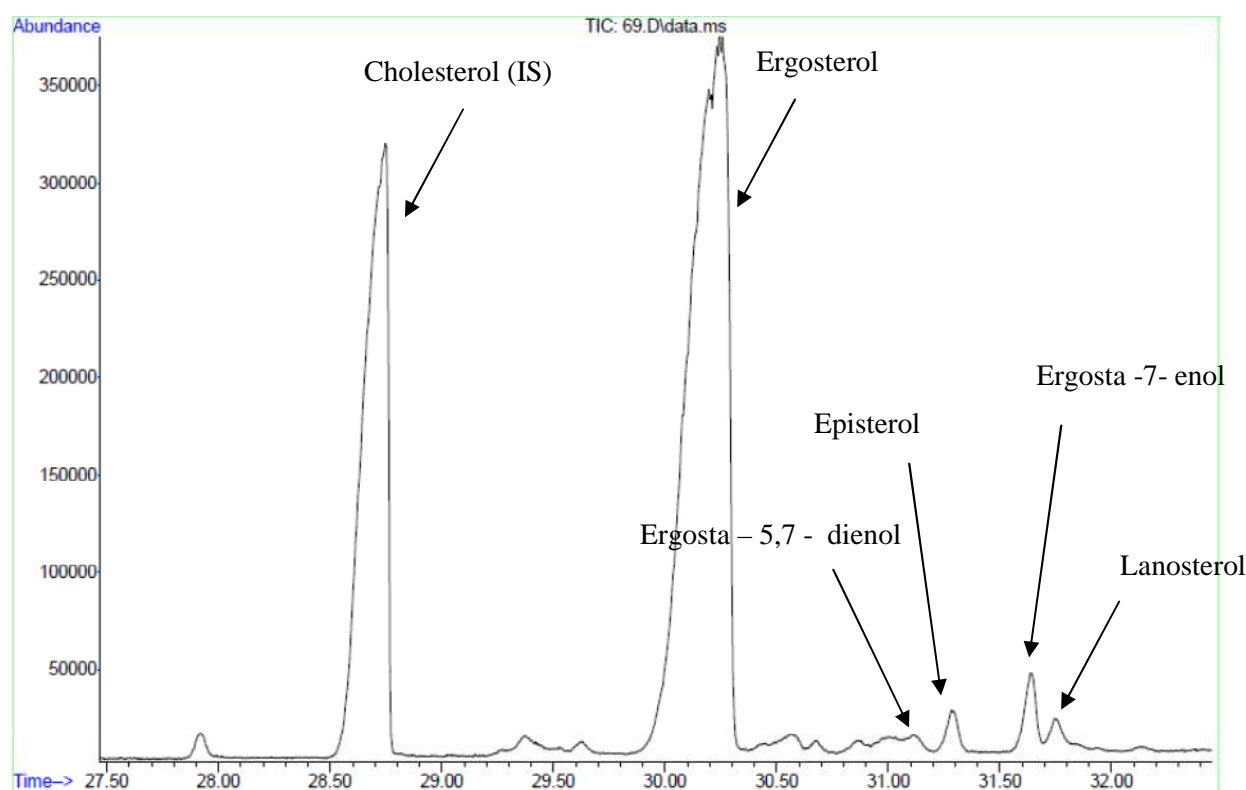


Figure 51: GC/MS chromatogram of the WT  $\Delta ku70his4$ . Cholesterol was added as an internal standard.

Table 78: Relative amounts of the main sterols in the GC/MS chromatogram of the WT strain

| Sterol                 | Relative amount (%) |
|------------------------|---------------------|
| Ergosterol             | 85                  |
| Ergosta – 5,7 - dienol | 0.6                 |
| Episterol              | 1.6                 |
| Ergosta – 7 - enol     | 2.9                 |
| Lanosterol             | 1.6                 |

As expected, the main sterol species in the *Picha pastoris*  $\Delta ku70his4$  strain (wild type) is ergosterol. Ergosterol, which is the end product of the ergosterol biosynthesis pathway, is followed by some of its precursors in the biosynthetic pathway, but none of them is nearly as



abundant as ergosterol. The GC/MS chromatogram shows a typical sterol pattern for a wild type strain.

#### 4.3.2 GC/MS chromatogram of the $\Delta erg5 - DHCR7$ strain

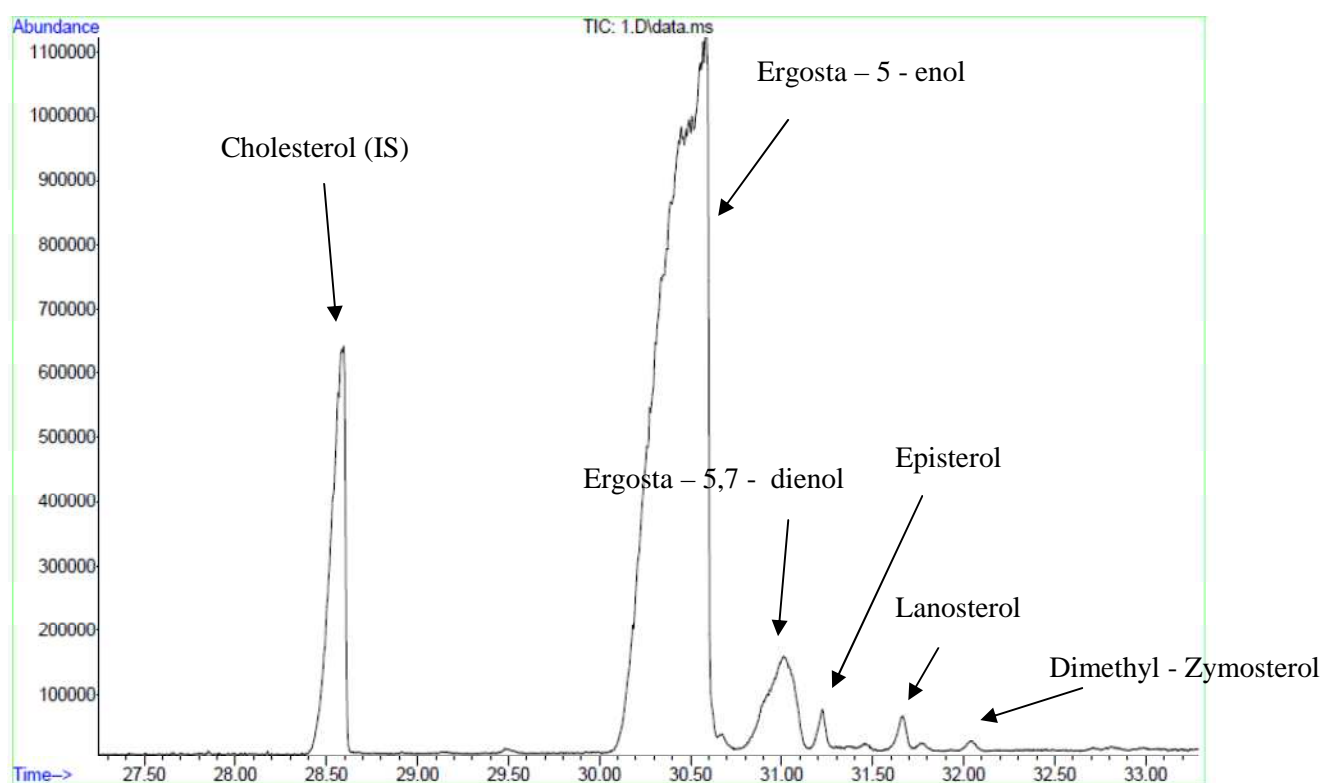


Figure 52: GC/MS chromatogram of the  $\Delta erg5 - DHCR7$  strain. Cholesterol was added as an internal standard.

Table 79: Relative amounts of the main sterols in the GC/MS chromatogram of the  $\Delta erg5 - DHCR7$  strain

| Sterol                 | Relative amount (%) |
|------------------------|---------------------|
| Ergosta – 5 – enol     | 90                  |
| Ergosta – 5,7 - dienol | 4.9                 |
| Episterol              | 0.9                 |
| Lanosterol             | 0.9                 |
| Dimethyl - Zymosterol  | 0.3                 |

In the case of the  $\Delta erg5 - DHCR7$  strain, ergosta–5–enol is the most abundant sterol species in the chromatogram. It shows perfectly, that the Erg5p, the C22 desaturase, has been knocked out and the Dhcr7p is functionally expressed under the control of the GAP promoter. However, a small amount of ergosta–5,7–dienol indicates, that the Dhcr7p could be more efficient.

### 4.3.3 GC/MS chromatogram of the $\Delta erg6$ - *DHCR24* strain

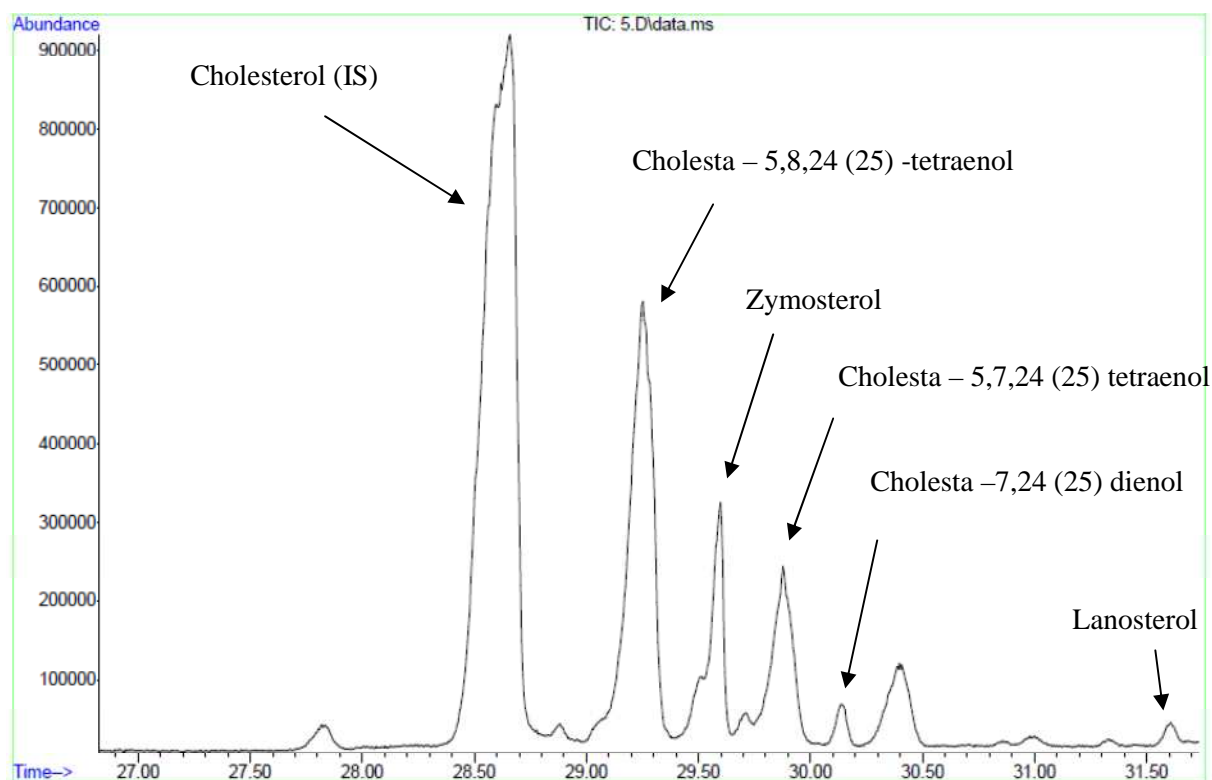


Figure 53: GC/MS chromatogram of the  $\Delta erg6$  - *DHCR24* strain. Cholesterol was added as an internal standard.

Table 80: Relative amounts of sterols found in the  $\Delta erg6$  - *DHCR24* strain

| Sterol                          | Relative amount (%) |
|---------------------------------|---------------------|
| Cholesta – 5,8,24(25) tetraenol | 46.6                |
| Zymosterol                      | 13.3                |
| Cholesta – 5,7,24(25) - trienol | 16.9                |
| Cholesta – 7,24 (25) - dienol   | 2.4                 |
| Lanosterol                      | 1.6                 |

The sterol spectrum of the  $\Delta erg6$  - *DHCR24* strain has shifted significantly compared to the wild type strain. Cholesta–5,8,24(25)–tetraenol is the main sterol species present in this mutant strain. The double bond at the C24 position of the sterol backbone is still present, which indicates, that the *Dhcr24p* is not active. Other sterols in this spectrum also show a double bond at the C24 position.

### 4.3.4 GC/MS chromatogram of the cholesterol producing strain

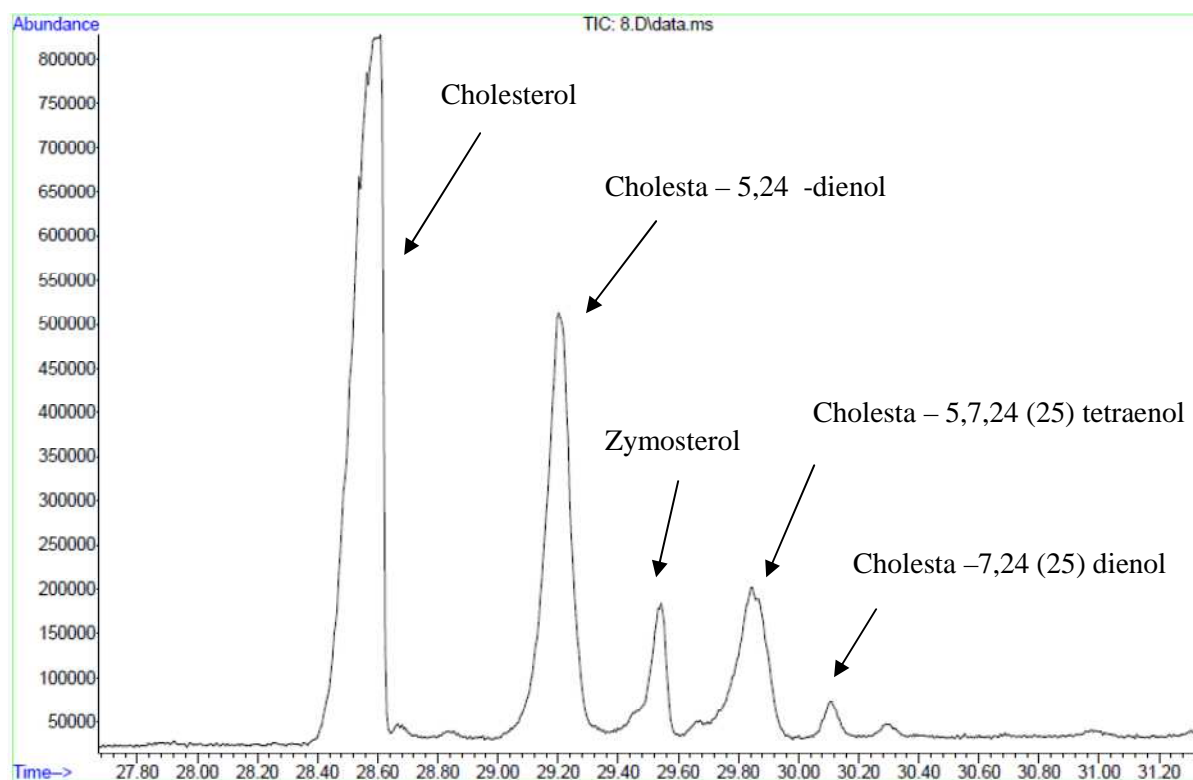


Figure 54: GC/MS chromatogram of the Cholesterol strain ( $\Delta erg5 - DHCR7 \Delta erg6 - DHCR24$ )

Table 81: Relative amounts of sterols found in the cholesterol strain ( $\Delta erg5 - DHCR7 \Delta erg6 - DHCR24$ )

| Sterol                          | Relative amount (%) |
|---------------------------------|---------------------|
| Cholesterol                     | 49.2                |
| Cholesta – 5,24 - dienol        | 25.4                |
| Zymosterol                      | 4.6                 |
| Cholesta – 5,7,24(25) - trienol | 11.7                |
| Cholesta – 7,24 (25) - dienol   | 1.6                 |

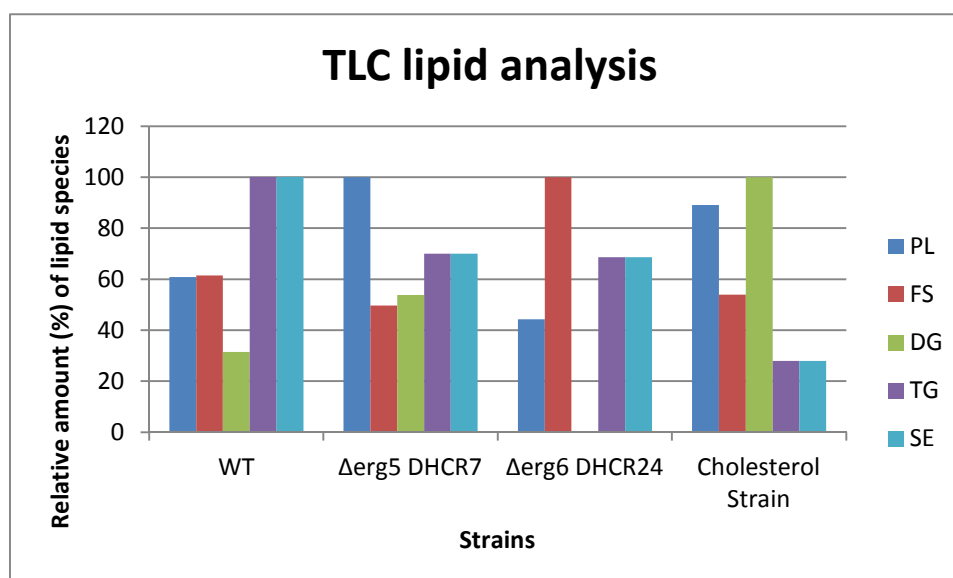
Almost 50 % of cholesterol is present in the  $\Delta erg5 - DHCR7 \Delta erg6 - DHCR24$  strain. The overall amount of cholesterol could be much higher, considering a greater activity of Dhcr24p. Finally, the sequential transformation experiments had worked out.

## 4.4 Lipid extraction and thin layer chromatography

After lipid extraction, the lipids were separated on a TLC plate and quantified using a TLC scanner. Sterols are either present as free sterols or steryl esters.

**Table 82: TLC analysis of lipid extracts of WT and strain variants showing steryl esters (SE), triglycerides (TG), diglycerides (DG), free sterols (FS) and phospholipids (PL) in percent**

| Lipids | WT    | $\Delta erg5$ <i>DHCR7</i> | $\Delta erg6$ <i>DHCR24</i> | Cholesterol strain |
|--------|-------|----------------------------|-----------------------------|--------------------|
| PL     | 14.13 | 23.22                      | 10.27                       | 20.69              |
| FS     | 31.07 | 25.10                      | 50.60                       | 27.29              |
| DG     | 4.09  | 6.98                       | -                           | 12.98              |
| TG     | 11.50 | 15.73                      | 7.69                        | 3.35               |
| ES     | 33.09 | 23.13                      | 22.68                       | 9.21               |



**Figure 55: TLC analysis of lipid extracts of WT and mutant strains showing steryl esters (SE), triglycerides (TG), diglycerides (DG), free sterols (FS) and phospholipids (PL)**

Figure 55 shows the significant change of the overall lipid composition of the created strain variants. The  $\Delta erg6$  - *DHCR24* contains an increased amount of free sterols, whereas the other strains comprise the same levels. The “cholesterol” strain shows very low levels of steryl esters compared to the wild type,  $\Delta erg5$  - *DHCR7* and  $\Delta erg6$  - *DHCR24* strains.

## 4.5 Fluorescein diacetate assay

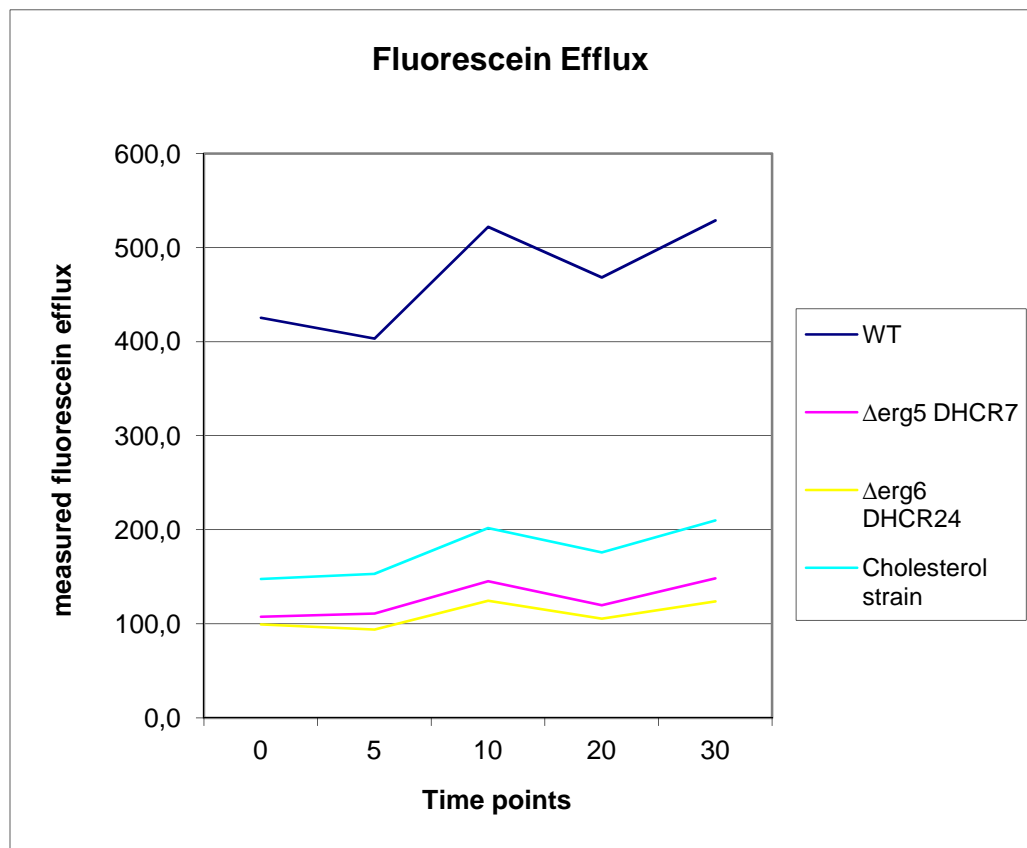
The FDA assay was performed as described in the methods section.

**Table 83: Strains used for the FDA assay**

| Number | Strain   |
|--------|--|
| 1      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 (WT)  |
| 2      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 $\Delta$ erg5-DHCR7   |
| 3      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 $\Delta$ erg6-DHCR24  |
| 4      | - <i>P. p.</i> CBS7435 $\Delta$ ku70his4 $\Delta$ erg5-DHCR7 $\Delta$ erg6-DHCR24 “Cholesterol strain” |
| 5      | - <i>S.c.</i> CEN.PK2 (generously provided by Birgit Ploier)   |

**Table 84: Measured fluorescein efflux after different time points**

| Timepoints | Fluorescein efflux |                     |                      |                    |
|------------|--------------------|---------------------|----------------------|--------------------|
|            | WT                 | $\Delta$ erg5 DHCR7 | $\Delta$ erg6 DHCR24 | Cholesterol strain |
| 0          | 425.4              | 107.5               | 99.2                 | 147.6              |
| 5          | 403.2              | 110.8               | 93.8                 | 152.9              |
| 10         | 522.1              | 145.3               | 124.4                | 201.9              |
| 20         | 468.4              | 119.7               | 105.4                | 175.7              |
| 30         | 528.8              | 148.4               | 123.8                | 210.0              |



**Figure 56: Overview of the fluorescein efflux in the wild type and the mutant strains**

Generally, the fluorescein efflux after the addition of 50 mM glucose was significantly lower in all the mutant strains than the efflux in the wild type strain. I observed, that most of the fluorescein had already been exported before starting the assay with the addition of glucose – especially in the case of the  $\Delta erg6 - DHCR24$  mutant and the cholesterol strain. Therefore, I conducted another experiment, to compare the efflux of fluorescein under glucose dependent and independent reaction situation.

#### 4.5.1 Glucose dependent/ independent efflux of fluorescein

The following figure gives an overview of the glucose dependent and independent efflux of fluorescein over the period of 2 h. The experiment also included the *Saccharomyces cerevisiae* CEN.PK2 wild type strain, generously provided by Birgit Ploier.

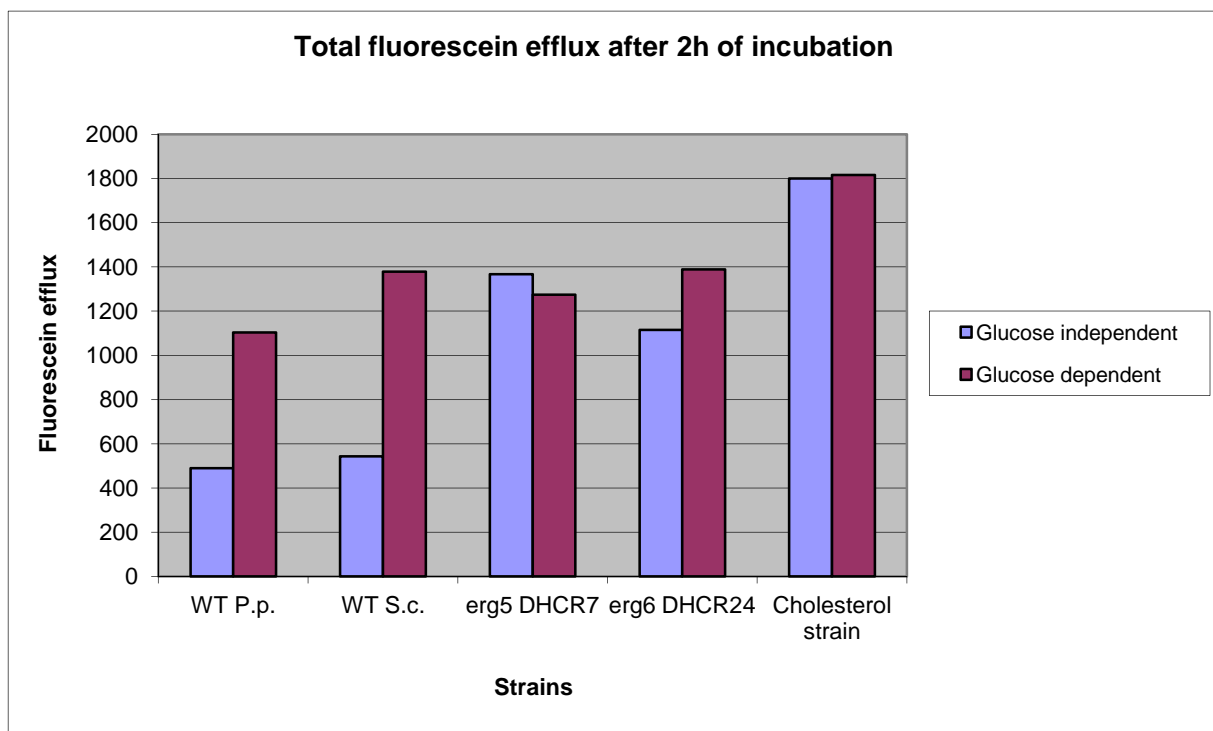


Figure 57: Overview of the total fluorescein efflux after 2 h of incubation

All of the constructed strains showed only a negligible difference between the induced and non - induced experimental setup. This turned out to be a very interesting discovery, as it suggests that Pdr12p of *P. pastoris* is strongly ergosterol dependent.

## 5 Discussion

### 5.1 Construction of the knockin cassettes

The construction of the knockin cassettes was a difficult task, because a variety of ligation experiments were involved and the screening of positive transformants was very time consuming.

The pGAPZ A vector was the basic starting point for the *DHCR7* knockin cassette and already carried the GAP promoter sequence and the zeocin resistance marker for selection. First, the *DHCR7* gene was inserted into the MCS and the 5' *ERG5* fragment was integrated upstream the GAP promoter. This construct was often denoted as the intermediate *DHCR7* cassette, simply because the 3' *ERG6* fragment was still missing.

The construction of the *DHCR24* knockin cassette started on the basic pPpKanSynPDI vector backbone. The vector already contained the kanamycin resistance marker, which refers to geneticin sulphate (G418) resistance in yeast. The methanol inducible *AOXI* promoter of the vector was replaced with the GAP promoter, which was obtained from the pGAPZ A vector system, for constitutive expression of the *Dhcr24p*. In the next step, the 5' *ERG6* fragment was integrated upstream the newly introduced GAP promoter. Finally, the *DHCR24* gene replaced the SynPDI sequence. This construct is described as the intermediate *DHCR24* cassette, the 3' *ERG6* fragment was also missing.

The final cloning steps were conducted with the pJET1.2/blunt vector system, which allowed the blunt-end integration of the 3' *ERG5* and 3' *ERG6* fragments followed by the introduction of both intermediate *DHCR7* and *DHCR24* cassettes afterwards. These cloning steps marked the final stages of the assembly of both knockin cassettes. The cassettes were amplified via PCR and transformed into the *Pichia pastoris* CBS7435  $\Delta ku70his4$  wild type strain.

## 5.2 Strain construction

The construction of the different mutant strains was achieved by homologous recombination of the constructed knockin cassettes into the *ERG5* and *ERG6* loci. Integration of the respective *DHCR7* and *DHCR24* cassettes yielded the single knockin strains  $\Delta erg5 - DHCR7$  and  $\Delta erg6 - DHCR24$ . The “cholesterol” strain,  $\Delta erg5 - DHCR7 \Delta erg6 - DHCR24$ , was derived from an already constructed  $\Delta erg5 - DHCR7$  strain variant, which was transformed with the *DHCR24* knockin cassette.

All of the strains I generated during this work, showed slow growth compared to the wild type strain. According to growth experiments, I conducted during my Master thesis, the slow growth is not associated with the inability of nutrient uptake. These results are not discussed in this work. The altered sterol composition apparently has an effect on the cell membrane properties in *Pichia pastoris* and is responsible of the slow growth.

## 5.3 Strain verification

Colony PCR experiments were performed in order to confirm the correct integration of the knockin cassettes in all constructed strains. In addition to that, GC/MS analysis was carried out for verification of the strains.

The main sterol of the wild type strain is, of course, ergosterol. All strain variants contain other main sterol species, which reflects the desired change of the native ergosterol biosynthesis pathway.

The main sterol of the  $\Delta erg5 - DHCR7$  strain is ergosta-5-enol with a total of 90 % of the overall sterol content, followed by ergosta-5,7-dienol with 4.9 %. All other sterol species are below the 1 % level. This shows perfectly that the *ERG5* gene was successfully knocked out, as the double bond at the C22 position was not present anymore. Removal of the double bond at C7 confirmed the activity of Dhcr7p.



In the case of the  $\Delta erg6 - DHCR24$  strain, I expected cholesta-5,7,22-trienol to be the most abundant sterol. Actually, the main sterol was cholesta-5,8,24(25)-tetraenol with 46.6 %, followed by cholesta-5,7,24(25)-trienol and zymosterol with 16.9 % and 13.3 %, respectively. The *ERG6* gene was knocked out, but Dhcr24p was not as active as I expected.

The *DHCR24* gene was codon optimized for the expression in *Saccharomyces cerevisiae*, hence the codon usage should be no limitation for proper expression of the protein in yeast. However, the activity of the expressed Dhcr24p may be elevated, through the integration of multiple copies into the genome of the *Pichia pastoris* CBS7435  $\Delta ku70his4$  wild type strain.

The cholesterol strain ( $\Delta erg5 - DHCR7 \Delta erg6 - DHCR24$ ) contains nearly 50 % of cholesterol, followed by cholesta-5,24-dienol with 25.4 %. The amount of cholesterol could have been greater if the Dhcr24p had been more active. In addition to that, the amount of cholesta-5,7,24-trienol (11.7 %) leads to the conclusion, that if the expression levels and the activity of both dehydrocholesterol reductases was optimized, the total percentage of cholesterol in this strain could be higher.

## **5.4 Strain characterization**

### **5.4.1 Thin layer chromatography**

Thin layer chromatography was performed to estimate the relative amount of lipid species in the constructed strain variants. Sterols are either present as free sterols and steryl esters. Both lipid species are discriminately present in the strains. It was confirmed that the altered sterol composition of the variants has a significant effect on the distribution of sterols between free sterols and steryl esters.

### **5.4.2 FDA analysis**

This assay was performed in order to characterize the Pdr12p function, but it turned out that the induction of the Pdr12p plus the energisation of the cells by glucose did not have the expected effect of a significant fluorescein efflux. As already shown in the results section, most of the fluorescein had left the cells before I induced the cells with the addition of glucose. The ideal situation would show almost the same levels of fluorescein in all the strains at time point zero, but all of the strain variants show much lower levels of fluorescein compared to the wild type at the start of the assay.

Therefore, the assay could not give any information of Pdr12p function in my mutant strains.

The glucose dependent/independent efflux measurements revealed that, there was only a negligible difference between the induced and non - induced experimental setup, which is interesting. Probably, there are other mechanisms involved, which lead to an improved uptake and export of fluorescein. It might also be the case that the cleavage of FDA is improved in the strain variants. On the other hand, it is possible that the cell membranes of the mutant strains are defective, due to the altered sterol composition and therefore fluorescein can diffuse freely without the need of an energy dependent mechanism.

## 6 Outlook

My Master thesis laid the foundation of creating a cholesterol producing *Pichia pastoris* strain. Future work on this project includes the increase of the overall cholesterol level, which has not reached its height yet. In addition to that, the cholesterol strain is an excellent organism for the investigation of protein - lipid interactions. The altered sterol composition enables us to study the activity of various membrane proteins under cholesterol surroundings. Therefore, membrane proteins have to be expressed in the cholesterol strain to make a clear statement on possible interactions with lipids.

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