Christoph Brandner, BSc.

Improvement of NADH-Regeneration in *Pichia pastoris*

MASTERARBEIT

zur Erlangung des akademischen Grades

Diplom-Ingenieur

Masterstudium Biotechnologie

eingereicht an der

Technischen Universität Graz

Betreuer

Ao.-Prof. Mag. Dr. Anton Glieder

Institut für Molekulare Biotechnologie

DI. Dr. Martina Geier / ACIB GmbH.

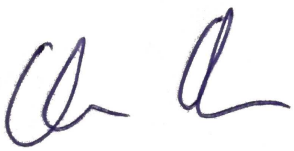
Graz, Mai, 2017

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Das in TUGRAZonline hochgeladene Textdokument ist mit der vorliegenden Masterarbeit identisch.

17.5.2017

Datum



Unterschrift

ABSTRACT

The methylotrophic yeast *Pichia pastoris* gathered much attention as whole-cell biocatalyst for the production of fine chemicals. Co-factor dependent bioreductions are often used for synthetic applications, whereby regeneration of the co-factor is a major cost factor. Whole-cells are a distinguished method for endogenous regeneration of co-factors, but the efficiency of the regeneration has to be further optimized. In order to improve the endogenous regeneration of NADH a new chassis strain based on *P. pastoris* CBS7435 was generated. Therefore, the yeast´s methanol utilization (MUT)-pathway was genetically engineered by deleting the two homologous genes *DAS1* and *DAS2* coding for the key enzyme (dihydroxyacetone synthase) of the assimilative pathway that is responsible for biomass production on methanol. As a consequence, the carbon flux was redirected into the dissimilative pathway, which increased the NADH-regeneration via methanol oxidation significantly. This was assessed in whole-cell conversions of acetoin catalyzed by the NADH-dependent 2,3-butanediol dehydrogenase (BDH1) from *S. cerevisiae*. Compared to the wild type strain producing BDH1, the amount of depleted substrate was up to 380% higher in the *Δdas1Δdas2* strain producing BDH1.

Further strain characterization was performed revealing that the knock-out strains did not show significant growth limitations on glycerol and glucose compared to the wild type strain. However, simultaneous deletion of both *DAS* homologs led to significant growth inhibition on methanol (μ = 0.01 h^{-1}) in comparison with the wild type strain (μ = 0.15 h^{-1}). Protein expression of the model protein eGFP driven by the two methanol inducible promoters (P_{AOX1} and P_{CAT1}) and the constitutive *GAP* promoter showed no limitations. In contrary, after induction with methanol *P. pastoris Δdas1Δdas2* showed significantly higher specific expression levels of eGFP under the control of P_{CAT1}.

Die methylotrophe Hefe *Pichia pastoris* erfuhr viel Bedeutung als Ganzzellen-Biokatalysator für die Produktion von hochwertigen Chemikalien. Co-Faktor abhängige Bioreduktionen werden oft für synthetische Anwendungen benutzt, wobei die Regeneration des Co-Faktors der wesentliche Kostenfaktor ist. Mikrobielle Ganzzellkatalysatoren sind eine ausgezeichnete Methode für die endogene Regeneration von Co-Faktoren, aber die Effizienz der Regeneration muss weiter optimiert werden. Um die endogene Regeneration von NADH zu verbessern wurde ein neuer Chassis-Hefe-Stamm, basierend auf *P. pastoris* CBS7435, hergestellt. Dafür wurde der Methanol-Verwertungs (MUT)-Stoffwechselweg gentechnisch verändert, indem die zwei homologen Gene *DAS1* und *DAS2*, die für das Schlüsselenzym (Dihydroxyaceton-Synthase) des assimilatorischen Stoffwechwechselweges, der für die Biomasse-Erzeugung auf Methanol verantwortlich ist, codieren, aus dem Genom gelöscht wurden. Infolgedessen wurde der Kohlenstoffffluss in den dissimilatorischen

Weg umgeleitet, was die NADH-Regeneration, durch die Oxidation von Methanol, erheblich verbesserte. Dies wurde mittels Ganzzellumsatz von Acetoin, katalysiert durch die NADH-abhängigen 2,3-Butandiol Dehydrogenase (BDH1) aus *S. cerevisiae*, evaluiert. Verglichen mit dem BDH1-produzierenden Wildtypstamm, war die Menge des verbrauchten Substrates bis zu 380% höher im BDH1-produzierenden *Δdas1Δdas2* Stamm.

Weiterführend wurde eine Stamm-charakterisierung durchgeführt, welche deutlich machte, dass die generierten Knockout-Stämme keine signifikante Wachstumslimitierung auf Glycerol und Glucose, verglichen mit dem Wildtyp-Stamm, zeigten. Allerdings führte die gleichzeitige Deletion beider Gene für die *DAS* homologen Proteine zu einer signifikanten Wachstumsreduktion auf Methanol (μ = 0,01 h^{-1}), verglichen mit dem Wildtypstamm (μ = 0,15 h^{-1}). Die Proteinexpression des Modell-Proteins eGFP unter der Kontrolle der zwei mit Methanol induzierbaren Promotoren (P_{AOX1} und P_{CAT1}) und des konstitutiven Promotors P_{GAP}, zeigten keine Limitierungen. Im Gegenteil, nach der Induktion mit Methanol, zeigte *P. pastoris Δdas1Δdas2* ein signifikant höheres spezifisches Expressionsniveau von eGFP unter der Kontrolle des P_{CAT1}.

TABLE OF CONTENTS

1. INTRODUCTION

1.1. History of biotransformations

In biocatalysis, contrary to classical catalysis (i.e. the use of inorganic substances to enhance reaction rates), enzymes and microorganism are applied for synthetic chemistry, whereby the properties of these natural catalysts are used for new objectives (1) (2).
Biotransformations in organic chemistry got their first attention in 1930 when Hilderbrandt et al. described the production of the precursor of L-ephedrine, (*R*)-phenylacetylcarbinol, via a condensation reaction of benzaldehyde and 2-oxo-propionic acid in the presence of *S. cerevisae* (3). In the early 30ies the activity of lipases present in the extract of porcine pancrease even in organic solvent was also already shown, but remained unnoticed in scientific society (4) (5). The wrong dogma that enzymes were only active in aqueous environments has held and prevented biocatalysis from a breakthrough for industrial application (6). Not before Zaks and Klibanov published papers in the 1980s considering enzymes were active in organic solvents, bioconversion was on the march. They deployed a 2-phase systems in their experiments to overcome limitations of dissolving hydrophobic substrates and products (7) (8). Further industrial applications were developed and biotransformation became a future field for organic synthesis and food industry, e.g. a method for producing a cacao butter substitute using lipases instead of classical catalysts as alkali metals, metal alkoxylates, or others (7) (8) (9) (10) (11).

1.2. Advantages of biocatalysts

There are several advantages using biocatalysts instead of classic catalysts. One of the most important one is chemical precision. This precision becomes most apparent in case of chemical selectivity which yields in an number of commercial benefits like better production of single stereoisomers, fewer side reactions, easier down-stream processing as separation of products gets simplified. Mild reaction conditions and lack of e.g. heavy metals as reaction catalysts should be pointed out as well, because these advantages lead to alleviation of pollution and also lead to lower production costs (12) (13).

1.2.1. Selectivity in bioconversion

As mentioned above selectivity is one of the major advantages of using enzymes as biocatalysts. Therefore electivity is elaborated in more detail in this section. Four discrete categories of chemical selectivity can be distinguished:

First the substrate selectivity, which is the ability of an enzyme to recognize, bind and transform concrete compounds, while it is relatively inert to a similar molecule. The two highly related tyrosine (eTATase) and aspartate (eAATase) aminotransferases from *E. coli* for instance, have similar rate constants for transamination of di-carboxylic amino acids, whereas eTATase has higher activity for the transamination of aromatic amino acids. Both enzymes show lower transamination activity with increasing side-chain length (from aspartate to glutamate to α-aminoadipate). The kinetic data supports one binding mode for all classes of substrates for eAATase and an additional mode in case of eTATase for increased affinity for hydrophobic amino acids (14).

Another important feature in biocatalysis is the ability to act on one location in a molecule called regioselectivity. As example, Jiong et al. described a novel synthetic route for synthesizing the corticosteroid rimexolone, which is used for the treatment of ocular inflammation without significant elevation of intraocular pressure. They used the cytochrome P-450 enzymes systems of *Curvularia lunata* AS 3.4381 for selective microbial transformation of 16α,17α-dimethyl-17β-(1-oxopropyl)androsta-1,4-dien3-one for the 11β-hydroxylation to rimexolone with a 50% biotransformation efficiency under optimized incubation conditions (15).

Production of fine chemicals also requires the separation of enantio-pure substances from racematic mixtures, which are most of the time the raw material for synthesis. This is often difficult to achieve with classical organic asymmetric synthesis. The stereo- and enatioselectivity of biotransformation overcomes in many cases this problem as e.g. production of 2-hydroxy acids. They are commonly used as further raw material for production of complex organic compounds. A method using 2 types of *Pseudomanas sp.* 2-halo acid dehalogenases (L-2-halo acid dehalogenase and DL-2-halo acid dehalogenase) was applied. These enzymes catalyse 2-halopropionic acid with inversion of configuration to L- and D-lactic acid and show the efficiency of enzymes with different enantioslectivity. They are employed in a gradually conversion of racematic 2-halopropionic acid, first L-2-halo acid dehalogenase converts L-2-halopropionic acid to D-lactonic acid and subsequently DL-2 halo acid dehalogenase transforms remaining D-2halopropionic acid to L-lactonic acid (16).

The last useful ability is the functional group selectivity of enzymes, allowing a chemical reaction on a desired functional chemical group within the molecule. In practice for instance *Rhodococcus rhodochrous* J1 shows a high benzonitrilase activity and is used for production of nicotinic acid with 3-cyanopyridine as base material. Nicotinic acid is also known as niacin or vitamin B3, an intermediate for pharmaceutical products and also important for feedstuff additives (17). Classically 3-cyanopyridine was oxidized with potassium di-chromate under high temperature to nicotinic acid or 3-cyanopyridine was hydrolysed with aqueous alkali hydroxide and obtained alkali nictotinate solution was crystallized with mineral acid at temperatures between 99°C and 135°C (18) (19).

1.3.Whole-cell conversion

Whole cells, cell free extract as well as isolated purified enzymes are now often used for different applications, whereby whole cells can be simpler and cheaper provided than isolated enzymes. The enzymes are protected within the cells e.g. from shear forces and can work in a habitant without external environmental influences and are hence more stable in long-term applications than free enzymes (20) (12). Another negative effect of removing enzymes from their natural environment, the microbial cell, may be the loss of activity or inactivation due to pH alteration, higher temperature, etc. Additionally whole cells (resting or growing) can be economically more feasible, if enzymes within the cell have no influence on purity of the product and a proper downstream processing is possible, i.e. enzyme isolation and purification can be expensive procedures and lead to significant costs in the production process. (21) (22).

If membrane-bound enzymes are employed in biotransformation whole-cells can be an interesting option. Membrane-bound proteins can lose all of their activity, when detached from the membrane, as in case of alkane hydroxylase, an integral membrane protein requiring phospholipids for its functionality (23). To set another example, the prominent enzyme class of cytochrome P-450 enzymes (CYP) should be mentioned. This enzyme class requires specific membrane conditions as well as a NADH-cytochrome P-450 reductase for catalytic activity. In case of the 3A gene family of cytochrome P-450, two reconstituted systems were tested. System I consisted of dilaurolylphosphatidylcholine (DLPC) in which low CYP activity was observable. System II was a mixture of phospholipids (DLPC, dioleoylphosphatidiylcholine and phospatidyl serine) and sodium cholate which yielded in high activities. Due to the high amount of lysine residues within the cytochrome P-450 3A enzymes, they are cationic and need the anionic phospholipid phosphatidyl serine for sufficient activity. In Sytem II cytochrome P-450 and the reductase also showed more interaction, which is necessary for reduction of the cytochrome P-450 (24).

Another application field for whole-cells is the implementation of multi-step reaction cascades, where two or more reactions are needed to obtain the final product. It is much easier to apply these steps within a single microbe than using a set of different isolated enzymes, substrates, cofactors and additional purification steps to synthesize the product, e.g. production of penicillin in a high yielding *Penicillium chrysogenium* strain (25).

Last and most important for the present study is the supply of cofactors (ATP, NAD(P)H or others) in whole-cell conversions by exploiting the cell metabolism. The NADH regeneration system is essential for reduction of ketones to chiral alcohols via oxidases and dehydrogenases. Different approaches can here be differentiated as well: cofactor regeneration with distinctive systems exploiting the already existing metabolic pathways of the cells, e.g. glycolysis or methanol utilization pathway in *P. pastoris* or engineered systems (engineering of pathways towards higher NAD(P)H regeneration

rate), enzyme coupled systems and substrate coupled regeneration (26) (27) (28) (29). An example for co-factor depending reactions or multi-step reactions is the NADH dependent butanediol dehydrogenase for reduction of acetoin to 2,3-butanediol, which has a high turnover-rate and is therefore one of the best candidates to test new generated strains for improved NADH-regeneration (27).

1.4. NAD(P)H-regeneration methods

The application of oxidation of C-H bonds and asymmetric reduction of carbonyl groups to alcohols and amines is significant for synthetic chemistry industry (30) (31) (32). Many oxidoreductases, oxidases, peroxygenases, dioxygenases, etc. require pyridine cofactors ($NAD(P)^+$ and NAD(P)H) for catalysing their reactions. Due to high costs of pyridine nucleotide cofactors, stoichiometric use of cofactors is economically not reasonable. On that account effective regeneration of cofactors needs to be taken in consideration for planning oxidation and reduction reactions. Different methods have been developed to allow the use of pyridine cofactors in catalytic quantities. Thereto two definitions pertaining the turnover numbers of cofactors are important. The “turnover number” (TN) describes the number of moles of product formed per mole of cofactor per minute and the “total turnover number” (TTN), which refers to the total number of moles of product formed per mole of cofactor for the whole reaction time. Both point out the costs whereby TN describes the cost per unit rate of product formation and TTN the total cost per mole product formed. A rule of thumb for economically sufficient process is to achieve TTNs of 10^3 to 10^5, depending on the value of the product. TTN also includes several factors of importance for the reaction as loss of cofactor through degradation or incorrect regiochemistry of regeneration (33).
Many different systems for regeneration of NAD(P)H are available in cell-free systems as well as in microbes: enzyme systems (coupled substrate, coupled enzyme regeneration, soluble pyridine nucleotide transhydrogenase (STH)), whole-cell regeneration machinery, chemical, photochemical or electrochemical regeneration (34) (35) (36).

1.4.1. Coupled-substrate-system

The coupled-substrate system can be applied for *in vitro* systems using purified enzymes as well as for whole-cell biotransformations. In both systems a co-substrate is added and either oxidized or reduced by the enzyme of interest to regenerate the oxidized or reduced form of $NAD(P)^+$/NAD(P)H. *Lactobacillus kefir* DSMZ 20587strain was used as biocatalyst for the asymmetric NADPH-dependent reduction of the prochiral alcohol ethyl 4-chloro acetoacetate to ethyl (*S*)-4-chloro-3-

hydroxybutanoate ((*S*)-CHBE) with the co-factor regeneration accomplished by oxidizing 2-propanol to acetone (Figure 1).

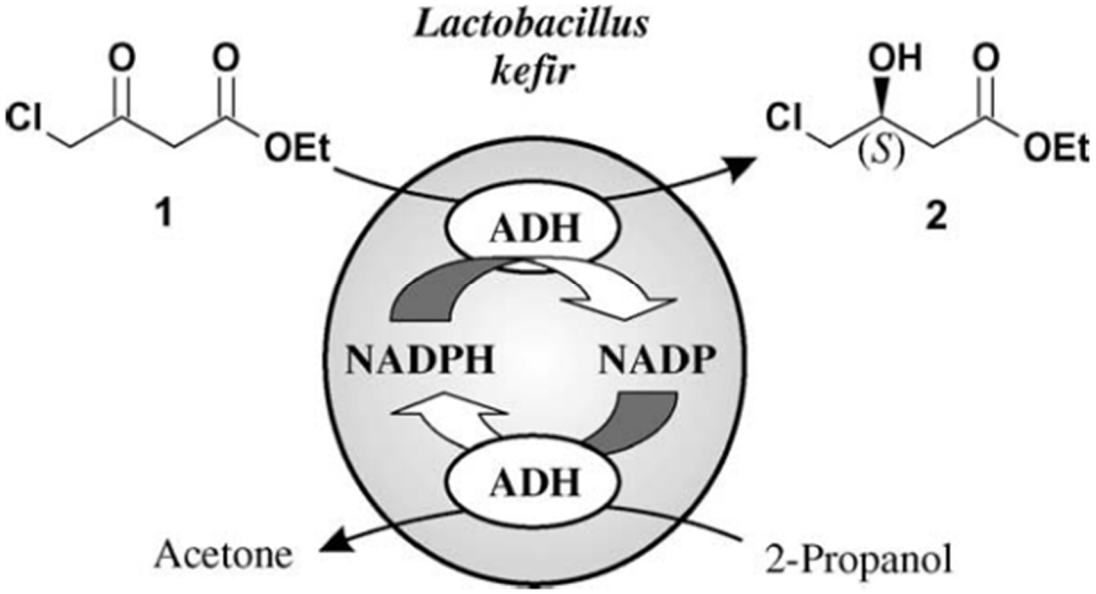


Figure 1 "Scheme of asymmetric reduction of ethyl 4-chloro acetoacetate **1** to ethyl (s)-4-chloro-3hydroxybutanoate **2** by *Lactobacillus kefir* with regeneration of NADPH using 2-propanol as co-substrate" **(29)**.

A yield of 100% was obtained when 2-propanol was added during the reaction, while a yield of 22% was observed with glucose as co-substrate after a 5h reaction time. The specific production of (*S*)-CHBE was 5.7 mmol per 1 g of dry cell weight (DCW) when 2-propanol was added and only 1.3 mmol/g_{DCW} with glucose as co-substrate. Additionally, the enantiomeric excess (ee) was increased from 87% with glucose to >99% with 2-propanol as co-substrate (29).

Operating with alcohol dehydrogenases (ADH) in cell free systems, the situation is the same as in whole cell catalysis: Cheap auxiliary substrates as 2-propanol or ethanol are added to regenerate NAD(P)H. E.g. Schubert et al. tested two commercial available and one heterologous expressed ADHs for NADH-dependent reduction of α-halogenated progargylic ketones to the respective progargylic alcohol: horse liver alcohol dehydrogenase (HLADH), *Thermoanaerobium brockii* ADH (TBADH) and *Lactobacillus brevis* ADH recombinantly produced in *E. coli* (recLBADH).

They used 2-propanol, ethanol or an ethanol/H_2O mixture as auxiliary substrate to regenerate NADH. Halogenated propargylic ketone shows low solubility in aqueous phase and therefore the reaction was performed in a solution with an amount of 25% short chain alcohol. recLBDH did not show much of a loss of activity after a reaction time of 24-36 h. >99% conversion of the halogenated progargylic ketone to the (*S*)-alcohol with an ee>99% and a TTN of the co-substrate of 20,000 was achieved (37).

1.4.1.1. Cross-linked enzyme crystals (CLECs) for enhancing stability of the enzyme and co-factor

To increase activity and stability of enzyme and co-factor towards organic solvents, crystallization and cross-linking became besides other methods (e.g. directed evolution or immobilization (38) (39)) a promising option. St. Clair et al. crystallized horse liver alcohol dehydrogenase (HLDH) in presence of reduced NADH, and further treated the crystals with glutaraldehyde to gain cross-linked enzyme crystals (CLECs). HLDH-NADH-DMSO-CLECs, HLDH-NADH-CLECs and HLDH-CLECs were compared to the free soluble HLDH with NADH added in catalytic amounts and no NADH added in the reduction of 6-methyl-5-heptene-2-one. The HLDH-NADH-DMSO-CLECs without NADH added had the highest activity of all tested CLECs. HLDH-NADH-DMSO-CLEC achieved 64% of the activity of the free enzyme approach where NADH was added. The inhibitor DMSO seemed necessary for correct folded HLDH, because the HLDH-NADH-CLECs showed only 18% of activity in the same approach. Moreover, the cross-linking increased the stability of HLDH towards the organic solvent isopropanol at elevated temperatures. Concerning the turnover number of co-factor regeneration CLECs yielded TTN greater than 12,000 with 1,4-butanediol as auxiliary substrate for co-factor regeneration (40).

1.4.2. Enzyme-coupled system

Another common method for regeneration of NAD(P)H is the use of an enzyme-coupled system for "*in vitro*" as well as for whole cell applications. Figure 2 shows the scheme of such a system. Two different enzymes are used. Enzyme 1 (E1) oxidizes substrate 1 and regenerates the NAD(P)H and E2 reduces substrate 2 and consumes NAD(P)H (41).

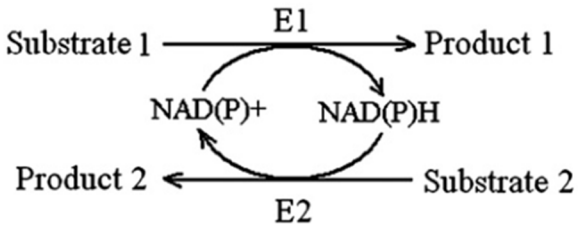


Figure 2 Scheme of enzymatic regeneration of NAD(P)H. E1 and E2 represent two different enzymes, whereby E1 is reducing $NAD(P)^+$ to NADH and E2 is using NAD(P)H as reduction equivalent **(41)**.

Wichmann et al. used L-leucine dehydrogenase (LEUDH) for the reductive amination of α-ketoisocaproate to L-leucine with formate dehydrogenase as co-enzyme to regenerate membrane-bound NADH in a continuous membrane reactor in bench scale. Ammonium formate is irreversibly converted to CO_2 and NH_3, whereby CO_2 can easily be separated. Through the separation, the desired regeneration reaction has a strong driving force (42). Beside the described LEUDH/NADH/FDH

system, lactate dehydrogenase (LDH)/NADH/FDH, alcohol dehydrogenase (ADH)/NADH/FDH, ADH/NADH/glucose dehydrogenase (GDH) and ADH/NADPH/GDH systems are applied in industrial applications (1).

Using whole cells for co-factor regeneration can also be very beneficial/advantageous, because the distinct metabolism of the cell often has enough capacity to regenerate the co-factor for different reactions (43). However, in some cases boosting the NADH-recycling rate is necessary. For example, Yamamoto et al. applied two different enzyme-coupled systems (GDH and FDH) in *E. coli* to regenerate NADH for a novel NADH-dependent carbonyl reductase (KaCR1) from *Kluyveromyces aestuarii*. They used these two systems for the synthesis of (*S*)-4-chloro-3-hydroxybutanoate (ECHB) by reducing ethyl 4-chloroacetate (ECAA) with KaCR1. Comparison of both enzyme-coupled systems in *E. coli* showed that the GDH-system produced 45.6 g/L (*S*)-ECHB from 50 g/L ECAA and the FDH-system produced 19.0 g/L, respectively. This study shows how important the choice of the NADH-regeneration system can be. Yamamoto et al. claimed the lower productivity of FDH was caused by low activity and instability at the used conditions (44).

In multi-step-reactions where both NADH- and NADPH-dependent enzymes are used, the regeneration could be limiting and, in whole-cell systems, the distinctive regeneration system could have, as in the above described single-step reaction, too low capacity. To overcome this problem NADH- and NADPH-regeneration systems e.g. enzyme-coupled systems as FDH and GDH are either added or overexpressed. Consequently, two co-substrates have to be applied, in *in vitro* as well as *in vivo* systems.

Enzyme engineering towards acceptance of both co-factors NADH and NADPH is a solution to handle the problem of co-factor regeneration in multi-step reactions, as Riebel et al. demonstrated with the NADPH/NADH oxidase from *Lactobacillus sanfranciscensis*, but in many cases this engineering is either not possible or too elaborate (45).

The soluble pyridine nucleotide transhydrogenase (STH) can simplify this system. It has the ability to catalyse the transfer of reducing equivalents according to the equation: $NAD^+ + NADPH \leftrightarrow NADH + NADP^+$. Boonstra et al. applied STH from *Pseudomonas fluorescence* in a cell-free and in an *E. coli*-based-system for the regeneration of NADH and NADPH for the production of the semisynthetic opiate drug hydromorphone. Conventional hydromorphone production was a whole-cell process with constitutively expressed $NADP^+$-dependent morphine dehydrogenase and a NADH-dependent morphinone reductase. Problems as co-factor depletion, unwanted by-product formation (i.e. dihydromorphine), poor solubility of opiate substrate and instability of the intermediate morphinone led to a low productivity of hydromorphone and decreased the usefulness of the system. The STH approach raised the yield of hydromorphone up to 84% with only 4.5% by-product accumulation in the cell free system after fine tuning of enzyme ratios. The original *E. coli* construct had a yield of

about 37.1% ± 2.4% and high levels of about 25.6% ± 0.5% dihydromorphine. Strains co-expressing STH improved the hydromorphone yield up to 51.7% ± 1.0% and less by-product (12.3% ± 2.7%) (35). For the production of poly-3-hydroxybuytrate (PHB) in *E. coli*, a STH (native *udhA* gene from *E. coli*) was co-expressed with the *phb* operon from *Alcaligenes eutrophus* H16 resulting in an increase of PHB yield from 49% to 66% g_{PHB}/g_{DCW} and an increase of PHB in final concentration from 3.52 g/L to 6.42 g/L compared to the control strain, not expressing STH (46).

1.4.3. Chemical co-factor regeneration

In a chemical regeneration of co-factors, a reaction has to be established where the catalyst stereoselectively transfers the reducing or oxidation equivalents to the co-enzyme from the added redox reagents. $Na_2S_2O_4$ for instance, a commonly used reducing agent, can be applied for such transfers in co-factor regeneration (47) (48). As easy as the usage is, enzymes suffer from inactivation of high salt concentrations and applicability in industries is not factual (49).

1.4.3.1. Electrochemical regeneration

Approaches for electrochemical regeneration have been long in the focus for biocatalystic applications as there are no by-products due to the fact that no co-enzyme(s) and co-substrate(s) are needed. The regeneration of NAD(P)H/ $NAD(P)^+$ is done by direct or indirect (via a mediator) oxidation/reduction of the co-factor performed by electrodes. However, various problems still limit this technology: high overpotentials are needed for direct oxidation or reduction of the co-factor, electrode fouling, generation of inactive cofactor-dimers and a limited efficiency range (i.e. only enzyme in direct physical proximity of the electrode are productive) (50).
Figure 3 shows the reduction principle of NAD^+ to NADH in an electrochemical approach. The first step is the transfer of one electron (e^-) to NAD^+ forming an NAD-radical, which is either further reduced and protonated to an enzymatically active 1,4-NADH (step 2a) or two neighbouring NAD-radicals form an inactive dimer (NAD_2) (step 2b). On unmodified electrodes step 2b is significantly faster and thus the major product of NAD^+ reduction is NAD_2 (51).

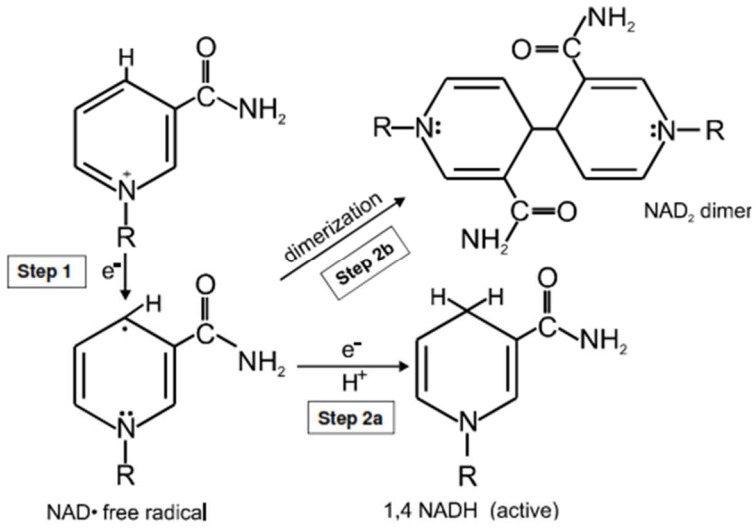


Figure 3 Scheme of NAD^+-reduction to NAD_2 and enzymatically active 1,4-NADH. Step 1: NAD^+ is reduced with an electron (e^-) to a NAD-radical. Step 2a: the NAD-radical is further reduced and protonated to give enzymatically active 1,4-NADH. This step is generally considered to be slow due to slow protonation of the NAD-radical. Step 2b: the slow protonation of the NAD-radical can result in dimerization of two neighbouring NAD-radicals leading to enzymatically inactive dimers (NAD_2).
R= adenosine diphosphoribose **(51)**

1.4.3.1.1. Mediators

Transition metal mediators were studied for this kind of co-factor regeneration, whereby the oxidation of NAD(P)H was more efficient as the reduction of $NAD(P)^+$ (52) (53).
Direct electrochemical regeneration, in which no mediators are needed stand in contrast to indirect electrochemical regeneration, where two single electrons are transferred from a mediator (usually viologen mediators) to a "helper"-enzyme (e.g. flavoenzymes), which reduces $NAD(P)^+$ in a "one-step" reaction to NAD(P)H (54). As avoiding a co-enzyme for regeneration was mentioned as an advantage of electrochemical regeneration, the development was directed to mediators, which can transfer two-electrons (or one hydride) in one-step to $NAD(P)^+$ to reduce NAD(P)H.
Two tasks should be fulfilled by such mediators: The two electrons (hydride) should be transferred directly to the co-factor and not reduce the substrate and the mediator has to operate at potentials less cathodic than -0.9 V (to avoid NAD-dimer formation) and more cathodic than the standard potential of the co-cofactor redox couple (E'= 0.59 V vs. saturated calomel electrode (SCE), pH 6.0 (55)) to make the reaction thermodynamically feasible (56). Tris(2,2'-bipyridyl) rhodium complexes ((57) (58) (59)) and substituted or non-substituted (2,2'-bipyridyl) (pentamethylcyclopentadienyl)-rhodium complexes (60) fulfil these requirements.

1.4.3.1.2. Electrode modification

Electrode modification can also increase the yield of 1,4-NADH without the use of mediators. As example, reticulated vitreous carbon (RVC) electrodes only gain enzymatically active 1,4-NADH below 1% at potentials of -0.75 V and -0.8 V. After a rhodium(III) bis-terpyridine complex with two covalently linked pyrrol groups was electro-polymerized on RVC, the conversion rate raised up to 30% enzymatically active NADH (at -0.85 V), compared to 0.6% with non-modified RVC (61).
Using Au- and Cu-electrodes also increases the yield of active NADH to 75% and 71% respectively, but at low negative potentials and low reduction rates, that are not feasible for industrial application. When raising the negative potential (relevant for operating on industrial scale) and hence reduction rate, the yield is low (28%) for Au and intermediate (52%) for Cu. Therefore, an Au electrode was modified with Pt which led to an increase to 63% of active NADH at industrial relevant potentials (62). Recently Ali et al. showed that non-modified glassy carbon (GC) electrodes produced at high cathodic potentials (-2.3 V vs. mercury-mercurous sulphate electrode (MSE)) an amount of 98% of 1,4-NADH in a batch electrochemical reactor (63).
In a next step, the glassy carbon electrode surface was patterned with platinum and nickel nano-particles to increase electrode efficiency of electrochemical regeneration. Pt and Ni provide “active” adsorbed hydrogen (Pt-H_{ads} or Ni-H_{ads}) for fast protonation of NAD^+, forming a NAD-radical. A 100% yield of enzymatically active 1,4-NADH could be generated at relatively low electrode potentials (-1.6 V vs MSE for GC-Pt and -1.5 V vs MSE for GC-Ni). In case of GC-Pt the NADH-yield decreased with potentials being more negative than -1.6 V. The efficiency was also dependent on the Pt nano-particle diameter. Nonetheless this achievement makes GC-electrodes and the modified GC-Pt and GC-Ni electrodes interesting for industrial bioreactors (64). However, due to the low surface area of these electrodes the corresponding conversion rate is rather slow. Employing a multiple electrode set-up might increase the regeneration rate, but with the disadvantage of increasing the reactor volume. Ali et al. applied electrodes with a carbon-based surface of very high roughness improving the surface-to-volume ratio. They used carbon nanofibres (CNFs), because they are carbon-based as glassy carbon and they have a very large surface-to-volume ratio, thus offering a large electro-active surface inferring a good electrochemical activity. CNFs were grown on stainless steel mesh as shown from Baddour et al. (65). Indeed, the CNF cathode showed faster electrochemical NAD^+ reduction kinetics and higher NAD^+ conversion in comparison to the glassy carbon and stainless steel mesh cathodes alone. CNF cathode had also a high yield (99.3% ± 0.6%) of enzymatic active 1,4-NADH (51).

1.4.3.2. Photochemical regeneration

A relatively new field of chemical regeneration is the photochemical approach using organic or inorganic photosensitizers and mediators (necessary for the electron transport to the co-factor) for reduction of NAD^+. These photosensitizers, i.e. porphyrin, mimic chlorophyll and therefore are excited by visible light. For industrial applicability, the photosensitizers as well as the used mediators have to be chemically stable in bioreactors. For example, Kim et al. used Zn-containing porphyrin as biomimetic light-harvesting molecule for artificial photosynthesis (under visible light) as shown in Figure 4.

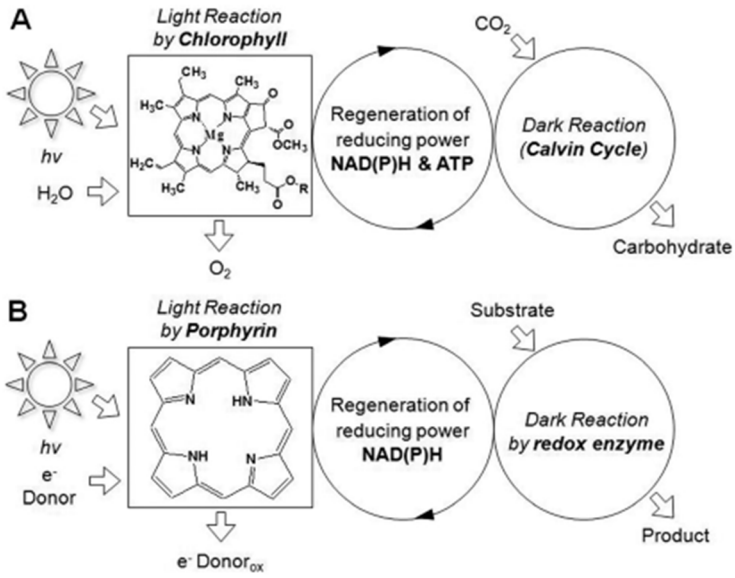


Figure 4 The graphic shows the scheme of the natural photosynthesis-system with chlorophyll (A) and an artificial biocatalyzed photosynthesis-system with porphyrin instead of chlorophyll (B). **(66)**

The regeneration system (see reaction mechanism in Figure 5) also contains a rhodium-based electron mediator (M) ($[Cp^*Rh(bpy)H_2O]^{2+}$) and triethanolamine (TEOA) as electron donor. Zn-porphyrin is first excited by irradiation with visible light and the rhodium of M binds non-covalently to the *meso*-functional ligand of the porphyrin. Thus, an intramolecular electron-transfer from the Zn-porphyrin to the M occurs due to the electronegative zinc at the porphyrin's core. The obtained reducing power of the M can reduce the NAD^+ to enzymatically active 1,4-NADH. Further, the electron donor TEOA is bound via weak axial coordination to the metal centre of the excited Zn-porphyrin and reduces the Zn-porphyrin through an inner-sphere electron transfer. Afterwards TEOA dissociates from the Zn-ion. This system was used to mediate the conversion from α-ketoglutarate to L-glutamate by glutamate dehydrogenase (66).

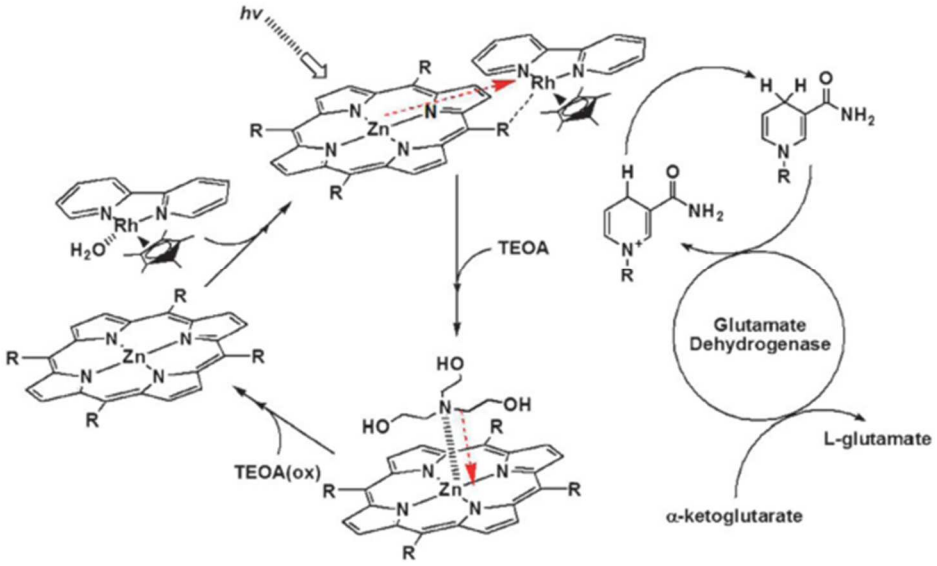


Figure 5 "Suggested mechanism of Zn-porhyrin-sensitized, biocatalysed artificial photosynthesis of L-glutamate. R is a meso-funtional group of the Zn-porphyrin. Red arrows indicate transfer between each component", by Kim et al. **(66)**

Besides organic photosensitizers there is a broad spectrum of different inorganic ones (e.g. nanostructured $CdIn_2S_4$ or CdS quantum dots (67) (68)) for production of solar hydrogen under visible light. Park et al. used the visible light photo-catalyst $W_2Fe_4Ta_2O_{17}$ and coupled it to a redox enzyme reaction for application in bioreactors as pictured in Figure 6. By visible light ($\lambda \geq 420$ nm) $W_2Fe_4Ta_2O_{17}$ promotes upon band-gap excitation an electron to the conduction band (e^-_{CB}). The rhodium based mediator (M) receives the e^-_{CB}, gets reduced and abstracts a proton from the aqueous solution. Subsequently M transfers the electrons and the hydride to NAD^+ and reduces it to 1,4-NADH. EDTA is the sacrificial electron donor, which fills the gap in the valence band (h^+_{VB}). NADH is consumed by glutamate dehydrogenase, producing L-glutamate from α-ketoglutarate. (69).

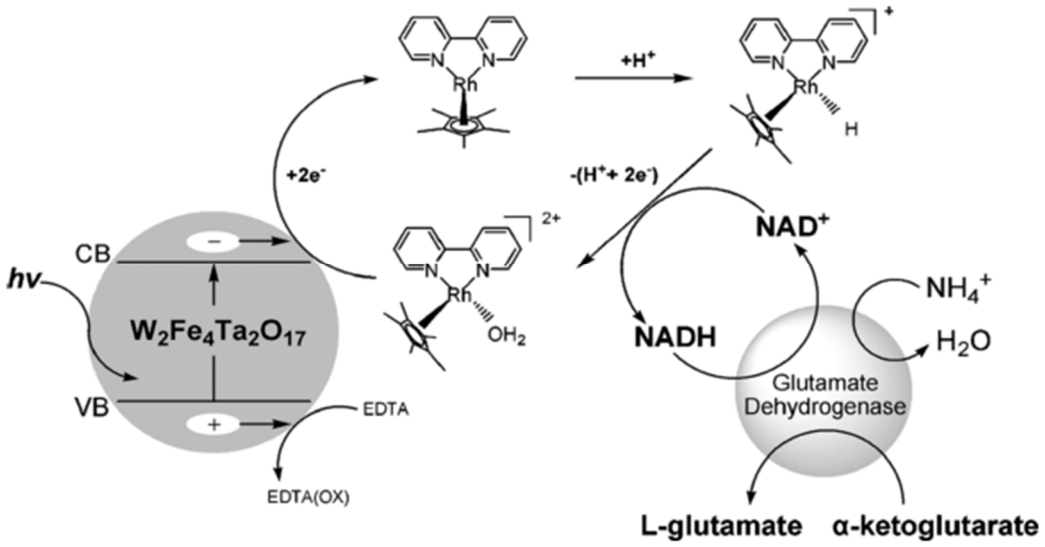


Figure 6 Schematic mechanism of the photocatalyst-enzyme coupled bioreduction of α-ketoglutarate to L-glutamate under visible light, by Park et al. **(69)**.

1.5. *Pichia pastoris* and the MUT-pathway

1.5.1. *Pichia pastoris* as recombinant expression system

The focus on the current work lies on improving the NADH-regeneration during whole-cell biotransformation with recombinant *P. pastoris* (*Komagataella phaffi*). This yeast harbours several advantages compared to other microorganisms as host for recombinant protein production as well as for biocatalytic applications. This methylotrophic yeast grows to very high cell densities, has available strong and tightly regulated promoters, and produces gram amounts of recombinant protein per litre culture both intracellularly and in secretory fashion (70).

Hasslacher et al produced 22 g/L of recombinant S-hydroxynitrile lyase (Hnl) from the tropical rubber tree (71)Mellitzer et al. were able to produce 18 g/L active secreted recombinant cellulose from *Trichoderma reesei* in a small scale bioreactor, which is the highest concentration of active secreted protein in *P. pastoris* published so far (72).Non-engineered *P. pastoris*' produces, as common for yeasts, hyper-mannosylated proteins which have a low bioactivity and are somewhat antigenic in humans (73). Glycan remodelling towards "humanized" glycosylation in yeast is a promising approach to use *P. pastoris* for production of pharmaceutical proteins instead of mammalian cell lines (74). For example Smith et al used the glycoengineered SuperMan$_5$ strain of GlycoSwitch® *P. pastoris* to produce bioactive human mast cell chymase (rhChymase) (75). Krainer et al. also accomplished a hypomannosylating strain of *P. pastoris* by knocking-out the *OCH1* gene. *OCH1* codes for the α-1,6-mannosyltransferase which triggers hypermannosylation of secreted proteins at great heterogeneity, significantly impending downstream processing and reproducibility. The *och1* knock-

out strain was able to produce homogenous glycoprotein species of recombinant horseradish peroxidase (used for applications in diagnostics, bioremediation and cancer treatment) with Man8 instead of Man10 N-glycans as dominant core structure. Though, the knock-out strain showed a growth impaired phenotype and considerable rearrangements of the cell wall components it achieved a volumetric productivity of 70% of the wildtype strain (76).

1.5.2. MUT-pathway

The methylotrophic yeast *P. pastoris* is able to use methanol as sole carbon source. For this metabolism it needs a set of enzymes to oxidize methanol, which builds the methanol utilization (MUT) pathway, see Figure 7. Two of the most important enzymes are alcohol oxidase (AOX) and dihydroxyacetone synthase (DAS), the expression of which is repressed while growing on e.g. glucose as sole carbon source, but highly induced by methanol. They are regulated at transcriptional level and can constitute up to 30% of the total protein content, when grown on methanol (77) (78). Methanol induced cells also show an up-regulation in genes encoding essential genes for peroxisome biogenesis and proliferation. The first step of the MUT pathway is the oxidation of methanol to formaldehyde within the peroxisomes via alcohol oxidase. The side product of this step, hydrogen peroxide, is subsequently reduced by catalase (CAT1) to H_2O and O_2. Formaldehyde stands at the branch point between the assimilation and dissimilation pathway. Following the dissimilation to carbon dioxide, formaldehyde diffuses in the cytoplasm and spontaneously reacts with reduced glutathione and is further oxidized by formaldehyde dehydrogenase (FLD1) and formate dehydrogenase (FDH) to CO_2, generating 2 equivalents of NADH per mole formaldehyde. Alternatively, formaldehyde stays in the peroxisome and enters the assimilation pathway reacting with xylulose-5-phosphate (Xu5P) via dihydroxyacetone synthase (DAS) to glyceraldhyde-3-phosphate (GAP) and dihydroxyacetone (DHA). DHA is further converted by dihydroxyacetone kinase to dihydroxyacetone phosphate (DHAP) and this facilitates the production of fructose 1,6-bisphosphate (FBP) via a reaction between GAP and DHAP by fructose 1,6-bisphosphate aldolase. FBP is dephosphorylated by fructose 1,6-bisphosphataseyielding in one molecule GAP per three moles of methanol (79) (77). It was assumed that the Xu5P gets recycled through not yet known rearrangement reactions in the pentose phosphate pathway (PPP), but Rußmayer et al. postulated a new recycle mechanism for the Xu5P, called the xylulose-monophosphat cycle, which utilizes a duplicated methanol inducible enzyme set and is totally localized to peroxisomes. There is not only an isoform of fructose-1,6-bisphosphate aldolase (Fba1-2) as reported by Küberl et al. (80), but also isoforms of the PPP enzymes transaldolase (Tal1-2), ribose-5-phosphate ketol-isomerase (Rki1-2), and ribulose-5-phosphate 3-epimerase (Rpe1-2). Each of these isoforms have significantly higher transcript and protein levels when methanol is present and also contain a PTS1 peroxisomal targeting

signal, indicating their involvement in a separate peroxisomal methanol assimilation pathway. Additionally sedoheptulose-1,7-bisphosphatase (Shb1) was found to be enriched in peroxisomes which hydrolyses sedoheptulose-1,7-bisphosphate (S1,7BP) to sedoheptulose-7-phosphate in a thermodynamically driven pathway for synthesis of pentose-5-phosphates alternative to PPP. Based on this data a novel carbon assimilation pathway was proposed that shares the concept of compartmentalization as in plants or cyanobacteria. DAS is responsible for C-C bond formation followed by a cyclic pathway for regeneration of the pentose phosphate substrate (Xu5P) of the carboxylation reaction. As Shb17 is driving the flux from erythrose-4-phosphate and DHAP towards ribose-5-phosphate in *S. cerevisiae*, it is proposed that Shb17 drives the flux towards Xu5P in a similar fashion in *P. pastoris* (81).

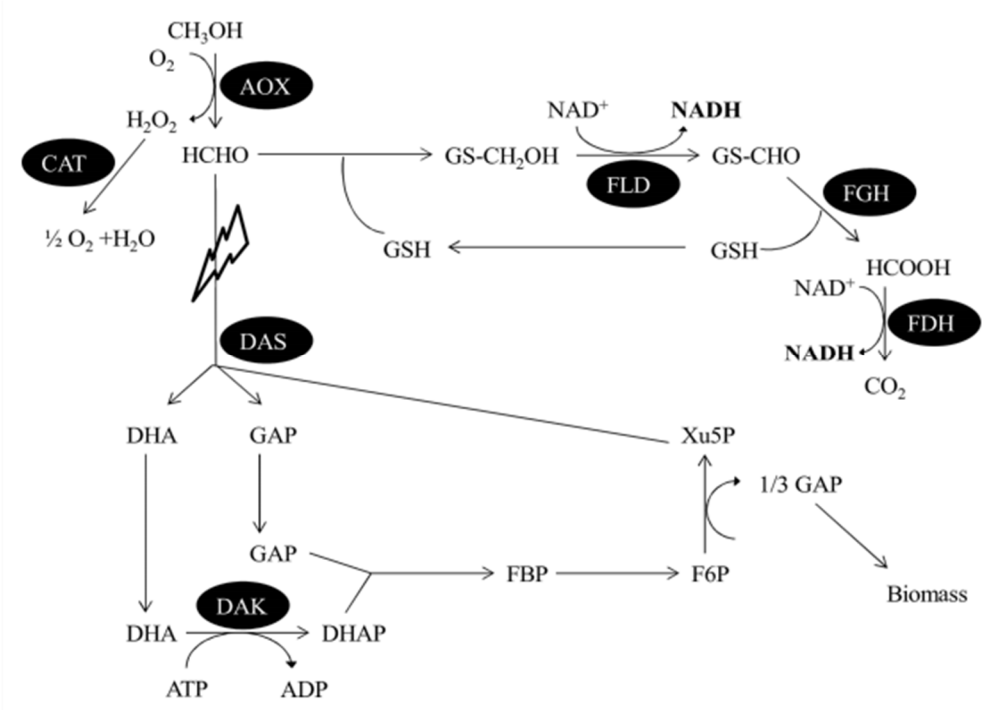


Figure 7 "Simplified schematic representation of the methanol utilization pathway in *Pichia pastoris*. The main pathways and the respective enzymes are shown. AOX: alcohol oxidase; FLD: glutathione-dependent formaldehyde dehydrogenase; FGH: S-formylglutathione hydrolase; FDH: formate dehydrogenase; CAT: catalase; DAS: dihydroxyacetone synthase; DAK: dihydroxyacetone kinase; DHA: dihydroxyacetone; GAP: D-glyceraldehyde- 3-phosphate; DHAP: dihydroxyacetone phosphate; FBP: D-fructose 1,6-bisphosphate; F6P: D-fructose 6-phosphate; Xu5P: D-xylulose 5-phosphate. By deleting dihydroxyacetone synthase the dissimilatory pathway leading to the formation of NADH is strengthened." Picture taken from Geier et al. (2015) **(82)**.

Two genes code for alcohol oxidase, *AOX1* and *AOX2*. Both genes are highly homologous in their DNA- and amino acid sequence, but the flanking regions share no homology. Especially the 5'UTR (promotor region) differs a lot leading to higher expression levels of the *AOX1* gene (77).
For heterologous protein production the tightly regulated *AOX1* promoter (P_{AOX1}) is often used, whereby methanol functions as carbon source and inducer. *P. pastoris* grows very slowly on methanol compared to other carbon sources. Thus, an alternative substrate is chosen for initial

growing phase to gain high cell densities. For induction and producing the protein of interest a switch to methanol is then performed (83).

Chung et al. analysed in an *in silico* approach the best feeding strategy for initial growth phase, when using the P_{AOX1}, to gain high growth rate with high capacity to synthesize amino acids in the early phase to have them available in the latter phase for heterologous protein production. Several carbon sources (glucose, glycerol, alanine, methanol, sorbitol, and trehalose) were tested for high growth yield of *P. pastoris*. Glycerol followed by sorbitol achieved best results concerning the gained biomass. Generally, high utilization of the central metabolism leads to increased production of various precursors applicable for biomass. However, flux distributions indicated that the utilization of central carbon metabolism is the highest under methanol conditions, but yielding in lowest growth rate. By examining the ATP flux-sum and the gaseous exchange rate in methanol utilization, a significantly higher turnover rate of ATP and gaseous exchange rates were observed, suggesting that much of the resources have been diverted to energy regeneration during methanol utilization. Concluding methanol utilization provides higher energy regeneration than other carbon sources (84). Solà et al. showed in a co-feeding experiment of glycerol/methanol, that on higher dilution rates the gaseous exchange rates are indeed higher (85), supporting the *in silico* analysis of Chung et al. (84). The MUT-pathway was further analysed in an *in silico* approach. A high turnover rate of NADH was observed when *P. pastoris* is grown on methanol minimal media, implying a potential for whole-cell biotransformation using NADH-dependent oxidoreductases. (86) (84). Moreover, the analysis of the MUT-pathway by Schroer et al. marked targets (possible bottleneck reactions) for improving the flux through the dissimilative pathway. The kinetic properties of alcohol oxidase (AOX), formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH) were studied and experimental derived kinetic data was applied for simulation of NADH-regeneration rates. FLD was determined as main bottleneck, which was further tested and confirmed experimentally. Therefore, NADH-dependent 2,3-butanediol dehydrogenase (BDH1) from *S. cerevisiae* and FLD, AOX1 and FDH were both over-expressed, respectively, in *P. pastoris* for whole cell biotransformation of acetoin to butanediol. BDH1 has a high turnover rate (k_{cat} = 98,000 min^{-1} (87)) and NADH-regeneration can be the limiting factor for the conversion. Thus, BDH1 is a good candidate to observe improved NADH regeneration. Methanol functioned as sole carbon source and as inducer for the expression of the actual biocatalyst 2,3-butanediol dehydrogenase. *P. pastoris* co-expressing FLD showed significantly higher turnover rates than the wild type strain. Strains co-expressing AOX and FDH showed even lower turn-over rates than the wild type. Regarding the high k_m values for FDH and the substrate formate this step could have be assumed as limiting, but it was not the case in the experimental results. The effect of FDH overexpression and hence increase of v_{max} was not comparable to FLD overexpression Higher

AOX concentrations were not beneficial for NADH regeneration due to inhibition of FLD by the AOX product formaldehyde.

FLD overexpression while whole-cell biotransformation with the strongly NADH-dependent BDH1 resulted in better conversion of acetoin to butanediol, concluding a better flux through the dissimilative pathway and thus a higher NADH-regeneration rate, which can be taken in advantage for NADH-dependent biotransformations (27).

1.6. Aim of the thesis

The aim of this thesis is to generate a new platform strain of *P. pastoris* CBS7435 with an improved NADH-regeneration without the overexpression of additional enzymes. Our approach was an enhancement of the flux through the dissimilative MUT pathway by shutting-down the assimilative pathway.

Dihydroxyacetone synthase (DAS) converts formaldehyde to GAP and DHA, the first reaction in the assimilative pathway. In the methylotrophic yeasts *Candida biodinnii*, *Hansenula polymorpha* DAS is encoded by the single gene *DAS1* (88) (89), whereas in *P. pastoris* dihydroxyacetone synthase is encoded by two homologs (*DAS1* and *DAS2*), which have a sequence similarity of 91% and are located close to each other on chromosome 3. *DAS1* and *DAS2* are orientated in opposite direction and the small gene, coding for a hypothetical guanosine nucleotide exchange factor, lies in-between, see Figure 8.

Figure 8 Genomic situation of *DAS1* and *DAS2* on chromosome 3 of *P. pastoris* CBS 7435.

Recently Küberl et al. discovered that in a former annotation of the genome of *P. pastoris* GS115 (FN392321.1) the region between the first 238 bp of both genes had been wrongly inverted, probably due to high similarity. Moreover, earlier annotations of *DAS1* (FJ752551.1) and *DAS2* (FJ752552.1) were incorrect (80).

By knocking out the 2 homologs *DAS1* and *DAS2* the flux should be directed to NADH-regeneration only and no biomass should be produced. Sakai et al. showed, that knocking-out *DAS1* in the methylotrophic yeast *Candida boidinii* led to no growth of it on methanol as carbon source (88). Näätsaari et al. already generated a double-knockout of *das1* and *das2* in *P. pastoris Δku70*, but they used the wrong annotation of the 2 genes, as mentioned above, for their approach. Despite using the

wrong sequences, they observed a growth limitation of the single *Δdas1*, *Δdas2*, and the double knock-out strains on methanol. Genome analysis of Küberl et al. also suggested a possible dihydroxyacetone synthase activity of transketolase 1 (TKL1) (80). Consequently, knocking-out of *TKL1* is desirable as well to completely redirect the methanol flux into the dissimilative pathway. Goal of this thesis is the construction of *P. pastoris* CBS7435 strains with single knock-outs of *das1*, *das2*, *tkl1* as well as the construction of a *das1das2* double knock-out and a *das1das2tkl3* triple knock-out strain. 2,3-butanediol dehydrogenase was chosen as model protein to proof improvement of NADH-regeneration in knock-out strains. The putative recycling of the two NADH, which are consumed by the model enzyme BDH1 by reducing one molecule acetoin to one molecule 2,3-butanediol, is achieved by two consecutive oxidations in the dissimilative pathway catalysed by the NAD-dependent enzymes formaldehyde dehydrogenase and formate dehydrogenase (27).

2. MATERIALS AND METHODS

2.1.Instruments, Devices, Software

2.1.1. Centrifuges and associated devices

- 5810 R centrifuge, Eppendorf
- 415 R centrifuge, Eppendorf
- JA-10 rotor, Beckman Coulter

2.1.2. Shakers and incubators

- Titramax 1000 platform shaker, Heidolph
- Thermomixer comfort, Eppendorf
- Drying oven, Binder
- HT Multitron incubator shaker, Infors

2.1.3. Polymerase chain reaction cyclers

- GeneAmp® 2720 thermal cycler, Applied Biosystems

2.1.4. Photometers and plate readers

- Spectrophotometer DU 800, Beckman Coulter
- Semi-micro cuvette, polystyrene 10x4x45 mm, Sarstedt
- NanoDrop 2000c spectrophotometer, Thermo Scientific

2.1.5. Gas chromatography devices

- Hewlett Packard 6890 instrument equipped with a FID
- Chirasil-DEX CB column (25 m x 0.32 mm, 0.25 µm film)

2.1.6. Electroporation devices

- Gene Pulser, Bio-Rad Laboratories
- MicroPulser™ Electroporator, Bio-Rad Laboratories
- Electroporation cuvettes, 2 mm, Bridge Bioscience

2.1.7. Pipettes and pipette tips

- Pipetman, adjustable single channel pipettes 0.2-1000 µL, Gilson
- Research® pipette, adjustable 0.1-2.5 µL, Eppendorf
- Pipette tips, 10, 200 and 1000 µL, with and without filter, GBO Greiner Bio-One
- Proline® multichannel pipettor, 8 channels 5-50 µL, Biohit
- Proline® multichannel electronic pipettor, 8 channels 50-1200 µL, Biohit
- Tips 300 µL, Single Tray, Biohit
- Tips 1200 µL, Bulk, Biohit

2.1.8. Reaction tubes

- Microcentrifuge tubes, 1.5 mL with lid, GBO Greiner Bio-One
- Polypropylene tubes, sterile 15 mL, GBO Greiner Bio-One
- Polypropylene tubes, sterile, 50 mL, GBO Greiner Bio-One
- Polypropylene tubes, sterile, 12 mL volume, GBO Greiner Bio-One
- Polypropylene tubes, sterile, 15 mL volume, GBO Greiner Bio-One
- Nunc™ Cryogenic tubes, sterile, 2 mL volume, ThermoScientific

2.1.9. Microplates

2.1.10. Web tools and software

2.1.11. Other Devices

- Vortex-Genie 2, Scientific Industrie
- inoLab® pH720 pH meter, WTW
- Polyplast Temp Din pH electrode, Hamilton
- MR 3000 and MR 2002 magnetic stirrers, Heidolph
- Millipore® VSWP01300 MF-Millipore™ DNA Filter Paper for Dialysis of DNA and Proteins, Plain White Mixed Cellulose Esters, 0.025 µm, Diameter 1.3 cm (Capitol Scientific)

2.2. Material for gel electrophoresis

- Biozym LE agarose, Biozym Scientific

- GeneRuler™ 1kb DNA-ladder, Thermo Scientific
- 6x DNA loading dye, Thermo Scientific
- Sub-cell GT, Bio-Rad Laboratories
- Power Pac™ Basic, Bio-Rad Laboratories
- Camera
- UV-device

2.3.Media and solutions

2.3.1. Antibiotics

- Ampicillin stock, 100 mg/mL: 5 g ampicillin / 50 mL dH2O, filter-sterilize (Ø 0,2 µm)
- Zeocin™ stock, 100 mg/mL: 2 g Zeocin® / 20 ml dH2O, filter-sterilize (Ø 0,2 µm)
- Antibiotic concentrations for E. coli 25 mg/L Zeocin®, 100 mg/L ampicillin (1 mL stock solution)
- Antibtiotic concentrations for *P. pastoris* 100 mg/L Zeocin® (transformation of expression constructs), 50 mg/L Zeocin® (transformation of knock-out constructs)

2.3.2. Media and solutions for *E. coli*

- Low salt Luria Bertani (LB): 10 g/L Bacto™ tryptone, 5 g/L Bacto™ yeast extract, 5 g/L NaCl, autoclave sterilization
- LB agar: 35 g/L LB agar (Lennox, Roth)
- Super optimal broth with gluose (SOC): 5 g/L Bacto™ yeast extract, 20 g/L Bacto™ tryptone, 0.58 g/L NaCl, 2 g/L $MgCl_2$, 2.46 g/L $MgSO_4$, 0.18 g/L KCl, 3.81 g/L α-D(+)-glucose monohydrate, autoclave sterilization

2.3.3. Media and Solutions for *P. pastoris*

- 10x D: 220 g/L α-D-glucose·H2O (20 %)
- 500x biotin: 200 mg/L D-Biotin, filter-sterilize (Ø 0,2 µm)
- 10x yeast nitrogen base (YNB): 134 g/L Difco™ yeast nitrogen base w/o amino acids, autoclave sterilization
- 1 M potassium phosphate buffer (PPB): 30.0 g/L K_2HPO_4, 118.0 g/L KH_2PO_4, pH 6.0, autoclave sterilization

- Yeast extract/peptone/dextrose (YPD) medium: 10 g/L Bacto™ yeast extract, 20 g/L Bacto™, 15g/L Difco Bacto Agar for solid medium, for 1 L medium dissolve in 900 mL dH2O and autoclave; add 100 mL/L 10x D (2%)
- Basal medium with 1 % dextrose (BMD1): 200 ml/L 1 M PPB, 100 ml/L 10x YNB, 50 mL/L 10x D, 2 mL/L 500x biotin, 650mL sterile ddH_2O
- Basal medium with 0.5% methanol dextrose (BMM0.5): 200 mL/L 1 M PPB, 100 mL/L 10x YNB, 5 mL/L methanol, 2 mL/L 500x biotin, 700 mL sterile ddH_2O
- Basal medium with 1% glycerol (BMG1): 200 mL/L 1 M PPB, 100 mL/L 10x YNB, 100 mL/L 10x GY (100 g/L), 2 mL/L 500x biotin, 600 mL sterile ddH_2O
- Basal medium with 1% methanol (BMM2): 200 mL/L 1 M PPB, 100 mL/L 10x YNB, 10 mL/L methanol, 2 mL/L 500x biotin
- Basal medium with 5% methanol (BMM10): 200 mL/L 1 M PPB, 100 mL/L 10x YNB, 50 mL/L methanol, 2 mL/L 500x biotin
- Minimal media with 1% methanol (MMMeOH1%): 100 mL/L 10x YNB, 2 mL/L 500x biotin, fill to 1 L with dH_2O; for agar-plates ad 15 g/L agarose before autoclaving, after autoclaving 10 mL/L methanol were added, when solution started to build flakes)
- 10x stocks for *TKL1* knock-out screening:
    - 10x glycerol (10% (w/v)) (10x GY): 5 g glycerol filled to 50 mL with ddH_2O, sterile filtered (0.2 µm Ø (Scientific Strategies))
    - 10x oleic acid (5% (w/v)) (10x OA): 2.8 g weighed and filled up to 50 mL with ddH_2O, sterile filtered (0.2 µm Ø (Scientific Strategies))
    - 10x sorbitol (10% (w/v)) (10x SO): 5 g sorbitol weighed and filled to 50 mL with ddH_2O, sterile filtered (0.2 µm Ø (Scientific Strategies))
    - 10x methanol (10% (v/v)) (10x MeOH): 5 mL were pipetted and then filled up to 50 mL with ddH_2O, sterile filtered (0.2 µm Ø (Scientific Strategies))
- Minimal and YP-media for *TKL1* knock out screening
    - Yeast extract/peptone/glycerol, oleic acid, sorbitol, methanol (YPGY, YPOA, YPSO, YPMeOH) medium:

      10 g/L Bacto™ yeast extract, 20 g/L Bacto™, 15g/L Difco Bacto Agar for solid medium, for 1 L medium dissolve in 900 mL dH2O and autoclave; add 100 mL/L 10x GY (10 %), 10x OA (5%), 10x SO (10%), 10x MeOH (10%)
    - Minimal medium with glycerol, oleic acid, sorbitol, methanol (MMGY, MMOA, MMSO, MMMeOH):

      10x YNB 12.5 mL/L, 1 M PPB 50 mL/L, 500x biotin 2 mL/L, 15g/L Difco Bacto Agar for

solid medium, for 1 L medium dissolve in 900 mL dH2O and autoclave; add 100 mL/L 10x GY (10%), 10x OA (5%), 10x SO (10%), 10x MeOH (10%)

2.3.4. Solutions for gDNA-isolation

- 10% sodium-dodecyl-sulphate (SDS): dissolve 5 g in 50 mL ddH_2O, filter-sterlize (Ø 0,2 µm)
- 5 M sodiumchloride: dissolve 14.61 g in in 50 mL ddH_2O, filter-sterlize (Ø 0,2 µm)
- 0.5 M ethylenediaminetetraacetic acid (EDTA): dissolve 9.31 g di-Na-EDTA.2H_2O in 50 ml ddH_2O, filter-sterlize (Ø 0,2 µm)
- Yeast lysis buffer: 2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)
- TE-buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

2.3.5. Other media, solutions and chemicals

- dNTP mix: 10 mM each, dATP, dTTP, dCTP, dGTP, Thermo Scientific
- 1 M dithiothreitol (DTT): 0,16 g/mL DTT, filter-sterilize (Ø 0,2 µm)
- Bicine/ethylene glycol/dimethyl sulfoxide (DMSO)/sorbitol (BEDS): 10 mM bicine-NaOH (pH 8.3), 3% (v/v) ethylene glycol, 5% DMSO (v/v) and 1 M sorbitol filter-sterilize (Ø 0,2 µm), freeze aliquots at -20°C
- Tris/HCl: 300 mM (pH 7) for pNPB-assay

2.3.6. Solutions for assembly cloning (acib protocol)

- 1 M Tris/ HCl pH 7.5 (dissolution of 60.57 g of Tris base (Roth, 4855.3) in 500 mL of ddH_2O; pH is adjusted with 32% HCl (Roth, P074.3); solution is autoclaved and stored at room temperature)
- 2 M $MgCl_2$ (dissolving of 20.30 g $MgCl_2$ x 6 H_2O (Merck, 5833) in 50 mL of ddH_2O; solution is sterile filtered 0.2 µm Ø (Scientific Strategies))
- 1 M DTT (dissolution of 3.08 g of DTT (Roth, 6908.1) in 20 mL of ddH_2O; sterile filtered 0.2 µm Ø (Scientific Strategies) and stored as 1 mL aliquots at -20°C)
- 100 mM NAD (dissolution of 0.66 g of NAD (Roth, AE11.2) in 10 mL of ddH_2O; sterile filtered 0.2 µm Ø (Scientific Strategies) and stored as 300 µL aliquots at -20°C))
- Before mixing all the ingredients (see Table 1) for the 5x ISO buffer out of the stock solution PEG-8000 was weighed in a 15 mL Falcon tube. The mixture is then filled up to 6 mLl end-

volume and vortexed till PEG-8000 was homogeneously dissolved. The solution is then sterile filtered (0.2 µm Ø (Scientific Strategies)) and stored in 100 µL aliquots at -20 °C.

Table 1: Preparation of 6 mL of 5x isothermal (ISO) reaction buffer by mixing following components in a 15 mL Falcon tube

component	stock solution	volume / amount
25% (w/v) PEG-8000	PEG-8000	1.5 g
500 mM Tris/Cl pH 7.5	1 M Tris/Cl pH 7.5	3000 µL
50 mM $MgCl_2$	2 M $MgCl_2$	150 µL
50 mM DTT	1 M DTT	300 µL
1 mM dATP	100 mM dATP	60 µL
1 mM dCTP	100 mM dCTP	60 µL
1 mM dGTP	100 mM dGTP	60 µL
1 mM dTTP	100 mM dTTP	60 µL
5 mM NAD	100 mM NAD	300 µL
	sterile ddH_2O	up to 6000 µL

- 1.2 mL of the assembly master was prepared with the components listed in Table 2 in a 1.5 mL Eppendorf tube, mixed via pipetting up and down, aliquot in 15 µL each and stored at -20°C.

Table 2: Preparation of the assembly master mix in a 1.5 mL Eppendorf tube

component	Volume to add [µL]
5x ISO reaction buffer	320
T5 exonuclease (10 U/µL)	0.64
Phusion® High-Fidelity DNA Polymerase (2 U/µL)	20
Taq DNA ligase (40 U/µL)	160
Sterile ddH_2O	699.36

2.4.Enzymes

Enzymes were applied according to manuals.

- FastDigest BamHI, 5'G^GATTC3', Thermo Scientific (# FD0054)
- FastDigest BglII, 5'A^GATCT3', Thermo Scientific (# FD0083)
- FastDigest EcoRI, 5'G^AATTC3', Thermo Scientific (# FD0274)
- FastDigest HindIII, 5'A^AGCTT3', Thermo Scientific (# FD0504)
- FastDigest NotI, 5'GC^GGCCGC3', Thermo Scientific (# FD0594)
- FastDigest SmiI (SwaI), 5'ATT^AAAT3', Thermo Scientific (# FD1244)
- FastDigest XhoI, 5'C^TCGAG3', Thermo Scientific (# FD0694)
- SfiI, 5'GGCCNNNN^NGGCC3', New England Biolabs® Inc. (# R0123S)
- T4 DNA Ligase, Thermo Scientific (# EL0014)
- EndoH, New England Biolabs (P0702S)
- FastAP thermosensitive alkaline phosphatase, Thermo Scientific (# EF0654)
- Dream Taq DNA polymerase, Thermo Scientific (# EP0701)
- Phusion high-fidelity DNA polymerase , Thermo Scientific (# F-530L)
- T5 exonuclease, Biozyme (162340)

2.5. Substrates and buffers for enzymatic assay

- 3-Hydroxy-2-butanone purum (acetoin), Fluka (00540)
- 4-Nitrophenyl butyrate (pNPB), Sigma (N9876)
    - pNPB-stock solution: a 8.4% pNPB solution in DMSO was freshly prepared before doing the assay (42 µL pNPB 98% was mixed with 458 µL dimethylsulfoxide (DMSO)
    - 300 mM TRIS/HCl, pH 7 (36.342 g were dissolved in dH_2O, pH was set with conc. HCl and then filled up to 1 L)
    - pNPB-assay solution (0.084 % pNPB): 500 µL of pNPB-stock solution was mixed with 49.5 mL ddH_2O in a 50 mL Greiner® tubes which was covered in aluminium foil to avoid autolysis.

2.6.Strains

P. pastoris CBS7435 was used as base for the knock-out experiments and *E. coli* Top 10 F' for all cloning approaches. Both were taken from the strain collection of ACIB GmbH/ Institute of Biotechnology. Other strains, which were provided by others (Thomas Vogl) and used for experiments are listed in Table 3.

All generated strains during the work on this thesis were stored at the strain collection and are listed in Table 30 in the Appendix section (7.3 Generated Strains).

Table 3 Strains used for different experiments in this work.

Host strain	Host strain designation	Plasmid	Collection No.	Internal No.
P. pastoris	CBS7435	pPICZ-BD1-BDH	3441	
P. pastoris	CBS7435 mutS	pPpT4mutZeoMlyI-intArg4-bidi-CalB_VTU-synPDImutBmrI-pAOX1+pGAP-fwd K1 [short 3]		638
P. pastoris	CBS7435 mutS	pPpT4mutZeoMlyI-intArg4-bidi-CalB_VTU-synPDImutBmrI-GAP+pCAT1-rev K1 [short 8]		643

2.7.Sequences

All sequences used for generation of knock-out vectors and vector sequences itself are listed in Table 29 in the 7.1 Sequences section.

2.8.Primers

Table 4: Primer list

Internal no.	Primer name	Sequence
C43	AOXTT_seq_rev	TCCCAAACCCCTACCACAAG
C54A	3UTRDAS1F	TCG GCC GAT CAG GCC ACG GGA AGT CTT TAC AGT TTT AGT TAG
C55	3UTRDAS1R	GCA TAT CGT AGT CCA ATT TAA ATT GTC ATA CAG ATC CAA TGC TGC
C56	5UTRDAS1F	ATG ACA ATT TAA ATT GGA CTA CGA TAT GCT CCA ATC C
C57	5UTRDAS1R	TCG GCC CTA GTG GCC GTT GTT TGT AAG TAA ACG AAT CAA GAT ACT G
C58	Up5UTRDAS1F	TACCCAATTCAGTGGAACCGTTC
C59	Down3UTRDAS1R	CTC TGC TAG TAA GGT ACA TCA TCA CGG TC
C60	3UTRDAS2F	TCG GCC GAT CAG GCC TTT TGA TGT TTG ATA GTT TGA TAA GAG TGA AC
C61	3UTRDAS2R	GGA ATA AGC AGA ACT GTA GAT TTA AAT CAA ACT CTT CAT CCA GAC TCT CAT C
C62	5UTRDAS2F	GAA GAG TTT GAT TTA AAT CTA CAG TTC TGC TTA TTC CCC C
C63	5UTRDAS2R	TCG GCC CTA GTG GCC GTA GAT TTG GCC ACT AAC GGG TTA G
C64	Up5UTRDAS2F	GAT GTA AGA CGT GAC GAT GAT TGG
C65	Down3UTRDAS2R	TAA TCC GGA AGT TCT TCT CCT GG
C66	3UTRDAS1&2F	TCG GCC GAT CAG GCC GTG ACG AGT TAG TAA AGA ACT GGA AAA AG
C67	3UTRDAS1&2R	CGA CAA ACT ATA AGT AGA TTT AAA TTG ATG GAG TCT ATA CTA GGC TAA C

C68	5UTRDAS1&2F	TAG TAT AGA CTC CAT CAA TTT AAA TCT ACT TAT AGT TTG TCG TGC TTT G
C69	5UTRDAS1&2R	TCG GCC CTA GTG GCC CGC TGC TCA CGG TTC
C70	Up5UTRDAS1&2F	CTT ACA CAA CTA CTA ACC CGT TAG TGG C
C71	Down3UTRDAS1&2R	TGG GAG TCC CTA TTC TAC TTA GTC TCA TAT C
C72	3UTRTKL1F	TCG GCC GAT CAG GCC TTT GTG CGC CTT TAG GTA GTT AG
C73	3UTRTKL1R	TAT CAA AGT TGA ATG CAT ATT TAA ATT GTC CAG ACT CAT TTG ATG CAG
C74	5UTRTKL1F	CAA ATG AGT CTG GAC AAT TTA AAT ATG CAT TCA ACT TTG ATA TTG TGT ATC GAC AC
C75	5UTRTKL1R	TCG GCC CTA GTG GCC TGT GTA GAG TGG ATG TAG AAT ACA AGT CTA GAG
C76	Up5UTRTKL1F	CCT ATG AAA TCT GTA GTC ACG TAC TGA TAC C
C77	Down3UTRTKL1R	CTT GAC GCT CCA ACA TCT GAA TCG
C78	3UTRTKL1_disF	TCG GCC GAT CAG GCC ACT CTC ACG AGC AAT TTG GTA TC
C79	3UTRTKL1_disR	AAT ATC AAA GTT GAA TGC ATA TTT AAA TTA ATA CGG CGA AGG AGT TGC
C80	5UTRTKL1_disF	TTC GCC GTA TTA ATT TAA ATA TGC ATT CAA CTT TGA TAT TGT GTA TCG
C81	5UTRTKL1_disR	TCG GCC CTA GTG GCC TGT GTA GAG TGG ATG TAG AAT ACA AGT C
C82	3UTRDAS1&2R_clean	AAG CAG AAC TGT AGA TTT AAA TTG TCA TAC AGA TCC AAT GCT GC
C83	5UTRDAS1&2F_clean	TTG GAT CTG TAT GAC AAT TTA AAT CTA CAG TTC TGC TTA TTC CCC
C84	3UTRDAS1F_sequencing	TCT GCC GTA GAT GTT ACC AGA CTG
C85	3UTRDAS2F_sequencing	GGT ATT TCT GTG GGG TAG CAT AGC
C86	3UTRDAS1&2F_sequencing	GAA AGC CAG ATG TGC AAC TGA TC
C87	3UTRTKL1F_sequencing	CTC CTG GGA AGC CAT GTC TC
C88	3UTRTKL1_disF_sequencing	TCC TTA CTA CGG CGG GTC TTC
C89	3UTRDAS1&2_cleanF_sequencing	GGG GGA GAC TTT TAC TGG ATT TGG
C108	CDSDAS1F	CAA AGC AGT TTC TTA CAA TGA TGA CAT CCA TGA C
C109	CDSDAS1R	AAA GGA ACT CTT GTC CCT CAC GAG GG
C110	CDSDAS2F	CTT AAC GTA CTC ATC CAA AAG TTT CTT CCA GTT AG
C111	CDSDAS2R	AGT ATC GAC ACA AGA TGA CAT TCA TGA ATT GG
C112	CDSTKL1F	TGG CCA AGG CTA ACT CCG G
C113	CDSTKL1R	GAC AGA CAT TAT TGG AAC ACC GTC TGG
C114	Primer_PGAP-fw	GAAACCACCAGAATCGAATAT
C115	PucSeqF	CTTTTTACGGTTCCTGGCCTTTTGC
C116	PAox1SeqR	GGTTTCATTCAACCTTTCGTCTTTGGATG
	DAS1_check_fwd	GCAGGATGCCTGATATATAAATCCCAGATGATC
	DAS1_check_rev	CATCAGATATTATCATCGCGGCTTACGTAATAAC
	DAS2_check_fwd	CCATCCCACCCTAGGATGTCCTACAGG
	DAS2_check_rev	CAAGTTCGTTTTAACTTAAGACCAAAACCAGTTACAAC
C119	EcoRI_CpXYL1opt_fwd	ATT TAA TTT ATT TGT CCC TAT TTC AAT CAA TTG AAC AAC TAT CAA AAC ACA GAA TTC GAA ACG ATG TCC ACT GCT ACT G
C120	NotI_CpXYL1opt_rev	AAA TGA AGC CTG CAT CTC TCA GGC AAA TGG CAT TCT GAC ATC CTC TTG AGC GGC CGC TTA CAC AAA GAC TGG AAT G

C121	EcoRI_ScBDH1_fwd	ATT TAA TTT ATT TGT CCC TAT TTC AAT CAA TTG AAC AAC TAT CAA AAC ACA GAA TTC GAA ACG ATG AGA GCT TTG GCA TAT TTC
C122	NotI_ScBDH1_rev	ATC AAA AAT GAA GCC TGC ATC TCT CAG GCA AAT GGC ATT CTG ACA TCC TCT TGA GCG GCC GCT TAC TTC ATT TCA CCG

2.9.Kits

- GeneJET™ Plasmid Miniprep Kit, Thermo Scientific. Changes to manual: Cells were taken off an agar plate, grown over night and directly resuspended in the resuspension solution.
- Wizard® SV Gel and PCR Clean-Up System, Promega
- CloneJET™ PCR Cloning Kit, Thermo Scientific: For the transformation into *E.coli* the whole ligation mix (20 µL) were directly used.

2.10. Methods

2.10.1. Agarose gel-electrophoresis

The concentration of agarose (Biozym) was 1% in TAE-buffer solution. The solution was heated in a microwave to dissolve the agarose. After cooling down the agarose solution ethidium bromide was added. GeneRuler™ 1kb DNA Ladder or GeneRuler™ DNA Ladder Mix (Figure 9) were used as a standard. The gel was run at 120 volts for 45 minutes for an analytical control gel and 1.5 hours at 90 volts for preparative purpose.

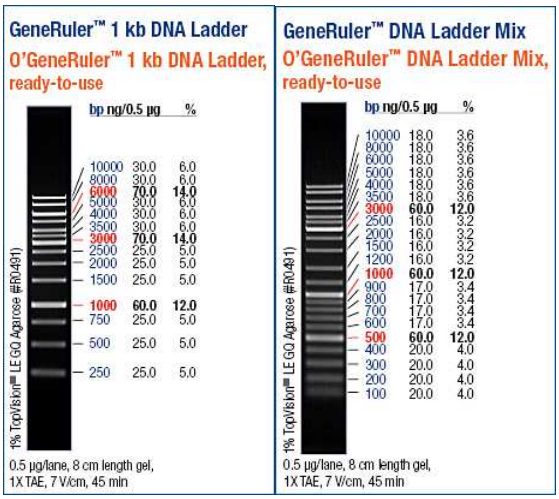


Figure 9: GeneRuler™ 1kb DNA Ladder and GeneRuler™ DNA Ladder Mix

2.10.1.1. Preparative DNA digests

For preparative DNA digest 0.5 µL of each restriction enzyme (FastDigest enzymes by Thermo Scientific) per 10 µl reaction mixture or 500 ng of DNA respectively were added. Whereby 1 µL of FastDigest enzymes is formulated to digest up to 1 µg of plasmid DNA in 20 min, 0.2 µg of PCR product in 30 min and 1µg of genomic DNA in 30 min. The Standard mixture (Table 5) is incubated at 37°C for 3 h. The volume of the approach was adjusted to the volume and/or the concentration of the DNA respectively.

Table 5 Standard reaction mixture for preparative DNA digest. The approach is incubated for 3 hours at 37°C.

Component	Volume [µL]
DNA (plasmid DNA, gDNA, PCR product)	Up to 50 µL
FastDigest restriction enzyme(s)	1 µL/µg DNA
FastDigest Buffer 10x	6 µL
ddH_2O	Fill up to 60 µL

2.10.1.2. Analytical DNA digests

The standard reaction mixture for analytical DNA digests with FastDigest restriction enzymes of plasmids is shown in Table 6. Depending of the concentration of the template, the volume of which was changed.

Table 6 Standard reaction mixture for analytical DNA digest. The mixture is incubated for 30-60 min at 37°C.

Component	Volume [µL]
DNA (plasmid DNA)	1 µL
FastDigest restriction enzyme(s)	0.5 µL
FastDigest Buffer 10x	1 µL
ddH_2O	Fill up to 10 µL

2.10.2. PCRs

2.10.2.1. Phusion High-Fidelity DNA polymerase (Thermo Scientific)

In Table 7 and Table 8 the standard procedures for PCR based on Phusion™ High Fidelity DNA Polymerase are listed.

Table 7: Standard mix for Phusion™ DNA polymerase

Component	Volume [µL]
5x Phusion HF buffer	10
dNTPs (2mM)	5
Forward primer (5 µM)	2
Reverse primer (5 µM)	2
Template (1ng/µL)	2
Phusion™ polymerase (2 U/µL)	0.3
ddH2O	Up to 50

Table 8: Standard program for PCR reactions using Phusion™ DNA polymerase

Temperature [°C]	Time [sec]	
98	30	
98	10	35 cyles
adjusted to primers	20	
72	30/1 kb	
72	10 min	
4	∞	

2.10.2.2. Overlap-extension-PCR (oePCR)

For oePCR a two-step procedure with Phusion™ High Fidelity DNA Polymerase was applied. In the first step overlapping fragments (fragment 1, fragment 2) with the standard PCR mix (see Table 9) was run for 15 cycles. The overlapping regions of fragment 1 and fragment 2 are annealing and elongated 5’ to 3’ during the PCR reaction constructing one DNA strand out of the 2 fragments.

Table 9: Standard mix for oePCR with Phusion™ DNA polymerase

Component	Volume [µL]
5x Phusion HF buffer	10
dNTPs (2mM)	5
Fragment 1 (2-15 ng/µL)	3
Fragment 2 (2-15 ng/µL)	3
Phusion™ polymerase (2 U/µL)	0.5
ddH2O	Up to 50

In a second step, 20 µL of fresh PCR mix containing the outer primers to amplify the full-length PCR product was added, (see Table 10). The program for a standard oePCR is shown in Table 11.

Table 10: Primer mix for oePCR which is added after the first 15 cycles of the PCR

Components	Volume [µL]
5x Phusion HF buffer	4
dNTPs (2mM)	2
Forward primer (5 µM)	4
Reverse primer (5 µM)	4
Phusion™ polymerase (2 U/µL)	0.5
ddH2O	Up to 20

Table 11: Standard program for oePCR with Phusion™ DNA polymerase

Temperature [°C]	Time [sec]		
98	30		
98	10	15 cycles without primers	20 cycles with primers
adjusted to primers	20		
72	15/1 kb		
72	10 min		
4	∞		

2.10.2.3. DreamTaq™ DNA polymerase (Thermo Scientific)

For PCR reactions conducted with DreamTaq™ DNA polymerase (Thermo Scientific), the standard protocol for a PCR reaction is described in Table 12 and Table 13. DreamTaq™ DNA polymerase was used for checking the correct size of several constructs, where proof-reading activity was not necessary.

Table 12: Standard mix for DreamTaq™ DNA polymerase

Component	Volume [µL]
10x Taq buffer	2.5
dNTPs (2mM)	2.5
Forward primer (5 µM)	2
Reverse primer (5 µM)	2
Template (10-50 ng/µL)	1
DreamTaq polymerase (5 U/µL)	0.2
ddH2O	Up to 25

Table 13: Standard program for DreamTaq™ DNA polymerase

Temperature [°C]	Time [sec]	
95	30	
95	10	35 cyles
adjusted to primers	20	
72	60/1 kb	
72	10 min	
4	∞	

2.10.3. Assembly Cloning – cloning by *in vitro* recombination (acib protocol)

Solutions were prepared as described in 2.3.6. for cloning by *in vitro* recombination. A 15 µL assembly master mix aliquot was thawed and mixed via pipetting with 5 µL DNA. DNA should be used in equimolar amounts of each fragment with a minimal concentration of 100 ng of each fragment. Equation 1 below describes the calculation for each fragment:

Equation 1: Formula for calculation of amount of linear DNA of the 2 fragments, which were cloned together.

$$\boldsymbol{amount}_{\,large\ fragment}[\boldsymbol{ng}] = \frac{\boldsymbol{amount}_{small\ fragment}\ [\boldsymbol{ng}] * \boldsymbol{size}_{large\ fragment}\ [\boldsymbol{kb}]}{\boldsymbol{size}_{small\ fragment}\ [\boldsymbol{kb}]}$$

After mixing the components, the reaction incubated on a thermo cycler at 50 °C for 60 minutes. If plasmids were assembled, the mix was desalted for 20 min and 5-20 µL were used to transform competent *E. coli* cells. Transformed cells were plated on selective plates and single clones were then analysed via restriction digest and sequencing (LGC Genomics).

2.10.4. Standard cloning protocol

For standard cloning purposes, the T4 DNA ligase (Thermo Scientific) was used. Before ligation the vector had to be linearized and the insert DNA had to be cut with the desired restriction enzymes (see 2.10.1.1). 100 ng of linearized vector was mixed with insert DNA (ratio vector to insert 1:3) using Equation 2.

Equation 2: Formula for calculation amount of insert DNA to certain amount of linearized vector DNA

$$ng\ of\ insert\ required = \frac{ng\ of\ vector\ \times kb\ size\ of\ insert}{kb\ size\ of\ vector\ \times insert : vector\ ratio}$$

The calculated amount was then mixed with T4 DNA ligase, T4 DNA ligase buffer 10x and filled up with ddH_2O to 20 µL (Table 14). The ligation mix was either incubated at 16°C overnight or at 22°C for 20-30 min in an incubator.

Table 14: Compounds for ligation of insert DNA into linear vector DNA with T4 DNA ligase (Thermo Scientific)

Compound	**Volume [µL] / amount [ng]**
Linear vector DNA	100 ng
Insert DNA	3:1 molar ratio over vector
T4 DNA ligase (5 U/µL)	1 µL
T4 DNA ligase buffer 10x	2 µL
H_2O	Up to 20 µL

After incubation the ligation mixture was deactivated at 70°C for 10 min and desalted for 25 min with a DNA filter paper. DNA was ready for electroporation into *E. coli*.

2.10.5. Construction of knock-out strains

2.10.5.1. Knock-out strategy

For knocking-out the desired genes in the genome of *P. pastoris* CBS7435 the vector-system pPpKC1 (Figure 10), based on the pPp_T4 plasmid, was used (placed at the disposal by Mudassar Achmad). pPpKC1 is a shuttle-vector for *E. coli* and *P. pastoris*, containing a Zeocin™-resistance marker under the control of the *ARG4* promoter and terminator for expression in *P. pastoris* and a EM72 Syn B promoter for expression in *E. coli*. For autonomous replication in *E. coli* the vector harbors a pUC origin of replication. There is a stuffer fragment flanked by two *SfiI* (5'-GGCCNNNN^NGGCC-3') restriction sites. Cloning with *SfiI* has a big advantage as some nucleotides of the recognition site can be varied. If designed smartly, two different overhangs are gained, which allows to clone the insert in the desired direction into the vector by using only *SfiI* as restriction enzyme. For the generation of knock-outs two homologous recombination sites flanking the gene of interest (GOI) are needed, which should be around 1 kb. Gene replacement events can be >50% of the total transformants population, when targeting fragments are each >1kb, but drop to <0.1% when fragment size is <0.5kb (92). The integration frequency is also strongly dependent of the locus where the replacement should happen and has to be considered as well (93). When linearizing the vector, as in the pPpKC1 approach, the frequency of the replacement events are also reduced (92). The empirical value for the size (~1kb) of the homologous regions for the pPpKC1 supported knock-out provided by Mudassar Achmad is in line with the data from literature. Other important features of the knock-out cassettes

are the FRT sequences flanking the stuffer fragment, which are recognized by a flippase required for the later marker recycling step. The flippase gene is under the control of the *AOX1* promotor, to initiate the vector recycling by the addition of methanol. For linearization of the vector (to obtain the linear knock-out cassette) a recognition site for a blunt end cutting enzyme (*SmiI*/*SwaI*) has to be introduced between the two homologues recombination sites.

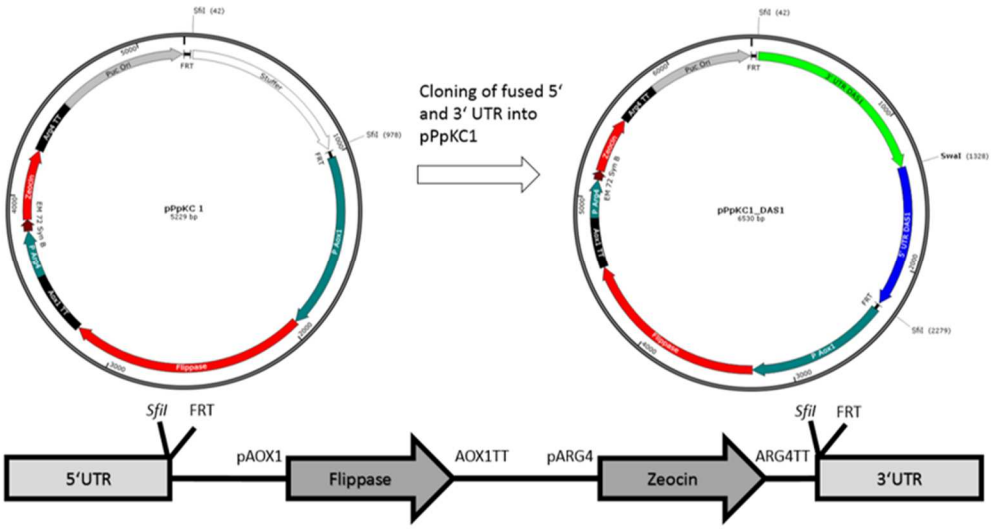


Figure 10: Schematic representation of knock-out strategy I
pPpKC1 vector with a flippase from *S. cerevisae* under the control of *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT, respectively). It also has a stuffer fragment flanked by 2 *SfiI* sites for cloning homologous sequences for recombination and 2 FRT sites. The FRT sites are recognized by the flippase for excision of the marker after successful replacement of the GOI. As selection marker zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used. The synthetic promoter EM72SynB for expression of the zeocin-resistance-protein and PucOri for replication in *E .coli* is present in the vector as well (adapted from Mudassar Achmad, data not published).

The first step was to design 4 primers (P1, P2, P3, P4) for each GOI that should be removed from the genome, as depicted in Figure 11. P1 is designed to be about 1 kb upstream of the gene in the 5'UTR (untranslated region) and P4 1 kb downstream of the gene in the 3'UTR. It was important to have half of the recognition site of the blunt-end cutting enzyme (*SwaI*) (ATTT or AAAT) at the 5'-end of the primer P1 and the other half at the 5'-end of the primer P4. That was a limitation for finding a suitable starting point for the PCR in the genome and determined the exact size of the homologous region, because this sequence had to be present in the 5'UTR as well as in the 3'UTR of the GOI. Primers P2/P3 start either within the gene, for partial gene disruption (pPpKC1_DAS1&2 and pPpKC1_TKL1_dis) or directly upstream of the start ATG and downstream of the stop-codon respectively for a clear knock-out. For later cloning into the pPpKC1-vector the introduction of *SfiI* recognition sites at the 5'-end of P2 and P4 with different variable nucleotides (see vector sequence) was necessary to assure the cloning happens in the right direction.

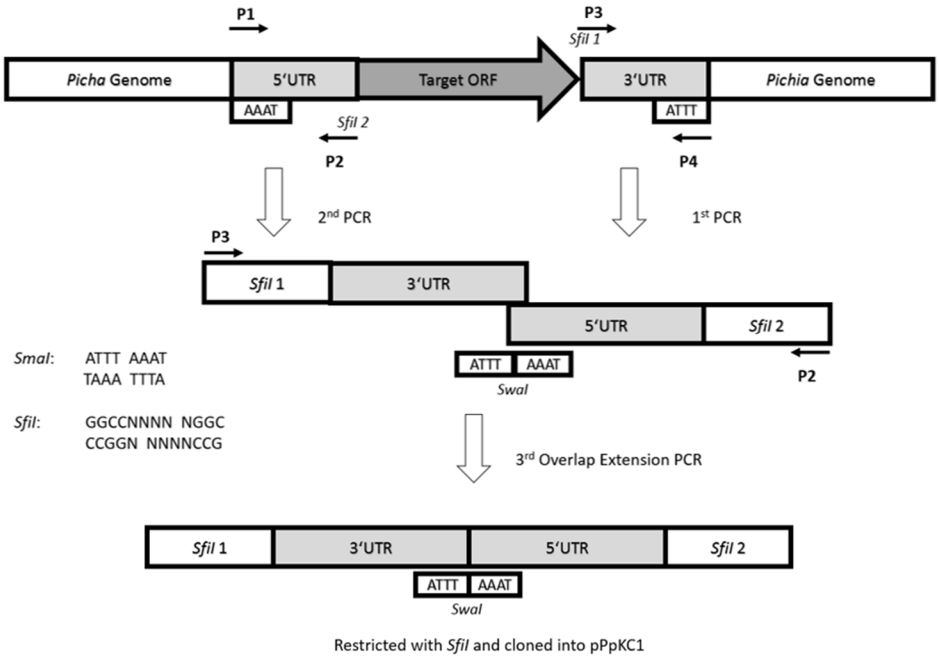


Figure 11: Schematic representation of knock-out strategy II
Two primer pairs (P1, P2 and P3, P4) amplifying the 5' and 3' UTR in 2 PCR reactions were designed. P1 and P4 had to start with ATTT or AAAT respectively for introducing a *SwaI* cutting side for later linearizing of the vector. To ensure cloning of the fused 5' and 3' UTR 2 *SfiI* sites with different overhangs had to be attached on the 5' end of P2 and P3. They all started upstream and downstream of the ATG and the stop-codon respectively, except cassettes for pPpKC1_DAS1&2 and pPpKC1_TKL1_dis. In a second step, an oePCR was performed with the fragments generated in the 1st and 2nd PCR and the resulting product was further restricted with *SfiI* for cloning into pPpKC1 (adapted from Mudassar Achmad, data not published).

After amplification of the homologous recombination sites in two separate PCR reactions using P1/P2 and P3/P4 according to the Phusion™ polymerase standard PCR protocol (2.10.2.1, 2.10.2.1 Phusion High-Fidelity DNA polymerase (Thermo Scientific)). *P. pastoris* CBS7435 gDNA was employed as template with a concentration of about 25 ng/µL. The isolated gDNA yielded in concentrations of 2.5-3.7 µg/µL and had had to be diluted 1:150. PCR products were separated on a preparative agarose gel, the desired bands (expected size of fragments are summarized in Table 16) were cut out, and purified via Wizard® SV Gel and PCR Clean-Up System (Promega). Concentrations of the purified DNA fragments were measured with NanoDrop 2000c spectrophotometer. As final step an oePCR, following the protocol for oePCR in section 2.10.2.2, with the fragments of PCR1 and PCR2 was performed to join these fragments. P2/P3 were used as outer primers for amplification of the joined fragments. The fragments were then separated on a preparative agarose gel, fragments were cut, cleaned up, and DNA concentration was determined. Subsequently, the fragments were cut with *SfiI* and cloned into the *SfiI* cut pPpKC1-vector. The pPpKC1 constructs containing the respective homologous recombination sites as insert were then used to transform *E. coli* Top 10F'. At least 4 clones were checked via restriction digest for the correct insertion of the insert. The sequence of the

final knock-out cassette was confirmed by Sanger sequencing (Microsynth AG) with primers listed in Table 15.

Table 15 List of used sequencing primers for constructed knock-out vectors

Construct	**Primer fwd**	**Primer binding in the homologous region of the fusion construct**	**Primer rev**
pPpKC1_DAS1	PucSeqF	3UTRDAS1F_sequencing	Seq_AOX1rev
pPpKC1_DAS2	PucSeqF	3UTRDAS2F_sequencing	Seq_AOX1rev
pPpKC1_DAS1&2_clean	PucSeqF	3UTRDAS1&2_cleanF_sequencing	Seq_AOX1rev
pPpKC1_TKL1	PucSeqF	3UTRTKL1F_sequencing	Seq_AOX1rev
pPpKC1_TKL1_dis	PucSeqF	3UTRTKL1_disF_sequencing	Seq_AOX1rev

The pPpKC1_HRS (homologous recombination site) was linearized with a blunt cutting restriction enzyme (*SwaI/SmiI*) and used to transform *P. pastoris* CBS7435. In this approach 2 homologous recombination events at the two HRS are necessary (see Figure 12) (94). Clones with integrated cassette were selected on YPD plates supplemented with 50 mg/L Zeocin®. To control the correct integration site as well as the full integration of the cassette colony PCRs were performed.

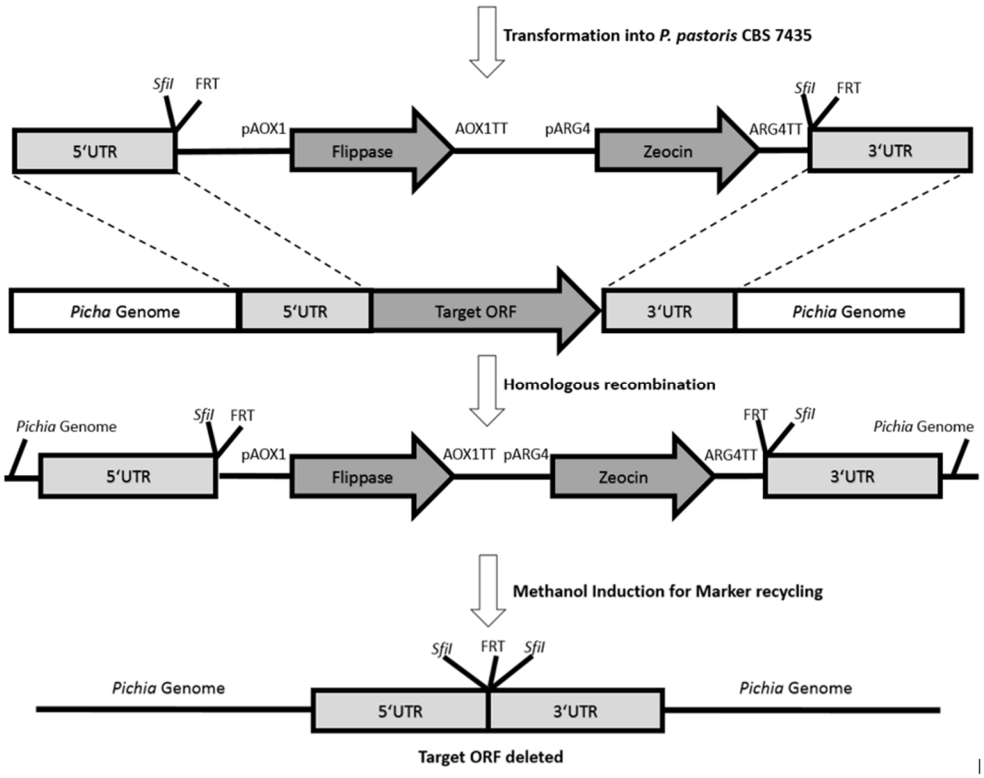


Figure 12: Schematic representation of knock-out strategy III
After construction of the knock-out cassette, the linearized vector was used to transform *P. pastoris* CBS7435. At the 5' and 3' UTR of the GOI in the genome a double homologous recombination event is supposed to happen, excising the part in between the homologous sites and integrating the knock-out cassette (94). The proofed knock-out clones still harbored the marker, which had to be eliminated. Therefore the flippase under the control of P_{AOX1} was induced by methanol addition. The activated flippase excised the marker cassette in between the HRS to receive a strain with deleted GOI (adapted from Mudassar Achmad, data not published).

As final step a marker recycling was performed. Therefore, a single colony from a single-streak-out of the positive clone was inoculated in 250 mL YPD media and incubated at 28°C and shaking at 90 rpm. After 24 and 48 hours of growth 0.5 mL methanol and after 60 and 72 hours 1 mL methanol was added to express the flippase from *S. cerevisae* under control of P_{AOX1}, which then recognizes the FRT sites and excises the marker cassette located in between them to gain a strain with a deleted GOI as seen in Figure 12. A single streak-out out of the flask was done after 96h of growth on YPD-plates which were then incubated for 48 hours at 30°C. About 24 single colonies were cultivated in 96-well DWP with 600 µL YPD at 28°C, 320 rpm and 80% humidity for 24 hours and then stamped on YPD-plates and YPD-plates supplemented with Zeocin™ 100 mg/L. These plates were incubated for 2 days at 30°C. If the marker recycling was successful the colony was growing on the YPD plates, but not on those containing Zeocin™.

2.10.5.2. Genomic DNA (gDNA) isolation from *P. pastoris* CBS7435: Bust´n´Grab

For gDNA isolation of *P. pastoris* CBS7435 a slightly altered protocol from Hariju et al. was applied (95). After two days of growth at 30°C on YPD plates, yeast colonies were resuspended in 100 µL yeast lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) and well mixed. Afterwards the suspension was frozen in liquid N_2 for about 2 minutes and then heated to 99°C in a thermomixer. This step was repeated once and the suspension was mixed in a vortex again. After adding 100 µL chloroform and 2 minutes of mixing the sample was centrifuged at 13 000 rpm for 3 minutes. The supernatant was transferred in 400 µL of ice cold ethanol (100%), incubated for 25-30 minutes at -21°C and centrifuged at 13 000 rpm for 5 minutes. The supernatant was decanted and the pellet was washed with ice cold ethanol (70 %) followed by centrifugation for 5 minutes at 13 000 rpm. The supernatant was removed using a pipette and the pellet was dried in an incubator at 37 °C. The dried pellet was dissolved in 20 µL of pre-warmed (55°C) ddH_2O and was stored at -21 °C. DNA concentration was determined with a NanoDrop 2000c spectrophotometer.

2.10.5.2.1. Knock-out cassettes

For construction of all knock out cassettes *P. pastoris* CBS7435 gDNA was used as a template (for isolation procedure, see 2.10.5.2). There are in total 5 cassettes planned (pPpKC1_DAS1, pPpKC1_DAS2, pPpKC1_DAS1&2, pPpKC1_DAS1&2_clean, pPpKC1_TKL1, pPpKC1_TKL1_dis), which were constructed in a 3 step process (see Figure 11). First the 5'-UTR and the 3'-UTR for each cassette (see Table 16 for used primers and Figure 13 for differences of pPpKC1_DAS1&2 and pPpKC1_DAS1&2_clean) were amplified in separate PCRs (2.10.2.1).

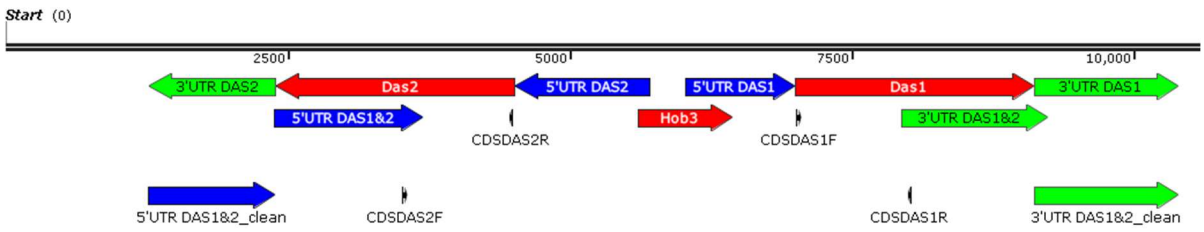


Figure 13 Schematic overview of the 5' and 3'UTRs used for the *DAS1*, *DAS2*, *DAS1/2* and *DAS1/2* clean knock-out cassettes. The used primers for generating 5' and 3'UTR are summarized in Table 16. The 5'UTR and the 3'UTR of pPpKC1_DAS1&2 are within the *DAS2* and the *DAS1* gene respectively. In contrary, the 5'UTR (3'UTR DAS2) and the 3'UTR (3'UTR DAS1) of pPpKC1_DAS1&2_clean are flanking the genes. Primer binding sites for verification of full marker recycling are highlighted with black arrows.

Table 16: Primers used for the construction of the knock-out cassettes. For amplifying the 5'UTR, and 3'-UTR regions *P. pastoris* CBS7435 gDNA was used as a template. 5'UTR and the 3'UTR have overlapping regions that were used to join the fragments in the subsequent oePCR.

Vector	Amplified fragment	Forward primer	Reverse primer	PCR-product size (bp)
pPpKC1_DAS1	DAS1 3' UTR	3UTRDAS1F	3UTRDAS1R	1281
	DAS1 5' UTR	5UTRDAS1F	5UTRDAS1R	978
pPpKC1_DAS2	DAS2 3' UTR	3UTRDAS2F	3UTRDAS2R	1200
	DAS 5' UTR	5UTRDASF	5UTRDAS2R	1125
pPpKC1_DAS1&2	DAS1&2 3' UTR	3UTRDAS1&2F	3UTRDAS1&2R	1300
	DAS1&2 5' UTR	5UTRDAS1&2F	5UTRDAS1&2R	1320
pPpKC1_DAS1&2_clean	DAS1&2_clean 3' UTR	3UTRDAS1F	3UTRDAS1&2_cleanR	1286
	DAS1&2_clean 5' UTR	5UTRDAS1&2_cleanF	5UTRDAS2R	1133
pPpKC1_TKL1	TKL1 3' UTR	3UTRTKL1F	3UTRTKL1R	1131
	TKL1 5' UTR	5UTRTKL1F	5UTRTKL1R	1228
pPpKC1_TKL1_dis	TKL1_dis 3' UTR	3UTRTKL1_disF	3UTRTKL1_disR	1086
	TKL1_dis 5' UTR	5UTRTKL1_disF	5UTRTKL1_disR	1228

The generated 5'-UTR and 3'-UTR fragments of each cassette had overlapping sequences, which were important for the second step, the overlap-extension-PCR (see 2.10.2.2). The overlapping sequences were used to fuse the fragments together. Outer primers, summarized in Table 17, were added to amplify the full-length fusion construct.

Table 17: Fragments and primers used for oePCR.

Vector	Fragment 1	Fragment 2	Forward primer	Reverse primer	oePCR product size (bp)
pPpKC1_DAS1	DAS1 3'-UTR	DAS1 5'-UTR	3UTRDAS1F	5UTRDAS1R	2272
pPpKC1_DAS2	DAS2 3'-UTR	DAS 5'-UTR	3UTRDAS2F	5UTRDAS2R	2327
pPpKC1_DAS1&2	DAS1&2 3'-UTR	DAS1&2 5'-UTR	3UTRDAS1&2F	5UTRDAS1&2R	2621
pPpKC1_DAS1&2_clean	DAS1&2_clean 3'-UTR	DAS1&2_clean 5'-UTR	3UTRDAS1F	5UTRDAS2R	2419
pPpKC1_TKL1	TKL1 3'-UTR	TKL1 5'UTR	3UTRTKL1F	5UTRTKL1R	2372
pPpKC1_TKL1_dis	TKL1_dis 3'-UTR	TKL1_dis 5'UTR	3UTRTKL1_disF	5UTRTKL1_disR	2334

After the oePCR, the obtained PCR product (5'UTR and 3'UTR) was cleaned up and cut with *SfiI*. The full-length fusion constructs were cloned into a *SfiI* cut pPpKC1 and further used to transform *E. coli* Top 10 F'. Phenotypically positive clones (growth on LB-Zeocin™ 25 mg/L) were analyzed with restriction digest and sequencing (LGC genomics). In Figure 14 an example for a knock-out-vector pPpKC1_DAS1) is shown. Each construct listed in Table 17 with the correct sequence was used to transform *P. pastoris* CBS 7435 cells as described in 2.10.9 and 2.10.10.

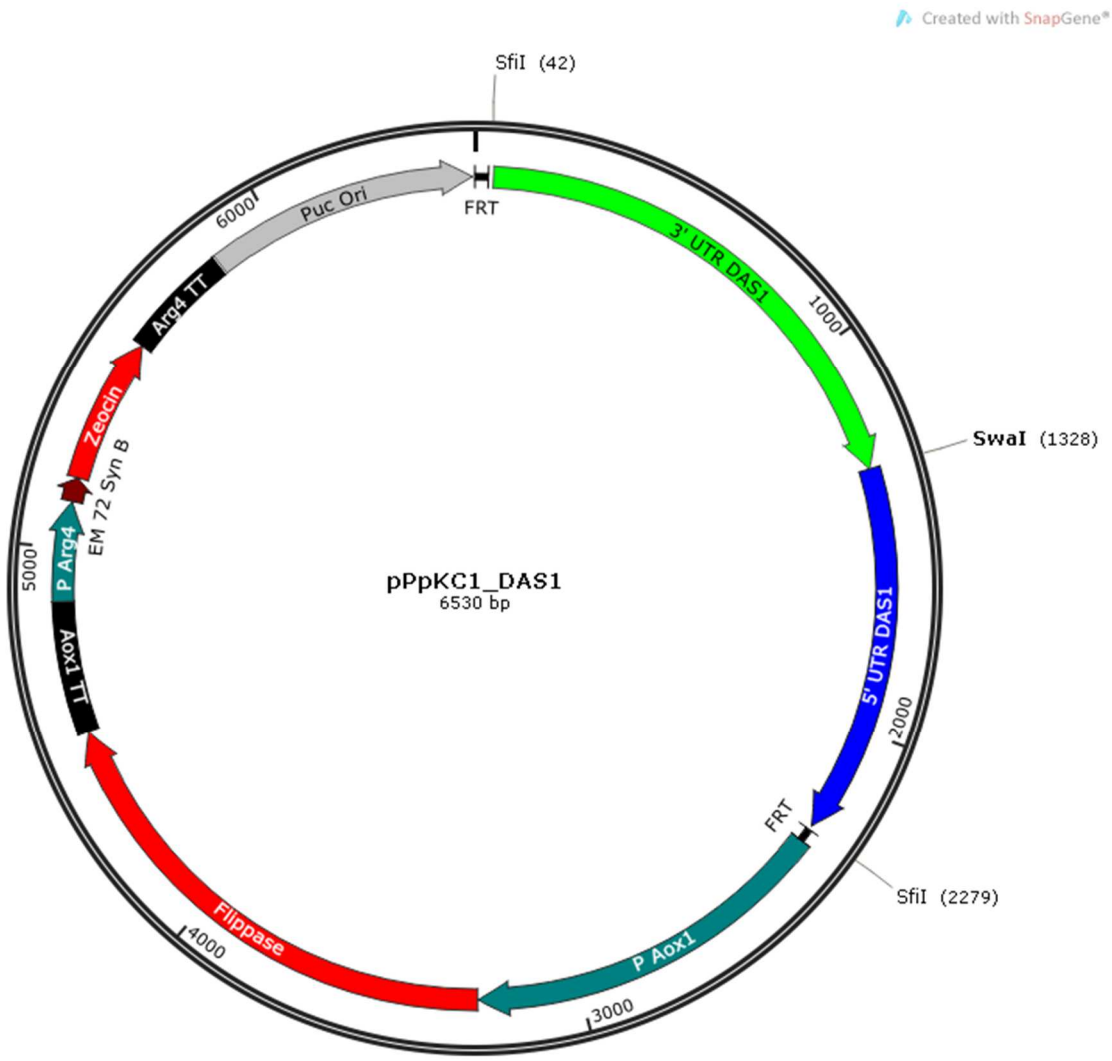


Figure 14: Plasmid map of pPpKC1_DAS1
pPpKC1_DAS1 vector harbors 2 homologous recombination sites located at the 5' UTR and 3'UTR of *DAS1* in the *P. pastoris* genome. In between these 2 regions a *SwaI*/*SmiI* cutting site has been introduced, which is required for later linearization of the vector. The 2 *SfiI* sites for cloning flank the 2 HRS as well as 2 FRT sites. The FRT sites are recognized by the flippase from *S. cerevisiae* which is under the control of the *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT respectively) for excision of the marker after successful deleting of the GOI. As selection marker zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used for expression in *P. pastoris*. The synthetic promoter EM72SynB was used for expression of Zeocin-resistance-protein expression. The plasmid also harbored a pUCori for replication in *E. coli*.

2.10.5.3. gDNA isolation *from P. pastoris* CBS7435 for knock-out screenings

For the isolation of genomic DNA from *P. pastoris* knock-out strains the protocol by Hofmann and Winston adapted by Mudassar Ahmad was appplied (96).

Single colonies of *P. pastoris* were cultivated for 24 hours at 28 °C, 320 rpm and 80 % humidity in deep well plates (DWP) containing 600 µL YPD media. Each culture was transferred into Eppendorf tubes and centrifuged at 13 000 rpm for 2 minutes. The supernatant was discarded and 150 µL yeast

lysis buffer, 150 µL of a phenol: chloroform: isoamyl alcohol (25:24:1) mixture and 0.3 g of glass beads were added sequentially. To enhance cell lysis, the mixture was vortexed for at least 8 minutes. Then 150 µL of TE-buffer were added into the tubes and centrifuged at 13 000 rpm for 10 minutes to separate the phases. The aqueous phase was then transferred into a new tube with 1 mL of 100% ice-cold ethanol. After incubation at -21 °C for 30 minutes, white coils (precipitated gDNA) were visible in the tube. Centrifugation at maximum speed at 4°C fixed the gDNA as pellet at the bottom of the tube. The supernatant was removed and the pellet was air-dried at 60°C and was dissolved in 200 µL of ddH_2O. Concentration of the gDNA was measured with NanoDrop 2000c spectrophotometer.

2.10.5.4. Verification of knock-out cassette integration and CDS deletion

The positive clones growing on YPD-Zeocin™ 50 mg/L plates after transformation of the knock-out cassette were verified on genomic level for proper integration and also for the complete absence of the coding sequence (CDS) of the GOI. Clones were cultivated and gDNA was isolated as described in 2.10.5.3. PCRs were done with DreamTaq DNA Polymerase Kit (see 2.10.2.3)
A three step screening procedure based on colony PCR was applied. The first step was checking for proper 5'-integration of the cassette performing a PCR with a forward (fwd) primer binding upstream of the 5'UTR and a reverse (rev) primer binding in the *AOX1* promotor region of the knock-out vector. Positive clones were then checked for proper 3'-integration via a forward primer binding at the pUCori and a reverse primer binding downstream of the 3'-UTR (used primers are summarized in Table 18, primer sequences are summarized in Table 4)

Table 18: Primers for 5'- and 3'-UTR integration check PCRs

***P. pastoris* CBS7435**	**5'-Integration primers**		**Band size (bp)**	**3'-Integration primers**		**Band size (bp)**
pPpKC1_DAS1	Up5UTRDAS1F	PAox1SeqR	1100	PucSeqF	Down3UTRDAS1R	1500
pPpKC1_DAS2	Up5UTRDAS2F	PAox1SeqR	1200	PucSeqF	Down3UTRDAS2R	1200
pPpKC1_DAS1&2	Up5UTRDAS1&2F	PAox1SeqR		PucSeqF	Down3UTRDAS1&2R	
pPpKC1_DAS1&2_clean	Up5UTRDAS2F	PAox1SeqR	1200	PucSeqF	Down3UTRDAS1R	1500
pPpKC1_TKL1	Up5UTRTKL1F	PAox1SeqR	1500	PucSeqF	Down3UTRTKL1R	1400
pPpKC1_TKL1_dis	Up5UTRTKL1	PAox1SeqR	1500	PucSeqF	Down3UTRTKL1R	1400

If both 5'- and 3'-integration events happened properly, there was a third PCR done to control if the CDS is still detectable, because of possible reintegration, with specific primers binding within the CDS of *DAS1*, *DAS2* and *TKL1* (Table 4). Expected sizes of the PCR product and the used primer pairs are listed in Table 19.

Table 19: Expected band sizes and primer pairs for CDS check PCRs for pPpKC1 transformants

***P. pastoris* CBS7435**	**Expected band size *DAS1* (bp)**	**Expected and size *DAS2* (bp)**	**Expected band size *TKL1* (bp)**
Primer pairs	**CDSDAS1F/CDSDAS1R**	**CDSDAS2F/CDSDAS2R**	**CDSTKL1F/CDSTKL1R**
WT	1010	973	1775
pPpKC1_DAS1	-	973	1775
pPpKC1_DAS2	1010	-	1775
pPpKC1_DAS1&2	-	-	1775
pPpKC1_DAS1&2_clean	-	-	1775
pPpKC1_TKL1	1010	973	-
pPpKC1_TKL1_dis	1010	973	-

2.10.5.4.1. Pool-screening

For the initial screening of correct 5'-integration a pool-screening method was applied. From each well (pPpKC1_DAS1, pPpKC1_DAS2, pPpKC1_DAS1&2_clean, pPpKC1_TKL1, pPpKC1_TKL1_dis) 150 µL of the ONC culture were pipetted into the well at the same position of a new sterile DWP and well mixed. This total amount of 750 µL was then transferred into an Eppendorf tube. gDNA was isolated from the obtained pellets (2.10.5.3) (Figure 15) and a colony PCR to verify 5'-integration of the knock out cassettes was performed (same procedure as mentioned in 2.10.5.4). Each gDNA sample was checked for proper integration of each knock-out cassette.

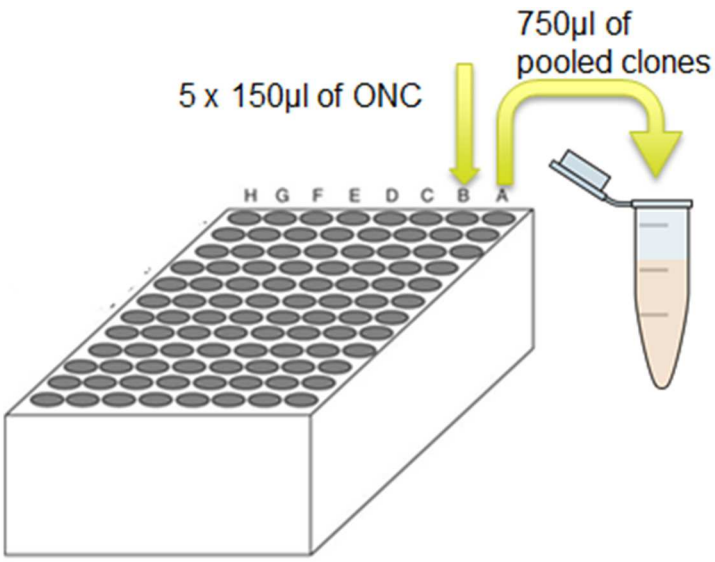


Figure 15: Pool screening schema for 5 different knock-out clones

The positive clones were then picked from stamped YPD-plates and inoculated in DWP as described in 2.10.5.3 and then screened for 5'-, 3'-integration and the deleted CDS.

2.10.5.5. Growing assay on MMMeOH1% plates

From fresh single-streak outs (30°C, 2 days on YPD-plates) of *P. pastoris* wild type strain and knock-out candidates a single colony was picked and single-streak outs were done from all strains on MMMeOH1%-square-plates and incubated for 6 days at 30°C.

2.10.5.6. Marker-recycling of knock-out clones

Marker-recycling was performed as described in 2.10.5.

2.10.5.6.1. Validation of marker-recycling

From each knock-out strain before and after marker-recycling and from the wild type strain, ONCs in 5 mL YPD (marker recycled clones) or YPD-Zeocin™ (100 mg/L) (clones before marker recycling) (28°C 90 rpm, in 50 ml Greiner® tubes) were done. 1 mL of this ONC was pipetted into Eppendorf® reaction tubes and centrifuged (13,000 rpm 10 min). Supernatant was discarded and the pellet was further used for gDNA Isolation (see 2.10.5.3). Again a PCR with primers (CDSDAS1F/CDSDAS1R, CDSDAS2F/CDSDAS2R, and CDSDTKL1F/CDSTKL1R) as described in 2.10.5.4 was performed to prove that the respective CDS (*DAS1*…) were indeed removed from the genome. For a second PCR, primers

binding at the edge of 5'UTR and 3'UTR of *DAS1* and *DAS2* were designed (see Figure 13 and Table 20) to check for a clean marker recycling.

Table 20: Primers and expected band sizes (pPpKC1 fully integrated, after marker recycling, wild type) of PCR products validate the marker recycling.

Knock-out	Cassette int. (bp)	After marker recycling (bp)	Wild type (bp)	Primers
Δdas1	4550	250	2400	DAS1_check_fwd; DAS1_check_rev
Δdas2	4500	200	2350	DAS2_check_fwd; DAS2_check_rev
Δdas1Δdas2	4600	270	7000	DAS2_check_fwd; DAS1_check_rev

2.10.5.7. Transformation of TKL1 and TKL1_dis knock-out cassettes

P. pastoris CBS7435 wildtype and *Δdas1Δdas2* were each 4 times transformed via electroporation (see 2.10.9 and 2.10.10) with 500 ng of pPpKC1_TKL1 and pPpKC1_TKL1_dis linearized with *SmiI*. Regeneration was done in 1 mL 1M Sorbitol and plated on selective minimal and full media with different carbon sources with 50 mg/L Zeocin™ (YPGY1%, YPMeOH1%, YPOA0.5%, YPSO1%, MMGY1%, MMMeOH1%, MMOA0.5%, MMSO1%) see 2.3.3 for details. On 2 plates of each media 250 µL of culture of each construct were plated.

2.10.6. Construction of model-protein vectors

2.10.6.1. Enhanced green fluorescence protein (eGFP)

The vectors (see Table 21 and the vector map of pPpT4mutZeoMlyI-intArg4-eGFP_pCAT1 in Figure 16 as an example) for eGFP screening were supplied by Thomas Vogl and used to transform *P. pastoris* CBS7435 wildtype, mutS, *Δdas1*, *Δdas2* and *Δdas1Δdas2*.

Table 21: pPpT4_eGFP vectors with different promoters. For sequences see Table 29.

Vector name	Promotor
pPpT4mutZeoMlyI-intArg4-eGFP_pAOX1BglII	*AOX1*
pPpT4mutZeoMlyI-intArg4-eGFP_pCAT1	*CAT1*
pPpT4mutZeoMlyI-intArg4-eGFP_pGAPshort	*GAP*short

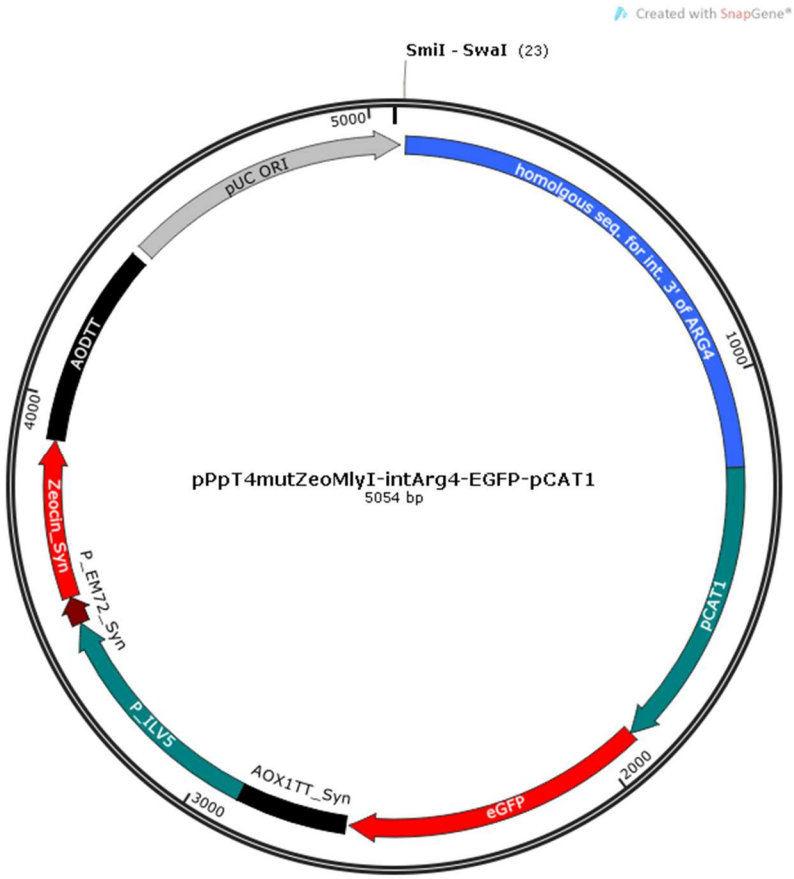


Figure 16 Vector map of the eGFP expression vector pPpT4mutZeoMlyI-intArg4-EGFP-pCAT1.
The vector is based on a pPpT4 vector with a homologous site for integration at 3' of *ARG4* in *P. pastoris* and the pUCori for replication in *E. coli*. Zeocin-resistance-protein under the control of the P_{ILV5} and AODTT for expression in *P. pastoris* and pEM72 for expression in *E. coli.* The expression of the model protein eGFP (enhanced green fluorescent protein) is under the control of P_{CAT1} (catalase promoter from *P. pastoris*) and the terminator of *AOX1* (AOX1TT).

2.10.6.2. *Candida antarctica* lipase B (CaLB)

From two *E. coli* Top 10F' strains provided by Tomas Vogl, one containing pPpT4-S_CAT1_calB and the other pPpT4-S_AOX1_calB (sequences are shown in Table 29), plasmid preparations were performed, DNA was linearized with *SwaI* FastDigest (Thermo Scientific) and used to transform (2.10.10) *P. pastoris* CBS7435 wild type and *Δdas1Δdas2* (2.10.9).

2.10.6.3. Butanediol1-dehydrogenase (BDH1)

A pPpT4_GAP-S vector (see Figure 17) containing *BDH1* from *S. cerevisiae* was constructed. Therefore, pPICZBd1-BDH1 (provided by Andrea Camattari, TU Graz) was used as template for a PCR (Phusion® High-Fidelity DNA polymerase) with assembly primers EcoRI_ScBDH1_fwd/ NotI_ScBDH1-rev. The PCR product had a length of 1150 bp and 5' and 3' overlaps with the vector. Subsequently the vector pPpT4_GAP-S digested with *EcoRI*/*NotI* (cut size: 3081 bp) was also prepared for assembly

cloning (protocol see 2.10.3). The assembled pPpT4_GAP-S_ScBDH1 vector was used to transform *E. coli* Top 10F' and transformants were selected on LB-Zeocin™ (50 mg/L) plates. A restriction digest with *EcoRI*/ *NotI* (Fast Digest, Thermo Scientific) was done to check for correct assembly. The sequence of the final construct was confirmed by Sanger sequencing (Primer_GAP-fwd/ AOXTT_seq_rev).

From the correct clone the vector pPpT4_GAP-S_ScBDH1 was isolated, linearized (with *SmiI*) and used to transform *P. pastoris* CBS7435 wildtype, *Δdas1*, *Δdas2* and *Δdas1Δdas2*.

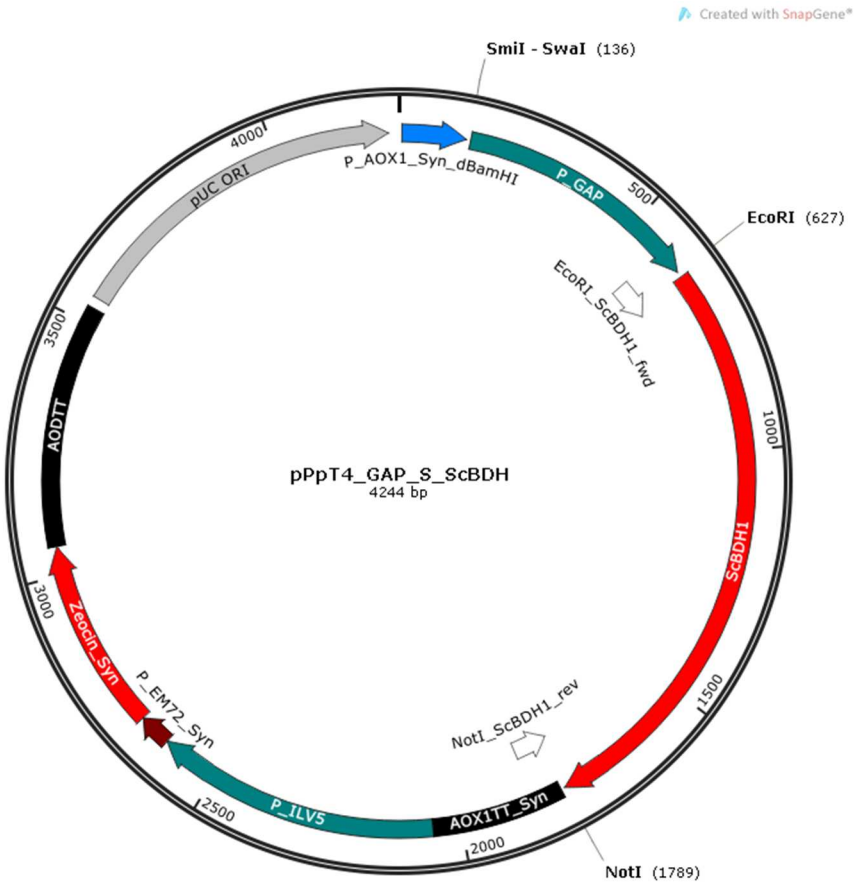


Figure 17: Vector map of pPpT4_GAP_S_ScBDH1
The pPpT4 vector has a homologous site for integration at P_{AOX1} in *P. pastoris* and the pUCori for replication in *E. coli*. Zeocin-resistance-protein under the control of the P_{ILV5} and AODTT for expression in *P. pastoris* and pEM72 for expression in *E. coli.* The expression of *BDH1* from *S. cerevisiae* (butanediol dehydrogenase) is under the control of the constitutive P_{GAP} (from *P. pastoris*) and the terminator of *AOX1* (AOX1TT). NotI_ScBDH1_rev and EcoRI_ScBDH1_fwd indicate the primers used to amplify *BDH1* gene from pPICZBd1-BDH1.

2.10.7. Preparation of electrocompetent *E. coli* cells

From freshly streaked-out *E. coli* Top10F' cells a single colony was inoculated as a pre-culture in 50 mL LB in 250 ml shaking flasks and incubated at 28-30°C, 120 rpm for at least 12 hours. A main culture (15 mL of pre-culture in 500 mL LB in 2 L flasks, 37°C, and 200 rpm) was grown to an OD_{600} of ~0.8. Then cells were transferred in sterile, pre-cooled centrifugation tubes (500 mL) and harvested

via centrifugation (2000x g, 15 minutes, and 4°C). The supernatant was discarded and the obtained pellet was washed twice, in a first step with 200 mL and in a second one with 100 mL of ice-cold ddH_2O. After each washing step, a centrifugation step followed at 2000x g, 15 minutes, 4°C. Subsequently 200 mL of ice-cold 10% (w/v) glycerol was added to the pellet. The mix was centrifuged again at 2000x g for 15 minutes at 4°C, this step was repeated. The cell pellet was then re-suspended gently in 1/50 of original culture volume (1 mL) ice cold 10% (w/v) glycerol and 80 µL aliquots were shock frozen in liquid nitrogen and stored at -80°C.

2.10.8. Electroporation of *E. coli*

80 µL of electro-competent cells were thawed on ice, mixed with approximately 100-200 ng of plasmid DNA, transferred in pre-cooled electroporation cuvettes and incubated on ice for 5 minutes. Instantly after pulsing (2.5 kV, 1 pulse) 1 mL of SOC media was added and the cells were regenerated (37°C, 850 rpm, 1 hour) and then plated on selective media.

2.10.9. Preparation of electrocompetent *P. pastoris* cells

P. pastoris cells were prepared according to the condensed protocol of Lin-Cereghino et al. (97)

2.10.10. Electroporation protocol for *P. pastoris*

100 µL freshly prepared electro-competent *P. pastoris* cells were mixed with 0.5-2 µg of linearized plasmid DNA and incubated on ice in pre-cooled electroporation-cuvettes for 10 minutes. Pulsing conditions for Gene Pulser® II electroporator (Bio-Rad Laboratories) were: cuvette gap, 2.00 mm; charging voltage 1.5 kV (single copy integration) or 2.0 kV (multi-copy-integration); resistance, 200 Ω; capacitance, 25 µF. After pulsing 0.5 mL cold 1 M sorbitol and 0.5 mL YPD-media were added and cells were incubated at 28°C, 110 rpm, and 1.5-2 hours for regeneration in 5 mL sterile tubes. 2x 100 µL, 200 µL and the rest were plated on selective media plates and incubated for 2 days at 30°C.

2.10.11. Deep well plate (DWP) cultivation

The DWP screening is based on the method of Weis et al. (98). 250 µL basal media containing a carbohydrate source (e.g. BMD1%) were filled in each well of the DWP, inoculated with single colonies and incubated at 28°C, 320 rpm, and 80% humidity. Before first induction cultures were stamped on selective or unselective agar plates, incubated at 30°C for 2 days and further stored at 4°C.

2.10.11.1. Induction protocol for eGFP screening

After about 60 hours of growth on glucose, 250 µL of induction media, BMM2 were added per well. After 12 h and 24 h after the first methanol induction 50 µL of BMM10 were added for eGFP expression under the control of the *AOX1* and *CAT1* promoter. For eGFP expression under the control of the *GAP* promoter BMD1% (250 µL after 60 h of initial growth and 50 µL after further 12 h and 24 h) was added at the same time points as for *AOX1* and *CAT1*.

2.10.11.2. Induction protocol CalB-screening

Cultures were grown as described in 2.10.11 with BMD1% and induced with 250 µL BMM2 after about 60 h of growth and then further induced after about 12 and 24 h with 50 µL BMM10.

2.10.11.3. Induction protocol for standard butanediol-dehydrogenase 1 (BDH1) screening

Cultures were grown for 60 hours in 250 µL BMD1%, induced with 250 µL BMM2 and then conversion was started by adding 50 µL of 250 mM rac-acetoin in BMM10 after 72h. Conversion time was 12 hours.

2.10.12. Enzyme Assays

2.10.12.1. eGFP-assay

Before first induction (after 60 hours of growth) and after 24, 48, 72 hours of the first methanol induction from the clones of the DWP (see 2.10.11.1) a sample of 10 µL was diluted in 90 µL ddH_2O in black 96-well-plates with clear, flat bottom. OD_{600} was determined after 30 seconds shaking in the plate reader. eGFP protein was excited with 488 nm and fluorescence was measured at 507 nm.

2.10.12.2. BDH-assay

P. pastoris was cultivated as in 2.10.11.3 for first screenings and 2.10.12.2.2 for time resolved screening of promising clones. To 300 µL of fresh or frozen supernatant 100 µL 50 mM *n*-butanol as internal standard (in BMM2) and 500 µL ethylacetate were added. The reaction mixture was well vortexed for about 5 minutes. For phase separation the DWP was spun down at 13,000 rpm, for 10 minutes. Through this extraction the organic compounds (*rac*-acetoin, *meso*-, and D-butanediol and *n*-butanol) are dissolved in the upper ethylacetetae phase. 270 µL of the organic phase were

transferred into 96-well plates (polypropylene) and then sealed with aluminium foil. Either the plates were stored at -20°C till measurement or immediately measured via GLC-FID (samples were always kept on 4°C during measurement).

Conversion of racematic acetoin to *D*-butanediol and *meso*-butanediol was determined via GLC GLC was performed using a Hewlett Packard 6890 instrument equipped with a FID (275°C) and a Chirasil-DEX CB column (25 m x 0.32 mm, 0.25 µm film) and H_2 as carrier gas (2.4 mL/min). Following program was applied (program name: CHIRASIL-DEX-CB_ACETOIN_STANDARD.M): heater 200°C; detector 250°C; splitless; constant flow 2.4 mL/min; 65 °C 6.5 minutes; 50 °C/min to 80 °C hold 0.70 min; 2°C/min to 85 °C hold 4 min; 50°C/min to 160 °C hold 3 min.

2.10.12.2.1. Standard curve

A standard curve was done to assign the peak areas of the chromatogram to a compound and its concentration. Acetoin was dissolved and diluted in BMM10 (250 mM, 100 mM, 50 mM and 25 mM) and then treated as the samples. 50 µL of each acetoin standard were mixed with 250 µL BMD1% and 250 µL BMM2. To these 550 µL 100 µL *n*-BuOH (50 mM) was added and then got extracted with 500 µL ethylacetate. Final concentrations of *n*-BuOH was 8.60 mM. Acetoin-stock solutions concentrations were 7.66 mM, 3.06 mM, 1.53 mM and 0.77 mM respectively. 270 µL of the organic phase were pipetted in ethylacetate resistant microtiter plates sealed with tin foil, cooled to 4°C and measured with GC-FID. For the calibration, each standard solution was prepared in triplicate.

2.10.12.2.2. Time-resolved BDH1 screening protocol

A single colony was incubated in 200 mL BMD1% (2 L baffled-flask) at 28°C, 90 rpm, 40% humidity (incubation room). After 24 h, 36 h and 48 h 50 mL of culture were withdrawn, and the OD_{600} was measured for calculation of a standardised OD_{600} of 15 in a final volume of 25 mL BM-media for each sample.

The calculated volume was spun down, supernatant was discarded and pellet was washed in 10 mL basal media without carbohydrate source (BM) (500x g, 5 min). After careful resuspension of the washed pellet in 25 mL BM-media, 250 µL of the culture in BM-media and 250 µL of BMM2 was pipetted into each well of the DWP (triplet determination) and incubated at 28°C, 320 rpm, 80% humidity for 12 hours. Then 50 µL of 250 mM *rac*-acetoin in BMM10 was added to each well and conversion took place for 12 hours under same conditions as for incubation. OD_{600} was measured in 96-well-plates. The DWP was then spun down (500x g, 5 min) and 400 µL of the supernatant was frozen at -20°C.

2.10.12.3. CalB-assay

A standard esterase assay for the CalB-screening was applied. In this assay *p*-nitrophenylbutyrate is hydrolysed by CalB to *p*-nitrophenol (PNP; coloured compound) and butyric acid (99). After cultivation in DWP (see 2.10.11.2) a sample of each well was taken for OD_{600} measurement in 96-well plates. DWP were spun down (4000 rpm, 4°C, 5 min) and 20 µL of supernatant were transferred to 96-well plates. 180 µL freshly prepared pNPB-assay solution was added to each well. Kinetic was measured at 405 nm for 5 min. CalB activity was calculated with the Equation 3.

Equation 3 Formula for calculation of calB activity
ΔE/min is the alteration of extinction per minute, extinction coefficient Ɛ for PNP in 300 mM Tris/HCl pH 7 is 9.594 mL/µmol/cm, the factor 1000 is for keeping units in mL.

$$\frac{U}{mL*OD600} = \frac{\frac{\Delta E}{min} * \frac{1000}{\varepsilon}}{OD600 * dilution}$$

2.10.13. Growth curves

For the determination of growth limitations of the *P. pastoris* CBS7435 Δ*das1*, Δ*das2* and Δ*das1*Δ*das2*, growth curves in three different media (BMD1%, BMM0.5% and BMG1%) were recorded. Per strain and per media 3 cultivations were done in parallel.
A single colony of each strain was freshly streaked out on YPD-plates and incubated at 30°C for 2 days. An overnight culture (ONC) of each strain in 50 mL BMD1% (250 mL baffled flasks) was grown for about 15 hours at 28 °C and 120 rpm. OD_{600} was measured (double determination) with a Beckman Coulter DW 800 spectrophotometer. Main culture (50 mL media in 250 mL baffled flasks) had a starting OD_{600} of 0.1. For this purpose, the volume of ONC was calculated via the formula $C1 * V1 = C2 * V2$, transferred into three 15 mL Greiner® tubes for each strain and each media, and centrifuged at 500x g for 5 min. Supernatant was discarded and each pellet was washed with 2.5 mL desired media for the main culture (BMD1%, BMM0.5%, BMG1%), spun down again and then re-suspended in 1 mL of media from the flask of the main culture and poured back into the flask. Before incubating at 28 °C, 120 rpm OD_{600} (t_0) was measured. Every 2 h an OD_{600} double measurement out of each flask was done till the stationary phase.

2.10.14. Preparation of cells for glycerol stocks

A single colony from a fresh single-streak out made on selective or none-selective agar plates (2 days, 30°C *P. pastoris*; 1 day 37°C *E. coli*) was incubated overnight in 5 mL full media supplemented or not supplemented with antibiotics (YPD, 28°C, 110 rpm, *P. pastoris*; LB, 37°C, 110 rpm, *E. coli*). 1 mL of

the ONC was then pipetted to 0.5 mL 60% (w/w) glycerol, well mixed and incubated for about 10 min in cryogenic tubes (2 mL). These were stored at -80 °C for further use.

3. RESULTS AND DISCUSSION

3.1. Construction of *P. pastoris* CBS7435 Δ*das1*, Δ*das2*, Δ*das1*Δ*das2* and Δ*tkl1*

3.1.1. Construction of knock-out cassettes

P. pastoris CBS7435 strains with improved NADH-regeneration were carried out by eliminating the assimilatory MUT- pathway. As mentioned in section 2.10.5 the pPpKC1 vector system was used for deleting the responsible genes *DAS1*, *DAS2* and *TKL1* (putative). 5' and 3'UTR regions of the respective genes were taken as homologous recombination sites for integration of the knock-out cassettes. In case of the *das1das2* double knock-out, the *HOB3* gene was deleted as well.
gDNA from *P. pastoris* CBS 7435 was isolated with the "Bust'n'Grab" protocol, described in 2.10.5.2, which yielded in concentrations of 2.5–3.7 µg/µL. for the first PCR reaction to amplify the 5' and 3'UTRs, as described in 2.10.5.2.1. In Figure 18 the control gel with the PCR products, after pooling four PCRs on a preparative gel and purifying with Wizard® SV Gel and PCR Clean-Up System, are shown. Most PCRs worked at once with yields (20–60 ng/µL) high enough to do the oePCR, except for the 5'UTR fragment of *DAS1* and 3' and 5'UTRs of *DAS2*. Hence the PCR was repeated with Phusion® buffer HF and GC, but again with the same annealing temperature.

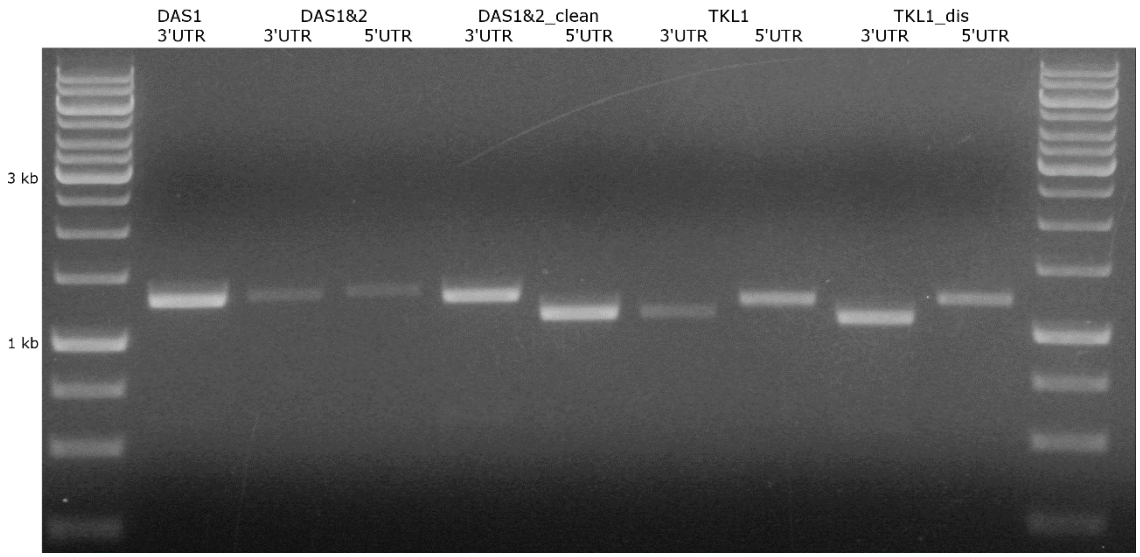


Figure 18 Control gel of amplified homologous 5' and 3'UTRs for knockout cassettes. 1 µL purified DNA was mixed with 9 µL ddH_2O and 2 µL 6x DNA loading dye and seperated on a 1% agarose gel at 120 V for 45 min. Every cleaned up product had a concentration of 20-63 ng/µL. As a standard 5 µL of GeneRuler™ 1kb DNA Ladder was used.

In Figure 19 the control gel of the second PCR with HF and GC buffer of the 3'UTR fragment of *DAS1* and the 3' and 5'UTR fragments of *DAS2* is shown after pooling and loading 4 µl of each. There was only a low yield for the 5'UTR fragment of *DAS1* and *DAS2* and a band for the 3'UTR of *DAS2* was

hardly visible. Still, the PCR products for each reaction were pooled, put on a preparative gel, cleaned up und concentrations were determined with NanoDrop spectrophotometer. Concentrations of the products were 5-12 ng/µL.

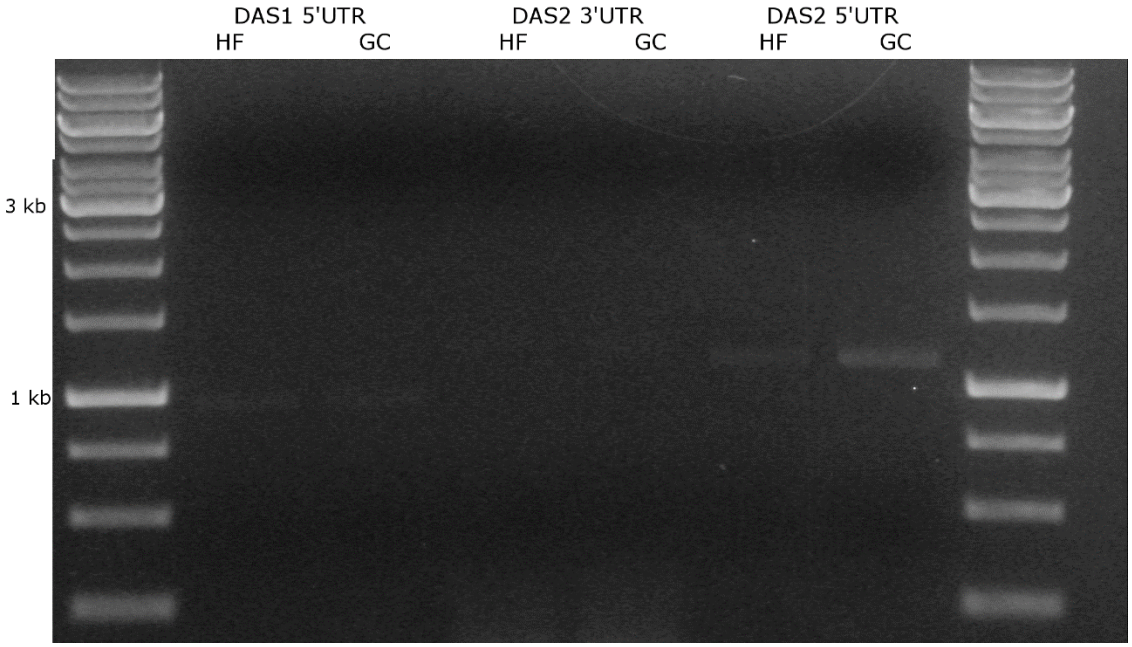


Figure 19 Control gel of amplified homologous 5'UTR of *DAS1* and 3' and 5'UTR of *DAS2* for knockout cassettes. 4 PCRs with Phusion® 5x buffer HF and 4 PCRs with Phusion® 5x buffer GC were done for each construct. The PCRs from each buffer and construct were pooled and 4µl of each was put on a control gel (1% agarose gel at 120 V for 45 min). As standard 5 µl of GeneRuler™ 1kb DNA Ladder was used.

The homologues 5' and 3'UTR fragments were diluted to a concentration of 2 ng/µL, except 3'UTR of *DAS1* and 3' and 5'UTR of *DAS2*, which had a quite lower concentration after clean-up, for oePCR as described in 2.10.2.2 and 2.10.5.2.1. Figure 20 A shows the control gel for the cleaned-up oePCR products, which have a band size between 2200 bp and 2400 bp. The yield for *DAS1&2*_clean wasn´t high enough for the preparative cut with *SfiI*, and the oePCR was repeated (four times each with Phusion™ buffer HF and buffer GC). The product after pooling all eight PCR reactions, separating on a preparative gel and clean up showed a brighter and thicker band on the control gel (Figure 20 B).

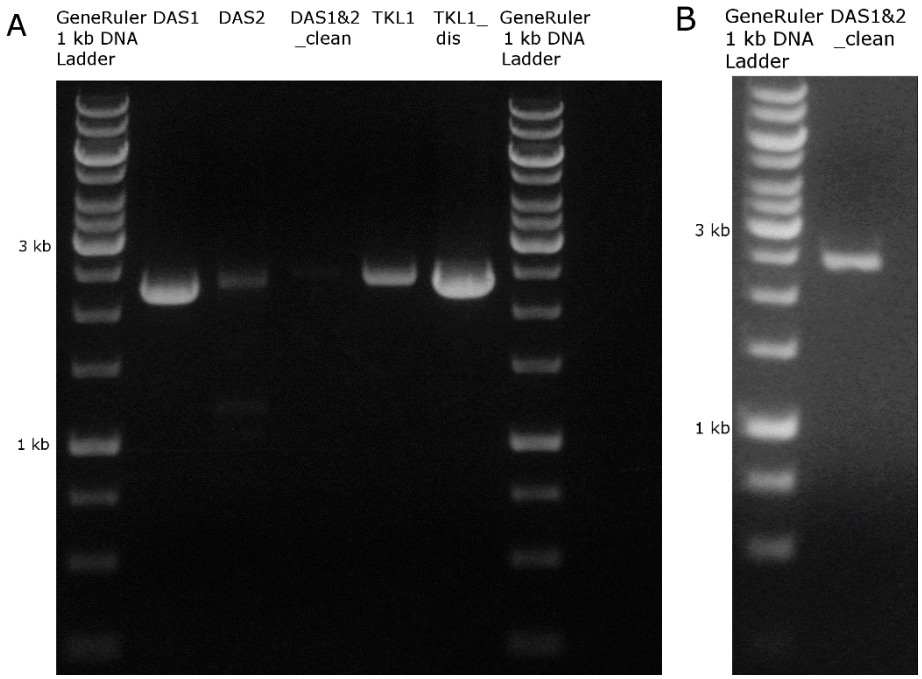


Figure 20 A Control gel of fused 5' and 3' UTR fragments of *DAS1*, *DAS2*, *DAS1&2*_clean, *TKL1* and *TKL1*_dis. **B** Control gel of fused 5' and 3' UTR of DAS1&2_clean via oePCR, after pooling approaches, preparative gel and clean-up (4 reactions each Phusion™ buffer HF and GC were pooled together).
1µl of cleaned up DNA mixed with 9 µl ddH_2O and 6x DNA loading dye. 1% agarose gel, 120 V, 45 min.

The oePCR for fusing the 5' and 3'UTR of *DAS1&2* didn´t work after repeating it under different conditions. As a clean knock-out of the genes *DAS1* and *DAS2* was preferred, only *DAS1&2*_clean was cloned into pPpKC1. DNA concentrations of the fused 5' and 3'UTR fragments after preparation cut with *SfiI* and clean-up were 21-27 ng/µL. The *SfiI* cut homologous regions were cloned into the *SfiI* cut pPpKC1 vector (see 2.10.4) and the resulting constructs were used to transform *E. coli* Top 10 F' cells (see 2.10.8).

Four clones of each construct were analyzed with a restriction digest (*SfiI*) and two vectors of the right size of each construct were further analyzed with Sanger sequencing (LGC genomics) as described in 2.10.5.1.

Correct knock-out constructs were linearized with *SmiI*, cleaned-up and 1 µg of the linearized vector was used to transform 100 µL P. *pastoris* CBS 7435 cells (see 2.10.9 and 2.10.10).

3.1.2. Screening for correct knock-out cassette integration

Initially, a pool screening (see 2.10.5.4.1) was done to screen about 100 clones of each construct for proper 5'UTR integration simultaneously (primers are summarized in Table 18). Thus, the number of gDNA isolation was drastically reduced (i.e. 100 instead of 500 for five different knock-out constructs).

Each gDNA sample was checked for every cassette. Here, some limitations have to be mentioned of this pool screening approach as the 5'UTR for pPpKC1_DAS2 and pPpKC1_DAS1&2 and for pPpKC1_TKL1 and pPpKC1_TKL1_dis are the same and the PCR reaction performed for this samples is the same. Positive clones of this PCRs have to be checked again. For example, if clone A1 from the pool-screening is positive for 5'UTR integration with primers Up5UTRDAS2F/ PAox1SeqR , this can either mean clone A1 with pPpKC1_DAS2 cassette or clone A1 with pPpKC1_DAS1&2 cassette or both are positive for 5'-integration of the cassette. Despite this limitation, it was far more efficient to do an initial pool screening. It had a higher throughput, because a whole DWP of each construct could be screened at once, which saved time at the gDNA isolation step.

45 positive clones from the pool screening were checked again due to these limitations. Positive clones (2-12 clones of each gene knock-out) from the pool screening were grown on new YPD plates containing Zeocin ™ (50 mg/L). These clones were then cultivated in 600 µL YPD DWP at 28°C, 320 rpm, 80% humidity overnight and gDNA was isolated for colony-PCR (2.10.5.3). Two colony-PCRs per screened construct were performed: one to check again proper integration at the 5'-end of the target and one for correct 3'-integration. Primers for the 5'-integration were binding upstream of the homologous integration site and the reverse primer binding within the knock-out cassette (*AOX1* promoter region). For checking proper integration at the 3' site of the locus a forward primer also binding within the cassette (at the pUCori) was used and the reverse primer binding downstream of the 3'UTR of the homologous recombination region (exact primer sets are listed in Table 18).

The whole PCR sample of each clone was analysed on a control gel. The gels in Figure 21 and Figure 22 show the results of the colony-PCRs. Clones growing on selection plates do not necessarily have the integration cassette in the right locus. This off-target integration is caused by non-homologous end joining (NHEJ) which a common problem in *P. pastoris*, filamentous fungi and other higher eukaryotes (100). NHEJ is initially a repair mechanism for double-strand breaks (DSB) caused by exogenous (e.g. UV-radiation) or endogenous (i.e. by products of the oxidative metabolism) factors which can lead to cell death (in unicellular organisms) or cancer (in multicellular organisms), but is also an important factor for integration of heterologous DNA (101).

Though no homologous sequences for repairing DSB are need in NHEJ it is far less specific than homologous recombination (HR), not necessarily accurate and deletion of a few nucleotides are often introduced at DSB-sites. Key player in NHEJ is the highly conserved Ku70p/Ku80p heterodimer which recognizes double-strand breaks (DSB) (100) (101). Gene replacement events in *P. pastoris* by HR occur with a frequency of <0.1% when homologous sequences are <500 bp or with an frequency up to 30% when sequences with about 1 kb homologous regions are used and are also locus depended (92) (93). In contrary HR is dominant in *S. cerevisiae* and NHEJ is hardly occurring by integration of heterologous DNA under laboratory conditions (102).

Figures A and C show products of 5'-integration PCRs and figures B and D show products of 3'-integration PCRs. For example, clone 2 (pPpKC1_DAS1) in Figure 21 A and B neither displayed a PCR product for proper integration at the 5' end of the locus nor a product for the 3'-integration PCR which implies off-target integration of the cassette leading to Zeocin™ resistant clones which are false positives, because the integration of the cassette occurred at the wrong locus. A proper 5'-integration did not imply proper integration at the 3' region of the locus, e.g. clone 31 (pPpKC1_DAS2) in Figure 21 C and D or clone 39 (pPpKC1_DAS1&2) in Figure 22 A and B. As well as for the false positives with integration of the whole cassette anywhere in the genome the NHEJ can occur only on one of the two DSB of the double cross-over event, which is done with the pPpKC1 cassettes. So 5' integration is happening via HR and 3' integration is a random event forced by NHEJ. As shown in Figure 21 A and B, 10 out of 12 clones of *P. pastoris* CBS7435 pPpKC1_DAS1 were positive for 5'-integration at the right locus (clear band at about 1100 bp), but only 8 clones showed the desired band of 1500 bp for the 3' integration at the right locus. The wild type control and the negative control (ddH_2O instead of gDNA sample) didn´t show any bands.
In summary, for each gene knock-out a clone having the knock- out cassette correctly integrated was identified: *P. pastoris* CBS 7435 pPpKC1_DAS2 (only one out of eleven clones showed proper 5' and 3' integration), pPpKC1_DAS1&2_clean (two of eleven clones were positive) and pPpKC1_TKL1/TKL1_dis (one of two clones showed proper integration).

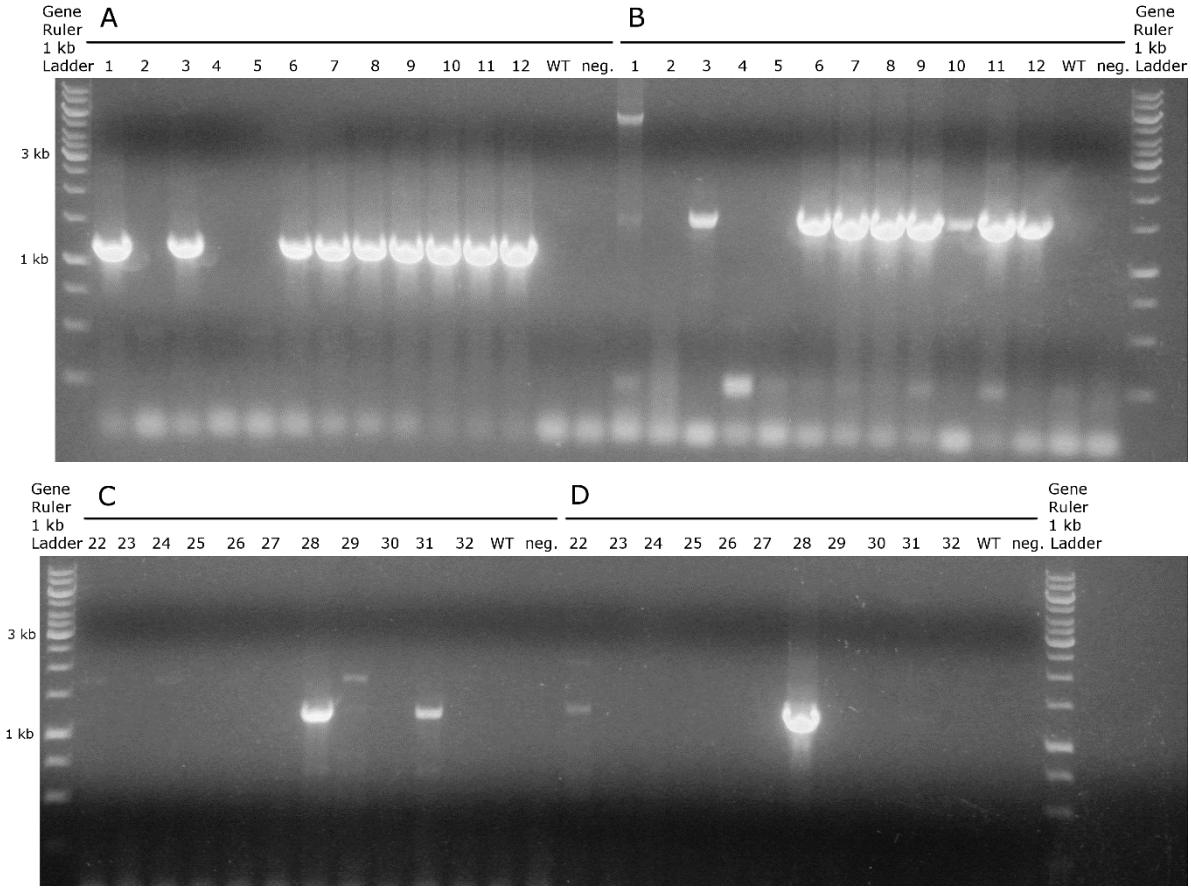


Figure 21 Screening for correct knock-out cassette integration into *P. pastoris* CBS7435. **A, B** Colony-PCR of 12 (1-12) clones of *P. pastoris* CBS7435 transformed with pPpKC1_DAS1. **C**, **D** Colony-PCR of 11 (22-32) clones of *P. pastoris* CBS7435 transformed with pPpKC1_DAS2.
The PCRs in A and C targeted 5' integration with one primer binding upstream 5'UTR (Up5UTRDAS1F and Up5UTRDAS2F) and one primer binding within the knockout cassette at the P_{AOX1} region (PAox1SeqR). In B and C respectively correct 3'-integration of the knockout cassette was checked. One forward primer was binding in the pUCori within the knock-out cassette (PucSeqF) and a reverse primer was binding downstream of 3'UTR region (Down3UTRDAS1R, Down3UTRDAS2R). product sizes: pPpKC1_DAS1 integrated 5'UTR: 1100 bp, 3'UTR 1500 bp; pPpKC1_DAS2 integrated 5'UTR: 1200 bp, 3'UTR: 1200 bp; 25 µl of PCR mixed with 5 µl 6x loading dye, 45 min, 120 V

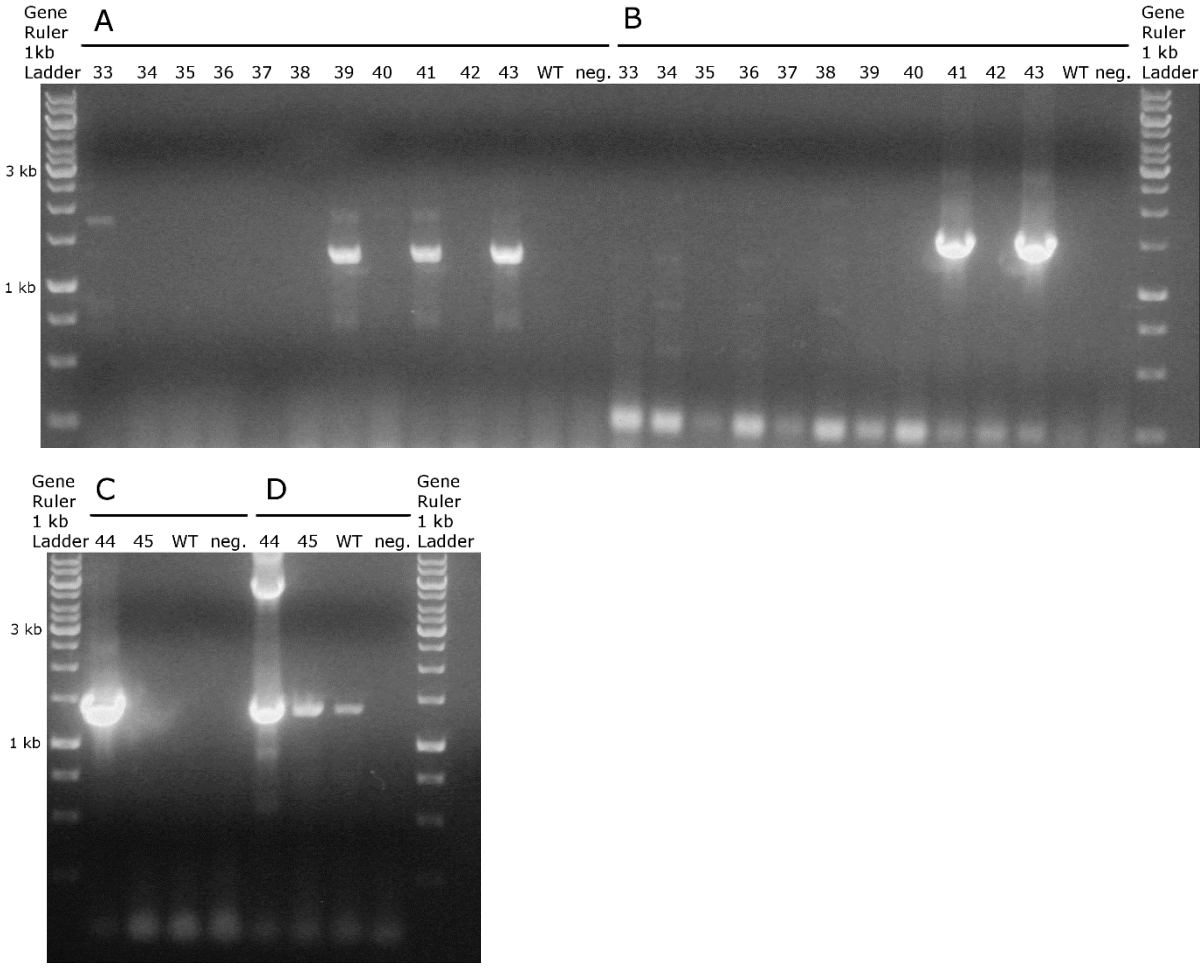


Figure 22: Screening for correct knock-out cassette integration into *P. pastoris* CBS7435. **A, B** Colony-PCR of eleven (33-43) clones of *P. pastoris* CBS7435 transformed with pPpKC1_DAS1&2_clean. **C**, **D** Colony-PCR of two (44-45) clones of *P. pastoris* CBS7435 transformed with pPpKC1_TKL1/TKL1_dis.
The PCRs in A and C targeted 5' integration with one primer binding upstream 5'UTR (Up5UTRDAS2F, Up5UTRTKL1) and one primer binding within the knockout cassette at P_{AOX1} region (PAox1SeqR). In B and C respectively correct 3'-integration of the knockout cassette was checked. One forward primer was binding in the pUCori within the knock-out cassette (PucSeqF) and a reverse primer was binding downstream of 3'UTR region (Down3UTRDAS1R, Down3'UTRTKL1R).
product sizes: pPpKC1_DAS1&2_clean integrated 5'UTR: 1200 bp, 3'UTR 1500 bp; pPpKC1_TKL1/TKL1_dis integrated 5'UTR: 1500 bp, 3'UTR: 1400 bp; 25 µl of PCR mixed with 5 µl 6x loading dye, 45 min, 120 V

3.1.3. Proof of eventual re-integration of the coding sequence (CDS)

Clones positive for 5' and 3' integration at the desired locus were further screened for the respective knocked-out coding sequence (CDS) with colony PCR. Due to not foreseeable re-integration events a control PCR was performed to ensure that the targeted CDSs are removed from the genome. Therefore, gDNA from clones with proper 5' and 3'UTR-integration served as template and primers binding in the CDS of *das1*, *das2* and *tkl1* were used (for primers and methods see 2.10.5.4).
Due the high homology of *DAS1* and *DAS2* of 92% (blastp from NCBI was used to align the two CDSs) primers were designed to bind in areas with less similarity. Consequently, the PCR did not amplify the whole gene, but the product of the colony-PCR only had about half of the size of the whole gene (91).

Expected size for *das1* colony-PCR was 1010 bp, for *das2* colony-PCR 973 bp and for *tkl1* colony-PCR 1700 bp.

The results of the colony PCR are shown in Figure 23. All clones with correct 5' and 3' integration of pPpKC1_DAS1 (3, 6, 7, 8, 9, 10, 11, 12) and one clone with pPpKC1_DAS1&2 (clone 43) showed only a weak band for *DAS1* within the colony-PCR compared to the wild type sample and clone 41 (pPpKC1_DAS1&2). The weak band compared to the wild type indicates that the CDS of *DAS1* was removed, but the PCR probably had a contamination with gDNA from other samples. The CDSDAS1F/R as well as the CDSDAS2F/R primers were also analysed by the "blastn"-tool at the NCBI homepage for non-specific binding in the CDS of *das2* and *das1* respectively – no binding areas in the opposite gene were identified and therefore, unspecific amplification can be excluded.

In case of the CDS of *DAS2* colony-PCRs of clone 28 (pPpKC1_DAS2) and clone 43 (pPpKC1_DAS1&2) show weaker bands compared to the wild type and clone 41 (pPpKC1_DAS1&2). The negative control also shows a weaker band of the desired size compared to the WT, probably due to a contamination with gDNA.

As seen in Figure 23 the colony-PCR for the CDS of *TKL1* clone 44 shows the expected band for the CDS as strong as the wild type. Concluding the CDS of *TKL1* is still within the genome.

Due to weak bands in colony-PCR of clones with probable knocked-out CDS and also the weak band in the negative control of *DAS2* CDS colony-PCR a growing assay on MMMeOH1%-square-plates was performed to make a definite statement concerning the deletion of the GOIs.

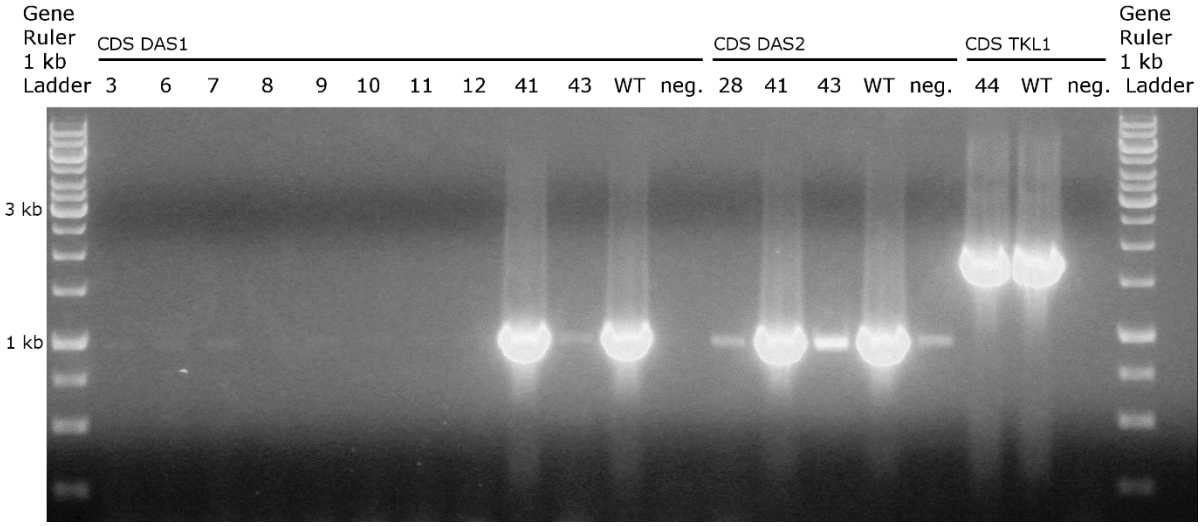


Figure 23: Colony PCR for proofing that CDS of *DAS1*, *DAS2* and *TKL1* is deleted in the genome of *P. pastoris* CBS7435 pPpKC1_DAS1 (3, 6, 7, 8, 9, 10, 11, and 12), pPpKC1_DAS2 (28), pPpKC1_DAS1&2_clean (41, 43) and pPpKC1_TKL1 (44). The results for the CDS *DAS1* PCR reaction clearly showed a strong band at the desired size for the CDS *DAS1* product (1010 bp) for clone 41 and the WT. The negative control showed no band. But also slight bands are visible for all the other clones. Similar results are seen in the CDS *DAS2* PCR, where clone 41 and the wild type show thick bands at about 1000 bp, whereas clone 28 and 43 show only weak ones. But also in the negative control a band is visible. The one positive *TKL1* knock-out (44) however showed a thick band at about 1700 bp as the wild type. The negative control showed no band. PCR product sizes: CDS *DAS1*: 1010 bp, CDS *DAS2*: 973 bp, CDS *TKL1*: 1775 bp; WT: *P. pastoris* CBS 7435 wild type, neg.: negative control

3.1.4. Growing Assay on MMMeoH1%

From each clone (*P. pastoris* CBS pPpKC1_DAS1 clone 8, pPpKC1_DAS1 clone 12, pPpKC1_DAS2 clone 28, pPpKC1_DAS1&2_clean clone 43 and a wild type strain as positive control) with proper 5', 3'UTR integration of the knock-out cassette and a negative colony-PCR targeting the CDS a growing assay on a MMMeOH1% agar plate (30°C, 6 days) was done. *P. pastoris* CBS7435 *Δdas1Δdas2* should not or hardly grow on methanol as a single carbon source due the lack of the 2 key enzymes of the assimilation pathway DAS1 and DAS2 (103).

In Figure 24, the growing assay showed nearly the same growth behaviour of clones from *P. pastoris* wild type, pPpKC1_DAS1 clone 8 and 12 and pPpKC1_DAS2 clone 28 after 6 days of incubation (big white colonies). The double knock-out clone *P. pastoris* CBS 7435 pPpKC1_DAS1&2_clean though had much smaller and more translucent than white colonies after the same time span. This result indicates that growth behaviour was altered in this strain. As Küberl et al. assumed that *TKL1* might have also a minor role in the MUT-pathway as dihydroxyacetone synthase, the observed growth could result from that enzyme`s activity (80).

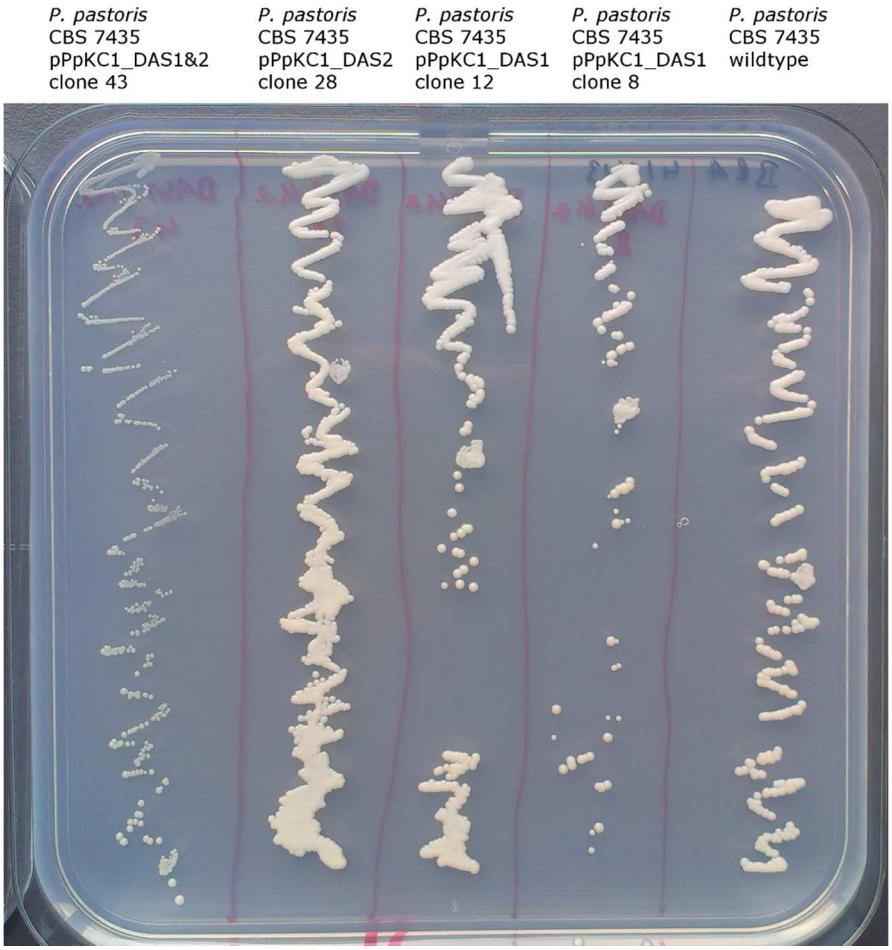


Figure 24: Growing assay of knock-out candidates on MMMeOH1% agar plates after 6 days of incubation at 30°C

3.1.5. Marker Recycling

Single colonies from single streak outs (YPD-Zeocin™ 100 mg/L) of *P. pastoris* CBS pPpKC1_DAS1 clone 8, pPpKC1_DAS1 clone 12, pPpKC1_DAS2 clone 28, pPpKC1_DAS1&2_clean clone 43 were grown in YPD media and MeOH was added at specific time points to induce the flippase under the control of P_{AOX1} for marker recycling, see 2.10.5.6.

Through induction with methanol the flippase under the control of the *AOX1* promotor is expressed. The flippase recognizes the FRT-sites on the expression cassette and excises the region located in between them, which contains the gene coding for the zeocin-resistance-protein and the flippase itself (compare Figure 12). After successful marker-recycling only one FRT-site from the knock-out cassette is left in the targeted region. Consequently, growth on Zeocin™ should be inhibited for the *P. pastoris* CBS7435 marker-recycled knock-out strains.

From single streak outs of these marker recycled clones on YPD plates, colonies were picked and cultivated in DWPs and stamped on YPD and YPD-Zeocin™ (100 mg/L) and cultivated (30°C, 2 days).

In Figure 25 (A on YPD plates without Zeocin™ and B on YPD-Zeocin™) the stamped clones from the DWP cultivation were displayed. All tested *P. pastoris Δdas1Δdas2* and *Δdas2* candidates (A1-B12 and C1-D12 in Figure 25 respectively) didn´t grow on YPD-Zeocin™ plates indicating a proper marker recycling. The recycling procedure for the *Δdas1* strain (2 different clones E1-F12 and G1-H6 respectively) didn't work as smooth. A few clones showed normal growth and many clones showed a weaker growth on the YPD-Zeocin™ plates. Only one of each namely the clone on position E8 had a proper removal of the integration cassette. It seemed likely that the pPpKC1_DAS1 integration cassette was harder to remove with the flippase. Maybe a longer methanol incubation would have been favourable in this case for a higher marker recycle efficiency.
From each construct one clone, growing on YPD and not growing on YPD-Zeocin™ was chosen, marked with a black circle in Figure 25 *(P. pastoris* CBS7435 *Δdas1das2* A1, *P. pastoris* CBS 7435 *Δdas2* C1, *P. pastoris* CBS 7435 *Δdas1* E8).

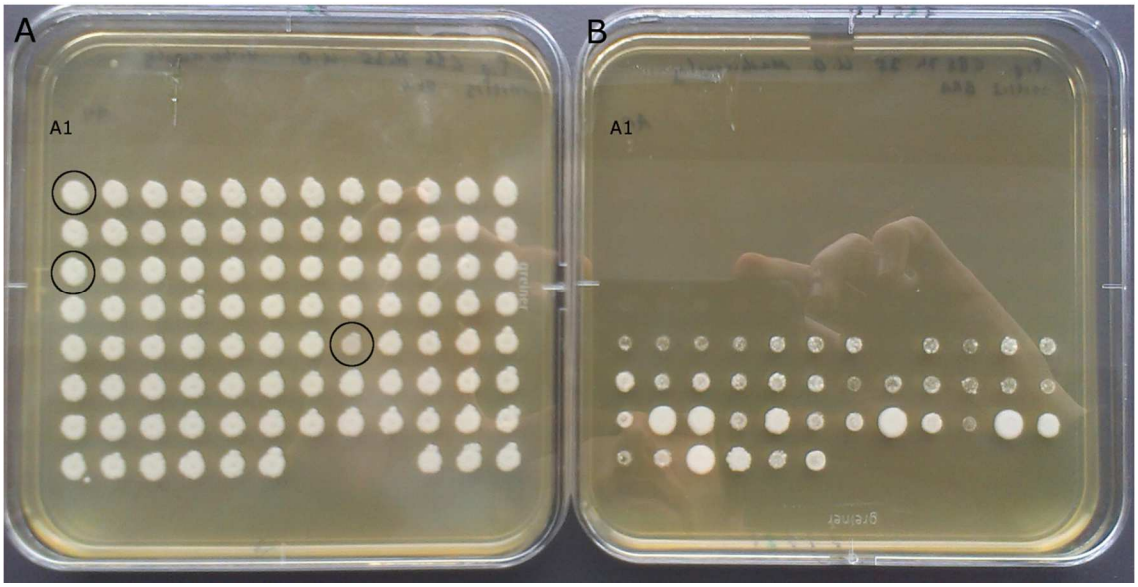


Figure 25: Marker recycling of *P. pastoris* CBS7435 knock-out candidates. Single colonies were cultivated in DWP (28°C, 320 rpm, 80% humidity) and then stamped on YPD (A) and YPD-Zeocin™ (100 mg/L) (B) (30°C, 2 days).
Correctly marker recycled clones didn´t grow on the YPD-zeocin100 plate, but grew normally on the YPD plate. All picked clones from the *das1das2* and the *das2* knock-out approach were positive. For the *das1* knockout one picked colony was positive as well. The black circle marked the colonies which were taken for further work. *P. pastoris* CBS 7435 Δ*das1das2* A1, *P. pastoris* CBS7435 Δ*das2* C1, *P. pastoris* CBS7435 *Δdas1* E8
A1-B12: DAS1&2_clean clone 43 K.O. candidate; C1-D12: DAS2 clone 28 K.O. candidate; E1-F12: DAS1 clone 12 K.O. candidate; G1-H6: DAS1 clone 8 K.O. candidate; H7-H9: sterile control; H10-H12: *P. pastoris* CBS7435

3.1.6. Verification of knock-out strains

To finally verify the generated knock-out strains 2 different experiments were done. For this purpose, single streak outs from marker recycled clones and clones containing the whole integration cassette were done and gDNA was isolated (2.10.5.3), confer Table 22.

Table 22: List of clones which were chosen for colony-PCRs

Clones containing whole integration cassette				
Label	**Clone**	**Origin**	**Name**	**Genomic situation**
1	8	single streak out	*P. pastoris* CBS7435 pPpKC1_DAS1	*Δdas1*; integrated cassette
2	12	single streak out	*P. pastoris* CBS7435 pPpKC1_DAS1	*Δdas1*; integrated cassette
3	28	single streak out	*P. pastoris* CBS7435 pPpKC1_DAS2	*Δdas2*; integrated cassette
4	43	single streak out	*P. pastoris* CBS7435 pPpKC1_DAS1&2_clean	*Δdas1Δdas2*; integrated cassette
5	43	G2, master plate	*P. pastoris* CBS7435 pPpKC1_DAS1&2_clean	*Δdas1Δdas2*; integrated cassette
Marker recycled clones				
6	E8	single streak out	*P. pastoris* CBS7435 *Δdas1*	*Δdas1*
7	C1	single streak out	*P. pastoris* CBS7435 *Δdas2*	*Δdas2*
8	A1	single streak out	*P. pastoris* CBS7435 *Δdas1Δdas2*	*Δdas1Δdas2*

3.1.6.1. Colony-PCR targeting CDS of *DAS1* and *DAS2*

After successful marker recycling, compare growing assay in section 3.1.5, an additional colony-PCR was performed to check the effective knock-out of the determined genes (*DAS1* and/or *DAS2*) on genomic level. This PCR was repeated, because in the initial CDS-control PCR (3.1.3) no clear results were achievable due to probable contamination of the PCR reactions.

Therefore, clones before and after the recycling step (Table 22) were analysed. To assure a proper working protocol, the sterile control (ster.) from the DWP was handled as a sample with colonies, in other words the gDNA isolation protocol was applied on the sterile control.

Figure 26 A shows the agarose gel electrophoresis picture of the colony-PCR targeting CDS of *DAS1* (band size: 1010 bp). As expected *P. pastoris* CBS7435 pPpKC1_DAS2 clone 28, *P. pastoris* CBS7435 *Δdas2* and *P. pastoris* CBS7435 wild type showed a band at this size. *P. pastoris* CBS7435 clones with pPpKC1_DAS1, pPpKC1_DAS1&2_clean, resulting in strains *Δdas1* and *Δdas1 Δdas2*, respectively, didn´t have these bands, concluding that the CDS of *DAS1* has been successfully removed in these ones.

In the gel picture of the colony-PCR targeting CDS of *DAS2* (Figure 26 B, band size: 973 bp) *P. pastoris* CBS 7435 pPpKC1_DAS1 clone 8, *P. pastoris* CBS 7435 pPpKC1_DAS1 clone 12, *P. pastoris* CBS 7435 *Δdas1*, *P. pastoris* CBS 7435 wild type showed the desired size as expected. In the other clones in which CDS of *DAS2* should be gone, no bands were detectable.

To summarize the deleted genes were not detectable in the genome of *P. pastoris* CBS 7435 *Δdas1*, *P. pastoris* CBS 7435 *Δdas2* and *P. pastoris* CBS 7435 *Δdas1Δdas2* anymore.

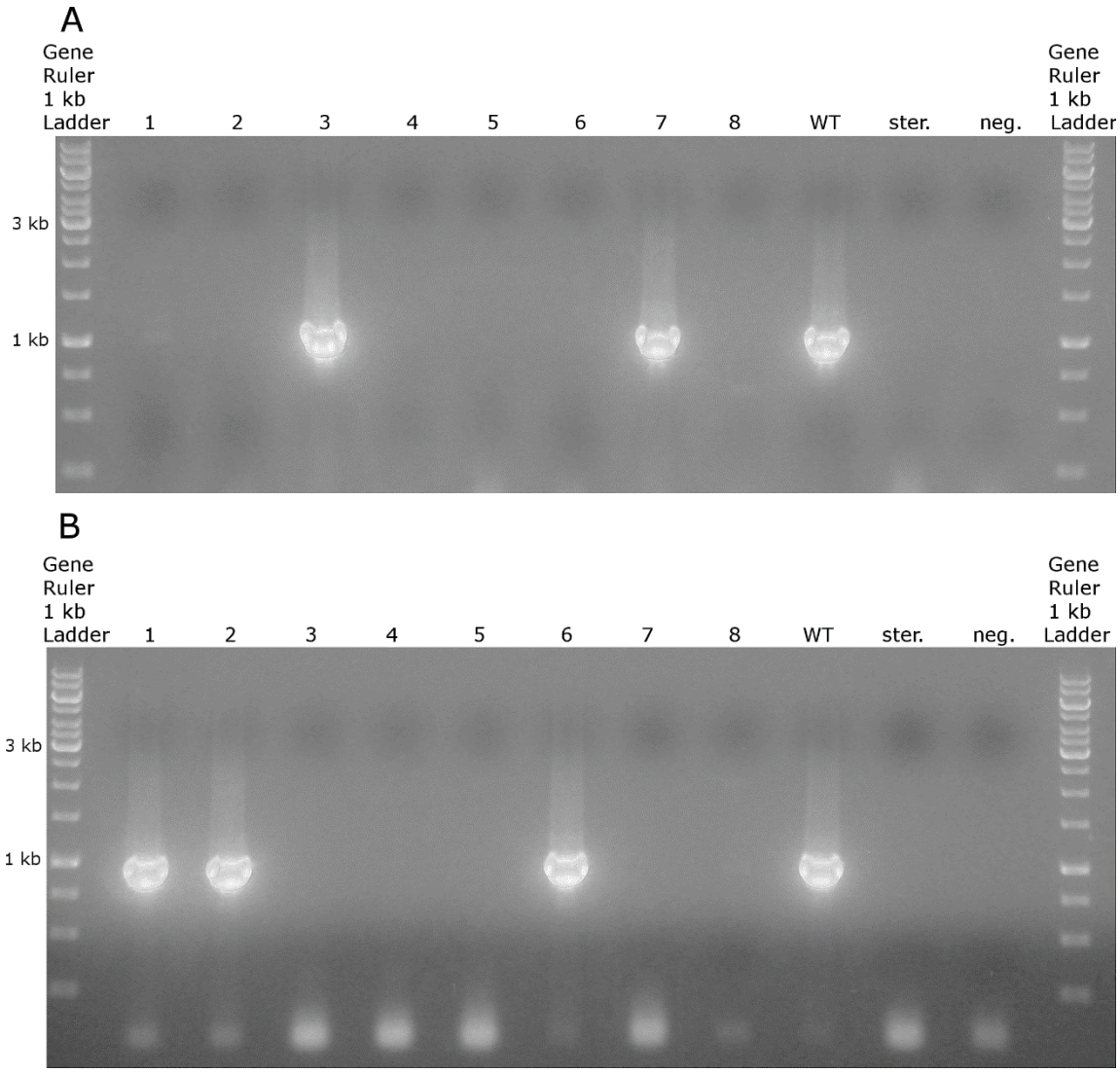


Figure 26: Colony PCR checking CDS of *DAS1* (**A**) and *DAS2* (**B**) in marker recycled clones and clones containing the whole integration cassette. Clones with deleted *das1* and *das2* didn't show a product for appropriate PCR.
1: *P. pastoris* CBS7435 pPpKC1_DAS1 clone 8; 2: *P. pastoris* CBS7435 pPpKC1_DAS1 clone 12; 3: *P. pastoris* CBS7435 pPpKC1_DAS2 clone 28; 4: *P. pastoris* CBS7435 pPpKC1_DAS1&2_clean clone 43 from single streak out; 5: *P. pastoris* CBS7435 pPpKC1_DAS1&2_clean clone 43 from master plate; 6: *P. pastoris* CBS7435 *Δdas1*; 7: *P. pastoris* CBS7435 *Δdas2*; 8: *P. pastoris* CBS7435 *Δdas1Δdas2*; WT: *P. pastoris* CBS7435; ster.: sterile control from DWP cultivation; neg.: ddH_2O
Primers: A: CDSDAS1F/CDSDAS1R; B: CDSDAS2F/CDSDAS2R; *DAS1* CDS: 1010 bp; *DAS2* CDS: 973 bp;

3.1.6.2. Colony PCR targeting the knock-out loci

To ensure a proper recycling of the marker on genomic level a second colony PCR was done, with primers binding at the inner site of 5' and 3'UTR (see Figure 13 and Table 20).
The PCR product sizes for properly recycled clones should be between 200 and 270 bp, for clones containing the whole integration cassette around 4.5 kb. For the wild type control, the PCR product

sizes are 2.4 kb for each *DAS1*- and *DAS2*-check PCRs, and 7 kb for the *DAS1&2*-check PCR (see Table 20).

In Figure 27 the gel picture of the *DAS*-check colony PCR is shown. As described above final/recycled single and double knock-out clones (6, 7, 8) show the desired band of about 200-270 bp, which approved the proper recycling of the marker, leaving 5'UTR, 3'UTR, 2 *SfiI* cutting sites and one FRT fragment left (which was also proved by sequencing of the PCR product).

The results for *DAS1*- and *DAS2*-check PCR for clones containing the whole integration cassette with the desired band sizes of 4550 bp for *DAS1*-check (clones 1 and 2) and 4500 bp for *DAS2*-check PCR (clone 3) and for the wild type control (2400 bp for *DAS1*-check and 2350 bp for *DAS2*-check PCR) were as expected.

For clones 4 and 5 (*P. pastoris* CBS 7435 pPpKC1_DAS1&2_clean) no band indicating the integrated knock-out cassette was observed, neither after repeating under different conditions for the PCR. The band size for the wild type control, which should be at 7 kb, was about 3.5 kb. DreamTaq DNA polymerase should be capable of producing 6 kb fragments with gDNA as template, but for longer fragments the extension temperature should have been lowered to 68°C (104). Despite these inexplicable band sizes for the *DAS1&2*-check PCR of *P. pastoris* CBS 7435 pPpKC1_DAS1&2_clean and the wild type, it was proven that the knock-out clones were successfully generated.

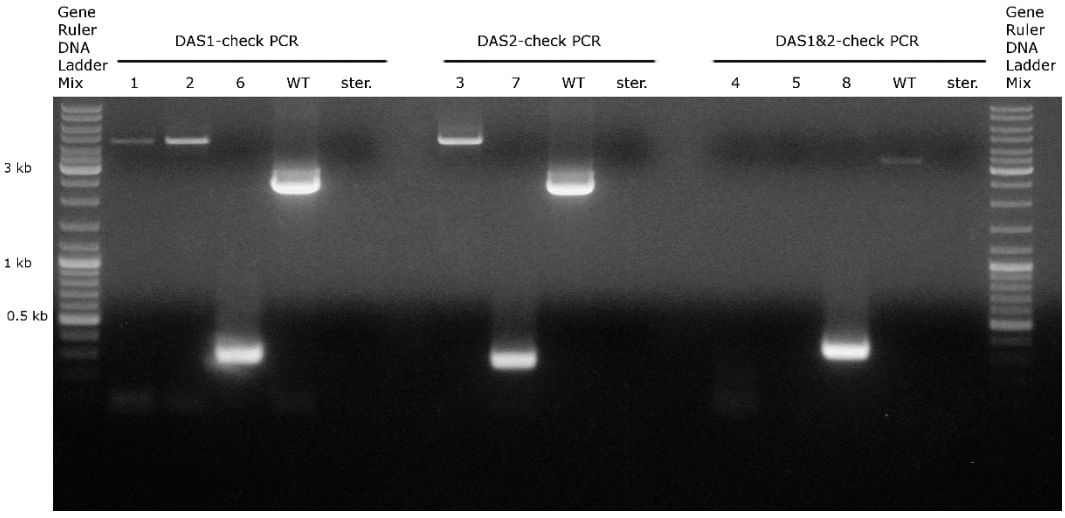


Figure 27: Colony PCR with primers binding at the 3'-end of 5'UTR (forward) and at the 5'-end of 3'UTR (reverse) to see if integration cassette is fully gone.
1: *P. pastoris* CBS7435 pPpKC1_DAS1 clone 8; 2: *P. pastoris* CBS7435 pPpKC1_DAS1 clone 12; 3: *P. pastoris* CBS7435 pPpKC1_DAS2 clone 28; 4: *P. pastoris* CBS7435 pPpKC1_DAS1&2_clean clone 43 from single streak out; 5: *P. pastoris* CBS7435 pPpKC1_DAS1&2_clean clone 43 from master plate; 6: *P. pastoris* CBS7435 *Δdas1*; 7: *P. pastoris* CBS7435 *Δdas2*; 8: *P. pastoris* CBS7435 *Δdas1Δdas2*; WT: *P. pastoris* CBS7435; ster.: sterile control from DWP cultivation
Primers: DAS1-check PCR: DAS1_check_fwd/ DAS1_check_rev; DAS2-check PCR: DAS2_check_fwd/ DAS2-check_rev; DAS1&2-check PCR: DAS2_check_fwd/ DAS1_check_rev

In Figure 28 the genomic situation of the *P. pastoris* CBS 7435 *Δdas1Δdas2* is shown. The PCR product of the DAS1&2-check PCR was cloned into pJET1.2/blunt. As planned in between the 5'UTR and

3'UTR, 2 *SfiI* sites and one FRT site were left after the marker recycling and the GOIs were deleted, compare Figure 12. The same result was obtained for the single knock-outs (genomic schemes not shown).

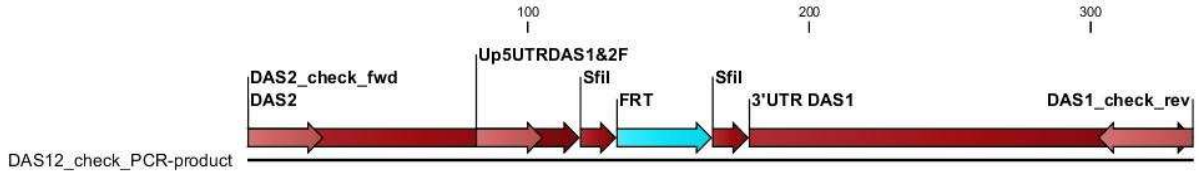


Figure 28: Example for the genomic situation of proper knock-out (*P. pastoris* CBS 7435 *Δdas1Δdas2*). After successful marker recycling only 5'UTR, 3'UTR, one FRT site and 2 *SfiI* remain at the locus. The band of about 270 bp from the DAS1&2-check PCR was cut and cloned into pJET1.2/blunt and further sent to sequencing.

3.1.7. Alternative *tkl1* knock-out strategy

Knocking out *TKL1* turned out to be an impossible task with the standard knock-out procedure applied in 3.1. Only a few transformants were gained by transforming *P. pastoris* CBS7435 and the screened clones had the *TKL1* gene still within the genome. Transketolase 1 is an important enzyme in the energy metabolism and for synthesizing aromatic amino acids and therefore the *TKL1* is more difficult to knock out. In contrary to *P. pastoris, S. cerevisiae* harbours 2 TKL homologs with different activity and knock-out of both genes resulted in total auxotrophy for aromatic amino acids (105). Alternative methods for the regeneration and selective media after the transformation of the pPpKC1_TKL1/_dis in *P. pastoris* CBS 7435 wild type and *Δdas1Δdas2* background were tried to generate a *TKL1* knock-out (2.10.5.7) with a view to reduce the effect of the carbon source on TKL1 necessity.

Transformants were only gained on the minimal media plates with glycerol, methanol, sorbitol and oleate as sole carbon source, respectively containing 50 mg/L Zeocin™ (Table 23). In contrary no transformants were gained on full media with the same carbon sources as in minimal media. Most transformants (96 colonies) were obtained on minimal media with oleate as sole carbon source. This media seemed most favourable to compensate the stress of deleting *TKL1* gene. Nearly no difference in transformation efficiency was recognizable between the wild type and the double-knock-out background or the two different knock-out cassettes. From growing clones gDNA was isolated following the protocol of 2.10.5.3. and a colony PCR for 5'UTR integration (2.10.5.4, for primers see Table 18) was done (Figure 29 A, B).

Table 23 List of grown colonies of the *pPpKC1*_TKL1/_dis in *P. pastoris* CBS7435 wild type and *Δdas1Δdas2* transformation on selective plates, which were used for gDNA isolation. No colonies were growing on full media plates.
MMGY: minimal media with 1% glycerol; MMMeOH: minimal media with 1%methanol; MMSO: minimal media with 1% sorbitol; MMOA: minimal media with 0.5% oleate.

	Wild type		***Δdas1Δdas2***	
Plates	**pPpKC1_TKL1**	**pPpKC1_TKL1_dis**	**pPpKC1_TKL1**	**pPpKC1_TKL1_dis**
MMGY	1	1	1	1
MMMeOH	1	-	1	-
MMSO	-	4	-	-
MMOA	24	20	33	19

Obtained transformants are summarized Table 23. From all 106 gained clones a colony PCR checking for 5'UTR integration was done. The PCR product should have a size of 1.5 kb, if integration at the 5'UTR locus was successful. In Figure 29 the products of these reactions was separated on a control gel. Only the gels of clones 1-24 and 76-99 are shown, because all the others were negative for proper 5'UTR integration. Clones 1-51 and 52-106 were transformants in the wild type and in the *Δdas1Δdas2* background, respectively. Out of these clones only clone no. 5 (Figure 29 A) from the wild type background with the pPpKC1_TKL1_dis integration cassette (colony grown on MMSO) and clone no. 99 (Figure 29 B) from the *Δdas1Δdas2* background with transformed pPpKC1_TKL1_dis (colony grown on MMOA) showed the 1.5 kb band for 5'UTR integration at the control gel and were thus further investigated.

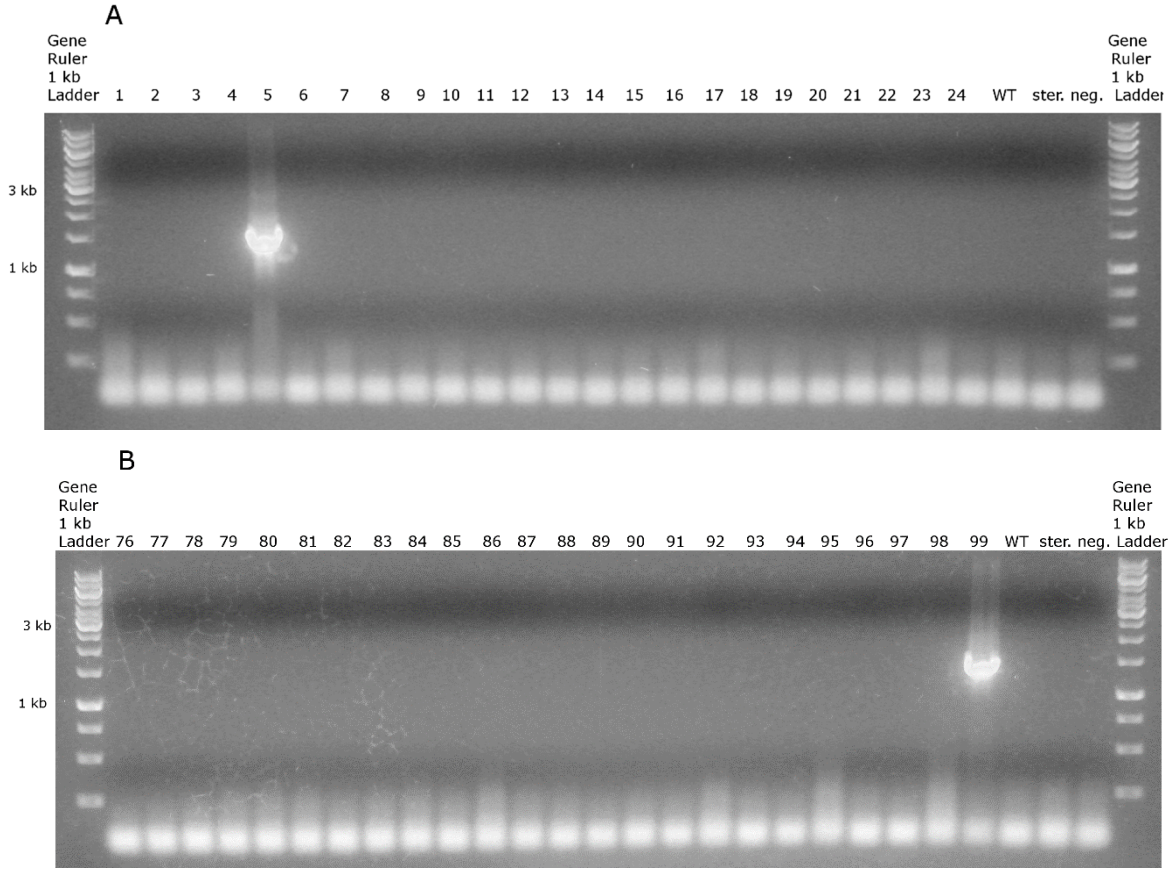


Figure 29 Screening for 5'UTR integration of pPpKC1_TKL1/-dis cassette in *P. pastoris* CBS7435 wild type (**A**) and *Δdas1Δdas2* (**B**). gDNA from transformants on minimal media plates with different carbon sources (Table 23) was isolated and checked with PCR for 5'UTR integration of the cassette.
1-51: *P. pastoris* CBS7435 pPpKC1_TKL1/_dis transformants; 52-106: *P. pastoris* CBS7435 *Δdas1Δdas2* pPpKC1_TKL1/_dis; WT: *P. pastoris* CBS7435; ster.: sterile control from gDNA isolation; neg.: negative control, PCR without template. Only two gel pictures with positive 5'UTR PCR products are shown.

Clones no. 5 and no. 99 were further tested again for 5'UTR integration, to confirm the former result, and also for proper 3'UTR integration of the cassette. Simultaneously deletion of CDS of *TKL1* was checked with a PCR reaction (protocol and primers are described in 2.10.5.4.).
5'UTR integration of the cassette was again approved by clear bands at the size of 1.5 kb (Figure 30 A). Consecutive the 3'UTR integration was checked as well (Figure 30 B). Here no clear results were seen for the *tkl1* knock-out candidate with the wild type background. Many bands with bigger size additionally to the wanted band at 1.5 kb were clearly detectable. The candidate with the double-knock-out background seemed quite promising, it had only one clear band of the desired size for 3'UTR integration. Suspiciously the wild type control showed also 2 bands of the size of 1.5 kb and one at the size of 1 kb, which was also seen in clone no. 5., maybe due to contamination of the PCR with genetic material. In advance CDS deletion was also checked in this step (Figure 30 C). The two knock-out candidates still had the CDS of *TKL1* (PCR product size was 1.8 kb) within the genome. The CDSTKL1F/R primers were also checked with "blastn" on NCBI for binding somewhere else in the

genome and unspecific bands could be excluded. No knock-out could be achieved with this strategy either.

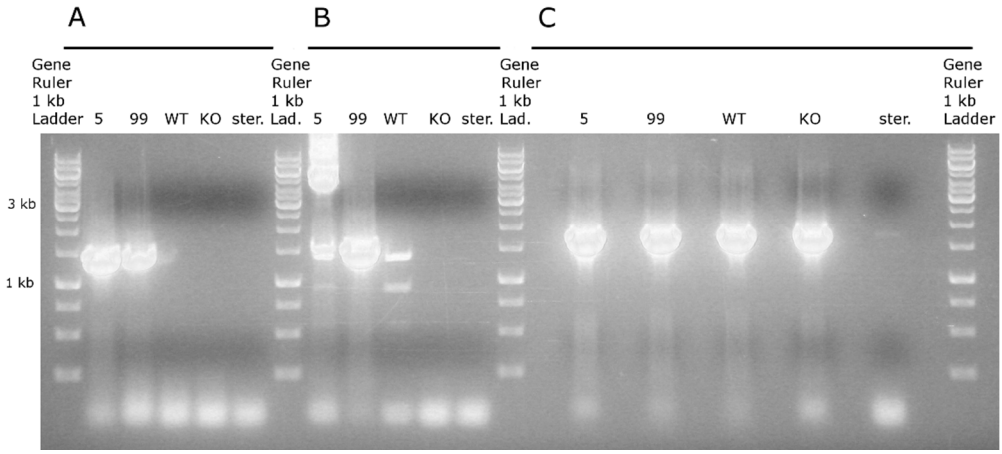


Figure 30 Control PCR for 5'UTR integration (**A**), 3'UTR integration (**B**) and CDS deletion (**C**) from the 2 positive clones of the 5'UTR integration PCR (Figure 29).
5: *P. pastoris* CBS 7435 pPpKC1_TKL1_dis (MMSO); 99: *P. pastoris* CBS743 *Δdas1Δdas2* pPpKC1_TKL1_dis (MMOA); WT: *P. pastoris* CBS 7435; KO: *P. pastoris* CBS743 *Δdas1Δdas2*; ster.: sterile control from gDNA isolation

The alternative strategy with different regeneration media after electroporation (i.e. regeneration in 1 mL 1 M sorbitol instead of 0.5 ml YPD and 0.5 ml 1 M sorbitol) and growing of the culture on different selective media resulted in no *tkl1* knock-out. However, it gave a clue how it could be possible. Eluding dextrose as sole carbon source and minimal media instead of full media increased the number of transformants. Most promising seemed oleate as carbon source. Keeping in mind that deletion of *TKL1/2* homologs in *S. cerevisiae* leads to a lethal strain, because of disruption of the synthesis pathway for aromatic amino acids and elimination of one of the key enzymes in the pentose-phosphate-pathway as well, probably it´s not possible to generate a *P. pastoris Δtkl1* strain (105) (106).

3.2. Characterization of knock-out strains

3.2.1. Growth rates

Growth rates of *P. pastoris* CBS7435 wild type, *Δdas1*, *Δdas2* and *Δdas1 Δdas2* in 3 different media with glucose, glycerol and methanol as sole carbon source (BMD1%, BMG1% and BMM0.5%) were determined. Each strain was grown threefold (biological triplicate) in each of the 3 different media and OD_{600} was measured every 2hours till stationary phase, for details see 2.10.13.
Glucose, glycerol and methanol are three of the most used carbon sources for expressing heterologous proteins in *P. pastoris* (107), therefore it was important to investigate possible growth limitations in the knock-out strains compared to the wild type. For *P. pastoris* CBS7435 *Δdas1Δdas2*

weak or no growth in basal media with methanol as sole carbon source was expected. The assimilative pathway should be blocked, because of lack of dihydroxyacetone synthase. Küberl et al found that *TKL1* might have a dihydroxacetone synthase activity as well and therefore growth on methanol as sole carbon source could be possible for the *P. pastoris* CBS7435 *Δdas1Δdas2* (80) (100). Knock-out strains and wild type strains showed nearly identical growth behaviour in BMD1% (see growth curve in Figure 31) within first 16 hours of growth. The logarithmic growth phase of *P. pastoris* CBS 7435 *Δdas1* continued to an OD_{600} of about 14 in contrast to the other strains, which only grew logarithmically till OD_{600} of about 7. It seems that knocking-out *das1* was beneficial for the biomass yield when growing on dextrose as sole carbon source. The reasons should be elucidated in further experiments.

There was no impact regarding the higher OD_{600} on the growth rate (µ) of the *Δdas1* strain conferring to the other strains (see Table 24). In all 4 tested strains µ was similar between 0.23 and 0.26 h^{-1}. In other studies, µ of the wild type strain was determined to be 0.30 h^{-1} (Centraalbureau voor Schimmelcultures, cited published data by Näätsari et al. (100)) thus higher than all values in this study. Overall no difference in growth was observable in basal media with dextrose as carbon source.

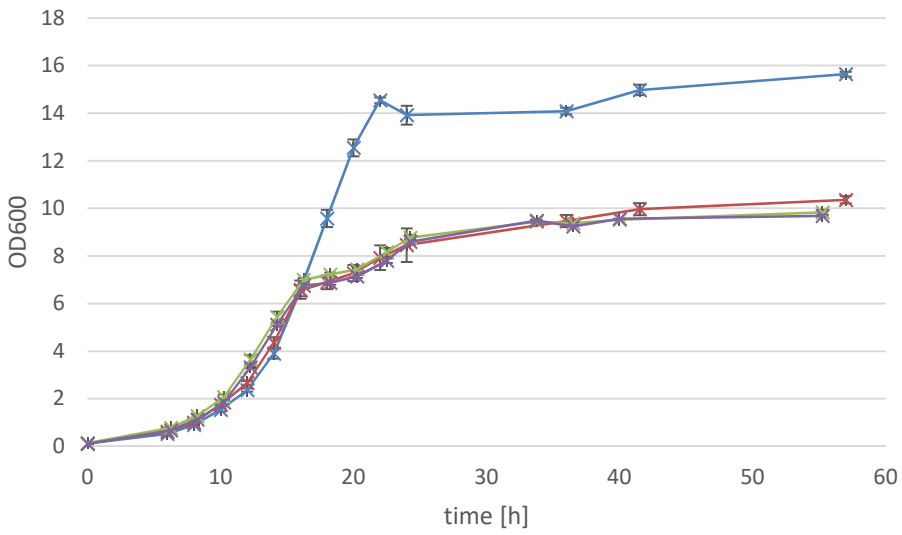


Figure 31 Growth curve *P. pastoris* CBS7434 wild type, *Δdas1, Δdas2* and *Δdas1Δdas2* in BMD1%.
blue: *P. pastoris* CBS7435 *Δdas1*; red: *P. pastoris* CBS7435 *Δdas2*; purple: *P. pastoris* CBS7435 *Δdas1Δdas2*; green: *P. pastoris* CBS7435

In the growth curves of the 4 tested strains in BMG1% hardly no difference was observable (Figure 32) during about 60 hours of growth. In Table 24 the calculated growth rate µ is shown, which was for all tested strains quite similar at about 0.20 h^{-1} or 0.21 h^{-1} respectively. This indicates no limitations in growth in minimal basal media containing glycerol as sole carbon source in the generated knock-out strains. The growth rate for the *Δdas1Δdas2* strain of this study was

comparable to the growth rate of *P. pastoris* CBS7435 *Δku70Δdas1Δdas2* determined by Näätsaari et al. (100). The growth rate for the wild type strain (0.20 h^{-1}) was lower compared to the data observed for the wild type strain by Centraalbureau voor Schimmelcultures (0.28 h^{-1}) (cited published data by Näätsari et al. (100)).

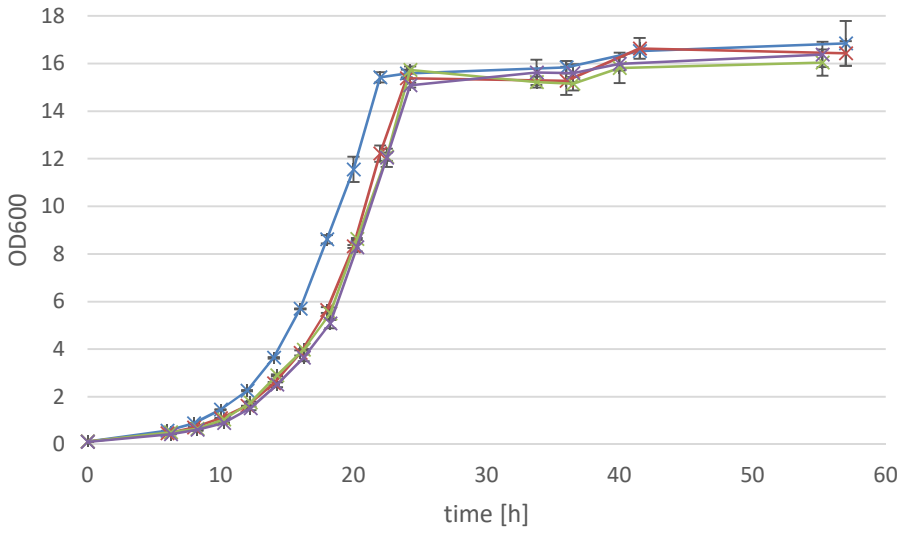
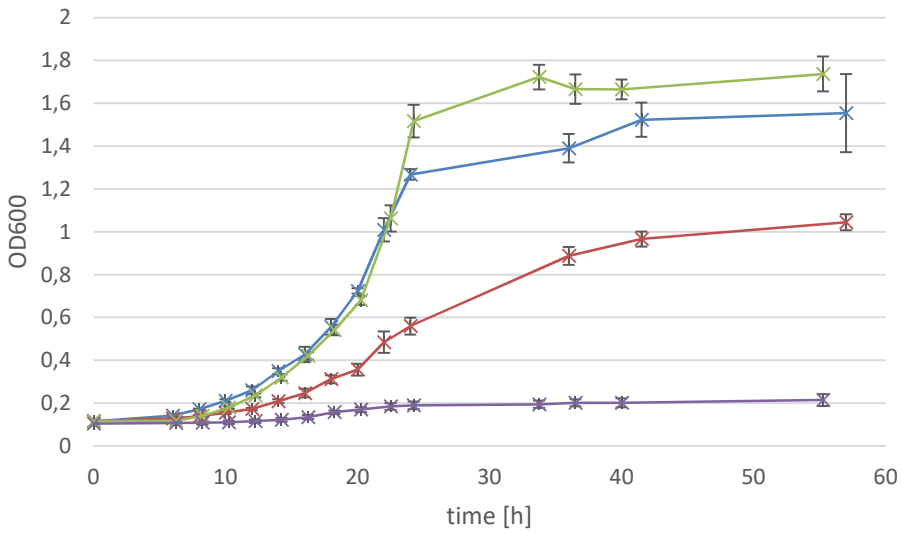


Figure 32 Growth curve *P. pastoris* CBS7434 wild type, *Δdas1, Δdas2* and *Δdas1Δdas2* in BMG1%
blue: *P. pastoris* CBS7435 *Δdas1*; red: *P. pastoris* CBS7435 *Δdas2*; purple: *P. pastoris* CBS7435 *Δdas1Δdas2*; green: *P. pastoris* CBS7435

In comparison to the other 2 investigated basal media (BMD1% and BMG1%) there were a few major differences in growth behaviour observable for BMM0.5%. Cell densities in BMM0.5% were significantly lower (OD_{600} = 1.0-1.8) compared to OD_{600} of 10.0-16.5 for all strains grown in BMD1% and BMG1% (confer Figure 31 and Figure 32). In BMM0.5% the highest OD_{600} was observed for the wild type strain (OD_{600} = 1.8). Metabolizing methanol as sole carbon source is not as effective as e.g. glucose, due to high oxygen utilization of the methanol metabolism. Oxidation of methanol to formaldehyde and further use of oxygen as final electron acceptor in the energy metabolism is energy consuming and leads to the lower final OD_{600}, as it was expected (108). The wild type strain also shows the highest growth rate (see Table 24) within the experiments. µ in this experiment was lower (0.15 ± 0.03 h^{-1}) than the cited published data from Näätsaari et al. (2012) (0.17 ± 0.00 h^{-1}) (100). The growth curves in Figure 33 shows that *Δdas1* and *Δdas2* strain had slightly lower OD_{600} and µ compared to the wild type strain, but still grew to a 15-fold and 10-fold higher OD_{600} in comparison to the start OD_{600} (0.1), respectively. It seems that knocking out *DAS2* had a more negative effect when grown in BMM0.5% than *DAS1* single knock-out. This could be at the bottom of the results of Krainer et al., who showed that *DAS2* gene transcription (99.1-fold) is slightly higher than *DAS1* (98.1-fold) with methanol induction (109), but should be further investigated.

The *P. pastoris* CBS7435 *Δdas1Δdas2* strain showed nearly no growth on methanol (μ= 0.01 ± 0.01 h^{-1}). OD_{600} was only doubled during 55 hours of growing from 0.1 to 0.2. In case that *DAS1* and *DAS2* were the only enzymes involved in the conversion of xylulose-5-phosphate and formaldehyde to dihydroxyacetone and glyceraldehyde-3-phosphate no growing should have been observed. Maybe little carbon resources were left after the washing-step of the pre-culture, which could be one way explain the growing phase. Genome sequencing of *P. pastoris CBS7435* from Küberl et al. suggested a hypothetical dihydroxyacetone synthase activity of *TKL1* within the MUT-pathway (80). Slightly growing of the double knock-out strain on BMM0.5% sustained this hypothesis (see Figure 33). As described earlier, it wasn't possible to generate a *tkl1* knock-out strain to prove this hypothesis.



It s

Figure 33 Growth curve *P. pastoris* CBS7434 wild type, *Δdas1, Δdas2* and *Δdas1Δdas2* in BMM0.5%
blue: *P. pastoris* CBS7435 *Δdas1*; red: *P. pastoris* CBS7435 *Δdas2*; purple: *P. pastoris* CBS7435 *Δdas1Δdas2*; green: *P. pastoris* CBS7435

Table 24 Growth rates of *P. pastoris* CBS 7435 knockouts and wild type in BMD1%, BMG1% and BMM0.5%

Strain	Growth rate μ [h^{-1}]		
	BMD1%	**BMG1%**	**BMM0.5%**
P. Pastorsis CBS 7435	0.25 ± 0.03	0.20 ± 0.05	0.15 ± 0.03
P. Pastorsis CBS 7435 *Δdas1*	0.23 ± 0.03	0.21 ± 0.03	0.12 ± 0.03
P. Pastorsis CBS 7435 *Δdas2*	0.24 ± 0.03	0.20 ± 0.02	0.09 ± 0.04
P. Pastoris 7435 *Δdas1Δdas2*	0.26 ± 0.03	0.20 ± 0.04	0.01 ± 0.01
P. Pastorsis CBS 7435[a]	0,30 ± 0.01	0,28 ± 0.02	0,17 ± 0.00
P. Pastoris 7435 Δku70Δdas1Δdas2[b]	0,21 ± 0.00	0,22 ± 0.01	0

a: cited published data by Näätsari et al. (2012) (100) (Centraalbureau voor Schimmelcultures.doi:10.1371/journal.pone.0039720.t001)
b: Näätsaari et al (2012) (100)

3.2.2. Recombinant protein expression in DAS knock-out strains

It is very important that the generated strains are applicable for production of heterologous proteins and therefore expression levels of the newly generated strains were compared to the wild type strain. As model protein eGFP under the control of three different promoters was chosen. All strains were transformed with pPpT4 vectors harbouring this eGFP expression cassettes and screened for their activity.

Vectors (pPpT4mutZeoMlyI-intArg4-eGFP_pAOX1BglII, pPpT4mutZeoMlyI-intArg4-eGFP_pGAPshort, pPpT4mutZeoMlyI-intArg4-eGFP_pCAT1) were, as provided by Tomas Vogl (see 2.10.6.1 and Table 29), used to transform (2.10.10) *P. pastoris* CBS7435 WT, *Δdas1*, *Δdas2*, *Δdas1 Δdas2* and mutS (only eGFP under the control of P_{CAT1}).

About 100 different clones from each transformation were picked and cultivated in DWP, using the protocol from 2.10.11.1. GFP-fluorescence at 507 nm and OD_{600} was measured (see 2.10.12.1) and compared to the wild type transformants and in case of pPpT4mutZeoMlyI-intArg4-eGFP_pCAT1 transformants in the mutS background as well.

To settle the different growing within the wells in the DWP the relative fluorescence units per OD_{600} (RFU/OD_{600}) was calculated for each clone. To show absolute expression of eGFP in the different clones RFU without growth settlement was plotted (Figure 36, Figure 37, Figure 38 D, E, F). The absolute RFU was shown to see differences of the protein yield during the induction phase with methanol, especially for the double knock-out, which showed hardly no growth in methanol as seen in the experiments above in comparison to e.g. the wild type strain (2.10.13).

In all knock-out strains eGFP under the control of 3 different promoters (P_{AOX1}, $P_{GAPshort}$ and P_{CAT1}) was functionally expressed.

The expression level of eGFP (RFU/OD_{600}) was depending on the promoter used to drive recombinant protein expression. Lowest expression levels were observed for clones expressing eGFP under the control of the constitutive promoter P_{GAP} (confer Figure 34 A for the landscape of *P. pastoris* CBS7435 *Δdas1Δdas2* and time resolved RFU/OD_{600} of Figure 36 to Figure 37 and Figure 38). Transformants expressing eGFP under the control of P_{AOX1} and P_{CAT1} promoters showed all similar expression levels in the eGFP assay, except in the *P. pastoris* CBS7435 *Δdas1Δdas2* background in combination with P_{CAT1}, which showed significantly higher expression (RFU/OD_{600}) in comparison to wild type (see Figure 34 C), *Δdas1* (Figure 35) and *Δdas2* transformants.

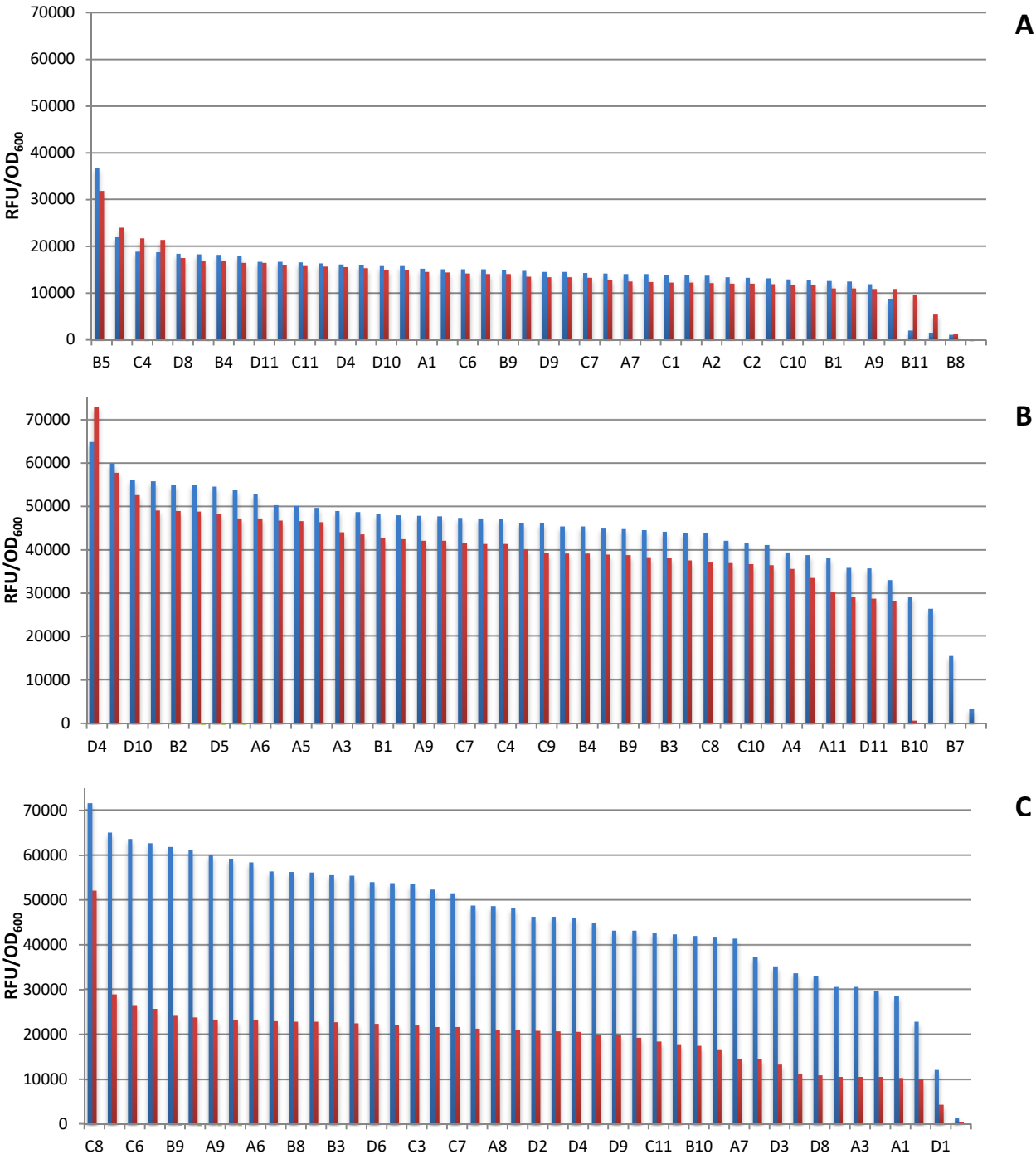


Figure 34 Landscapes of eGFP expression levels obtained in *P. pastoris* CBS 7435 *Δdas1Δdas2* and wild type transformants using P_{GAP} (**A**), P_{AOX1} (**B**), P_{CAT1} (**C**). After the growth phase of 60 h on glucose independent of the harbouring promoter, the cells were first induced with glucose (P_{GAP}) or methanol (P_{AOX1}, P_{CAT1}) and again after 24, 48 and 72 hours, see 2.10.11.1. This graph shows the landscapes after 72h of induction with glucose or methanol respectively.
blue: *P. pastoris* CBS7435 *Δdas1Δdas2*; red: *P. pastoris* CBS7435

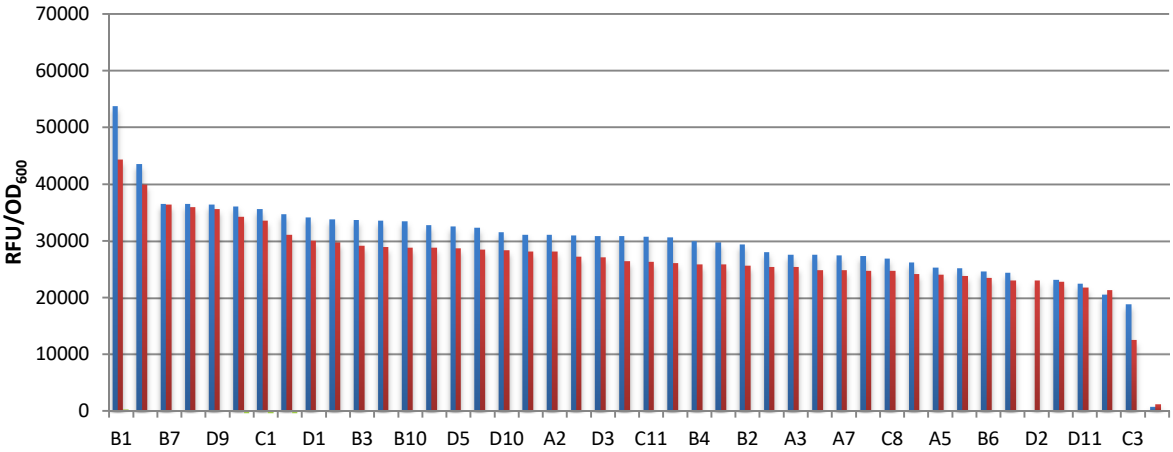


Figure 35 Landscape of eGFP expression levels obtained in *P. pastoris* CBS7435 *Δdas1* and wild type transformants using P_{CAT1}. After the growth phase of 60 h on glucose independent of the harbouring promoter, the cells were first induced with glucose (P_{GAP}) or methanol (P_{AOX1}, P_{CAT1}) and again after 24, 48 and 72 hours, see 2.10.11.1. This graph shows the landscape after 72h of induction with methanol.
blue: *P. pastoris* CBS7435 *Δdas1*; red: *P. pastoris* CBS7435

The mean values for the eGFP fluorescence in all knock-out strains and the wild type with eGFP under the control of P_{GAP} promoter had an average of about 20,000 RFU/OD_{600} and was constant before and during the induction period for the time-resolved analysis of the DWP fermentations (Figure 36 A, B, and C). Copy number of the integration cassette was not determined; outliers were considered as multiple integration clones for mean value calculation.

The constant expression level ensued from the constitutive P_{GAP} promoter, which controls the glyceraldehyde-3-phosphate dehydrogenase at a high basal level (110). Though eGFP under the control of P_{GAP} was expressed from the start of cultivation in the DWP, the highest expression levels could have been achieved within the 60 h growing phase with high glucose levels (no measurements done) before first measurement point/“induction point”. To clarify expression levels in the first 60h of growing measurements within this phase have to be considered for further experiments. In comparison the absolute RFU values showed no significant differences as well (Figure 36 D, E, and F). Concluding growth and expression of eGFP under the control of P_{GAP} in BMD1% seemed to have no difference at all tested strains on expression and growth level.

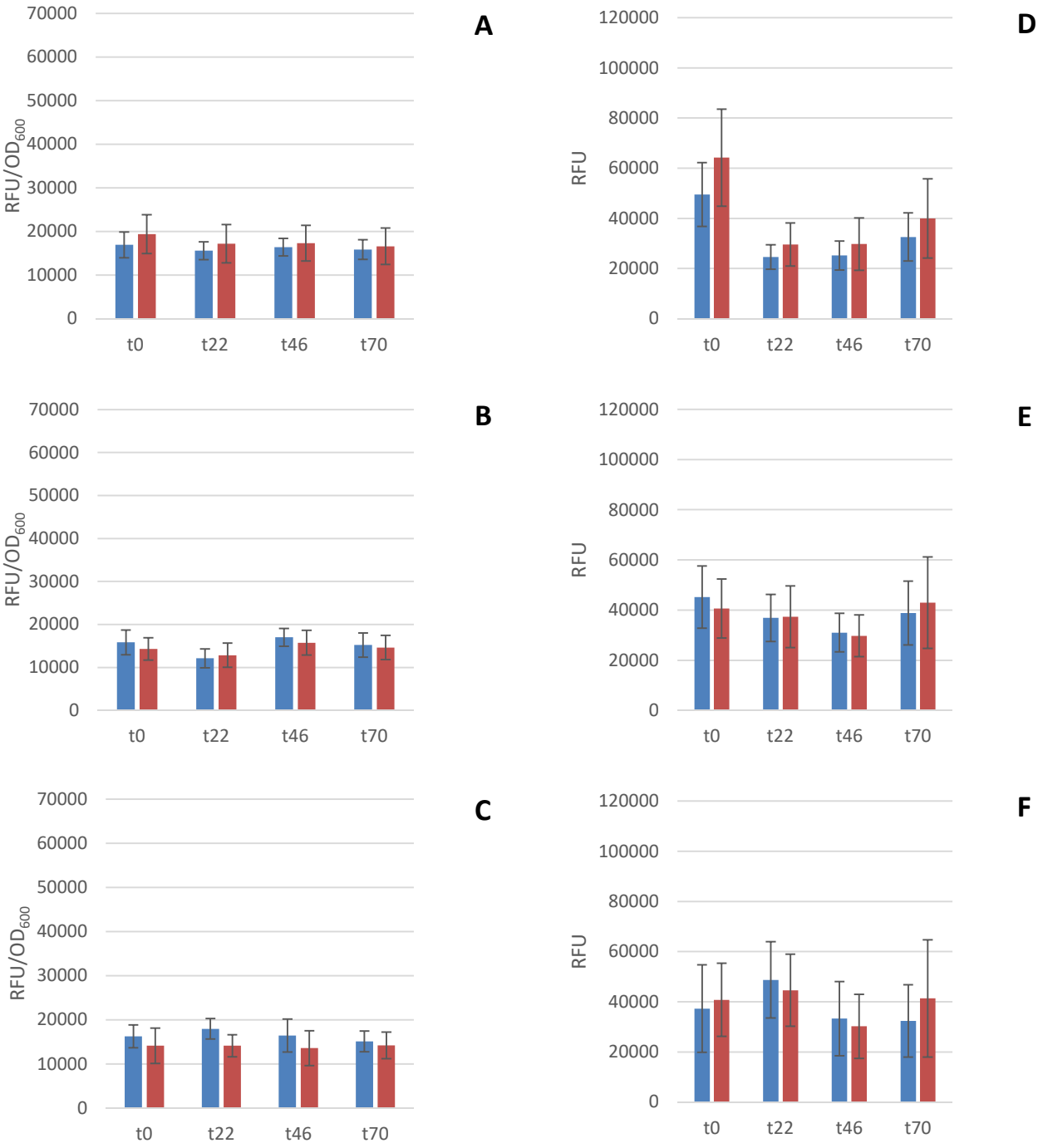


Figure 36 Time resolved comparison of eGFP expression under the control of P_{GAPt} in *P. pastoris* CBS7435 *Δdas1* (RFU/OD_{600} A and RFU D), *Δdas2* (RFU/OD_{600} B and RFU E) and *Δdas1Δdas2* (RFU/OD_{600} C and RFU F) to wild type transformants. Blue: mean of respective knock-out clones at respective time point; red: mean of wild type clones; black bars indicate standard deviation based on all clones of the landscape at the respective time points.

Clones which expressed eGFP under the control of the alcohol oxidase 1 promoter (P_{AOX1}), showed hardly any expression before induction with methanol (Figure 37). P_{AOX1} is strongly repressed by glucose, glycerol and ethanol. It gets de-repressed by depletion of glucose, but this doesn´t lead to

full induction of the promoter. Full promoter strength is only achieved through addition of methanol (111). The GFP signal before induction resulted from the de-repression.

Highest expression levels in all three knock-out clones and in the wild type strain were achieved after 70 hours of methanol induction (Figure 37 A, B, and C and Figure 37 D, E, and F). The knock-out clones seemed to gain slightly higher expression levels (RFU/OD_{600}) than the wild type. In the single knock-out clones where growth rate in BMD1% and BMM0.5% is nearly the same as for the wild type (confer Table 24) the absolute eGFP expression was in case of the *Δdas1* strain about 44% better than in the wild type transformants (after 70 h of induction). *Δdas2* transformants had still 12% (70 h) to 21% (40 h) better expression than the wild type and nearly same absolute expression levels as the wild type. In Figure 37 C and F RFU/OD_{600} and absolute expression of double knock-outs and wild type clones were plotted. These two graphs show on the one hand that the relative expression was up to 50% higher (after 46 h of induction with methanol) for the *P. pastoris Δdas1Δdas2* compared to the wild type. On the other hand that the absolute expression level was much weaker (only about 23% of the wild type niveau) keeping in mind the slow growth of the double knock-out on methanol and thus lower OD_{600} and less total protein.

The fact that the maximum of the expression levels in between the 3 different experiments varied from 30,000 to 45,000 RFU/OD_{600}, in the knock-out transformants and in wild-type transformants as well, made clear that different DWP are not comparable with each other and reproducibility within this system is restricted, excluding that clones weren´t genetic duplicates.

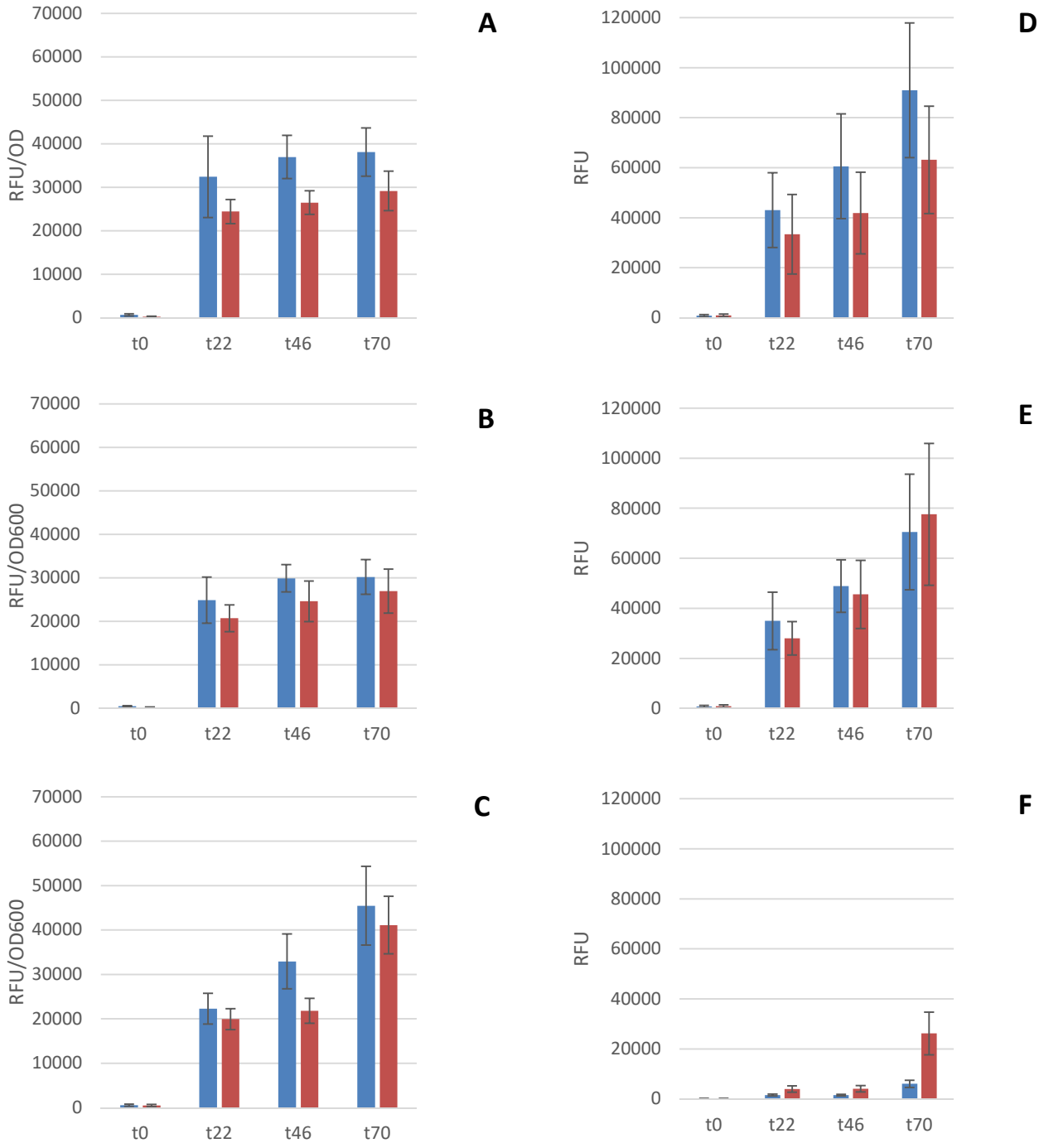


Figure 37 Time resolved comparison of *P. pastoris* CBS7435 *Δdas1* (RFU/OD_{600} **A** and RFU **D**), *Δdas2* (RFU/OD_{600} **B** and RFU **E**) and *Δdas1Δdas2* (RFU/OD_{600} **C** and RFU **F**) transformants expressing eGFP under the control of P_{AOX1}.
Blue bar: mean of respective knock-out clones at respective time point; red bar: mean of wild type clones; black bars indicate standard deviation based on all clones of the landscape at the respective time points.

The third promoter tested for recombinant protein production was the one of catalase 1 (P_{CAT1}) from *P. pastoris*. Catalase, located in the peroxisome, breaks down the side product H_2O_2 in H_2O and O_2. Hydrogen peroxide is a highly toxic molecule, which is produced during oxidation of toxic compounds as well as during the oxidation of methanol to formaldehyde in the methanol metabolism (112)

(113). Catalase is associated with many oxidases in the peroxisome producing hydrogen peroxide. When the carbon source is switched from glucose to methanol, oleate or D-alanine, cytosolic enzyme expression, like formate dehydrogenase, as well as peroxisomal protein expression (i.e. catalase, alcohol oxidase) and peroxisome proliferation itself are induced (114) (115).

eGFP expression based on integrated pPpT4mutZeoMlyI-intArg4-eGFP_pCAT1 worked in knock-out and wild type transformants. No significant differences between *P. pastoris Δdas1*, *Δdas2* and wild type were observable. The single knock-outs had about 9-20% better expression considering the OD_{600} (Figure 38 A, B), which is shown in a time resolved plotting of mean RFU/OD_{600}. Absolute RFU were slightly weaker at different time points, but reached 95% (*Δdas1*) and 85% (*Δdas2*) respectively of the wild type expression level after 70 h of induction (Figure 38 D and E). These results indicate no alteration of protein expression driven by P_{CAT1} in strains having a single knock-out at the *das1* or *das2* locus compared to the wild type.

According to Figure 38 weak expression of the reporter protein before induction with methanol was seen. Former work pointed out, when *P. pastoris* is grown on glucose, peroxisome proliferation is weak as well as induction of associated proteins, i.e. catalase concluding a low P_{CAT1} driven eGFP expression when *P. pastoris* is cultivated on glucose (114; 116).

In contrast to the wild type and single knock-out strains *P. pastoris Δdas1Δdas2* transformants with integrated pPpT4mutZeoMlyI-intArg4-eGFP_pCAT1 showed far higher RFU/OD_{600} values (35%, 75%, 150% and 100% higher expression compared to the wild type transformants, respectively) (Figure 38 C). Growth limitations of *Δdas1Δdas2* strains in induction medium with methanol as sole carbon source became important for the absolute RFU (Figure 38 F). Before induction expression level of eGFP was at the same niveau as the wild type, afterwards it sank from 63% to 37% compared to the wild type. The total amount of protein is less, because of the lower cell densities, but the amount of heterologous protein per cell is higher in the double knock-out transformants, as RFU/OD_{600} results showed. For further investigations another induction/de-repression method with e.g. oleate should be tested to ensure same growth rate as the wild type and probably also same absolute expression level (115) (117) (116).

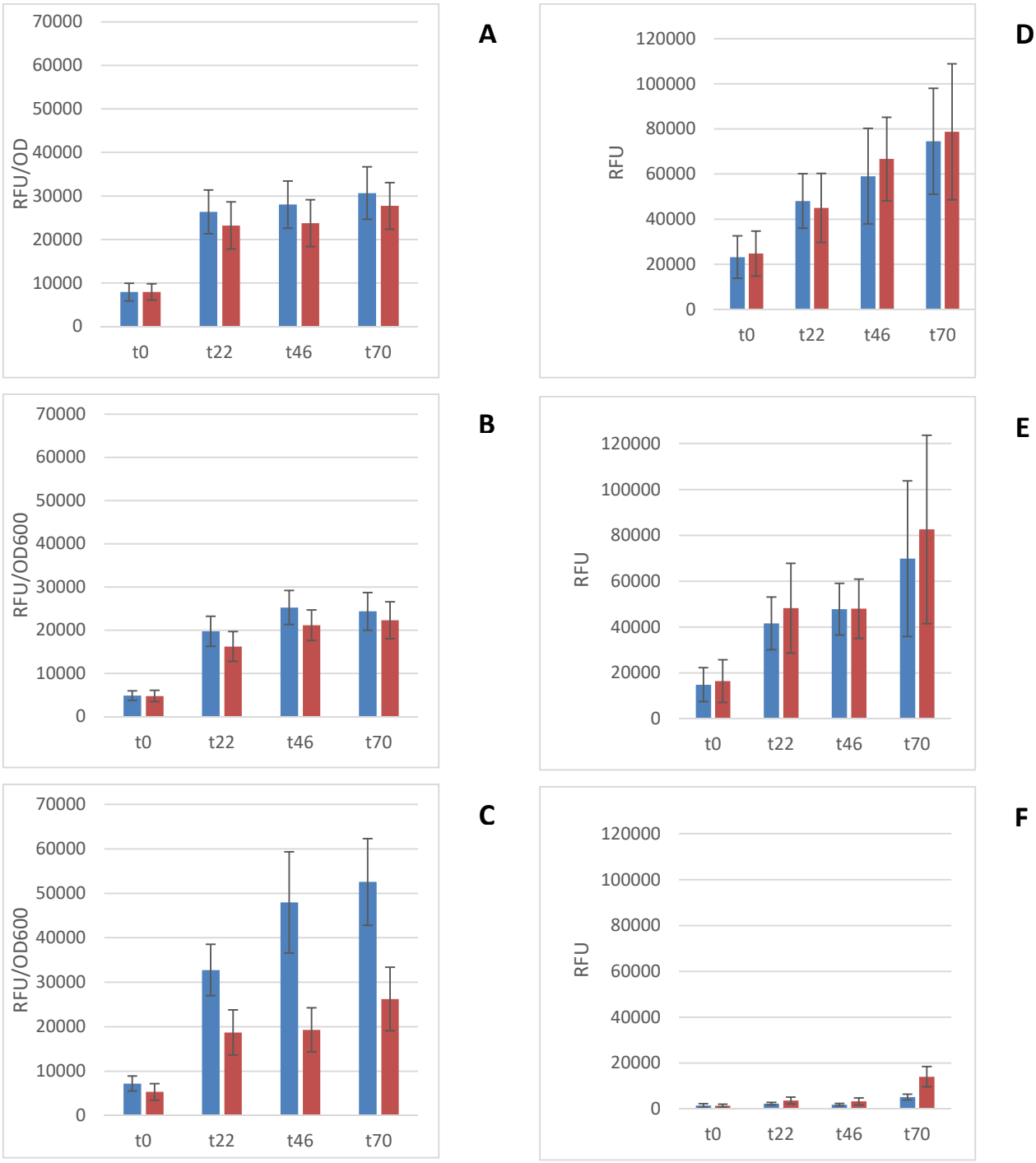


Figure 38 Time resolved comparison of *P. pastoris* CBS7435 *Δdas1* (RFU/OD_{600} **A** and RFU **D**), *Δdas2* (RFU/OD_{600} **B** and RFU **E**) and *Δdas1Δdas2* (RFU/OD_{600} **C** and RFU **F**) transformants expressing eGFP under the control of P_{CAT1}.
Blue: mean of respective knock-out clones at respective time point; red: mean of wild type clones; black bars indicate standard deviation based on all clones of the landscape at the respective time points.

To further investigate the higher expression levels derived from P_{CAT1} in the double knock-out strains, an experiment with a methanol utilisation slow strain (mutS), wild type and *Δdas1Δdas2* transformants with pPpT4mutZeoMlyI-intArg4-eGFP_pCAT1 integrated was done. The mutS strain lacks the faster homolog of the first enzyme in the MUT-pathway, namely alcohol oxidase 1 (*AOX1*).

This strain relies on the much weaker alcohol oxidase isoform *AOX2* and therefore grows much slower on methanol. In many cases employing the *P. pastoris* mutS strain leads to higher protein production, for example as shown for hepatitis B surface antigen (117).

This experiment should proof, if the higher P_{CAT1} driven protein expression is obtained from the slower growth on methanol, which arises in case of the mutS from the weaker expressed *AOX2* gene and in the Δ*das1Δdas2* strain from deleting the assimilatory pathway. The mutS strain is known for diminished toxic metabolic compounds, as formaldehyde when grown on methanol (118). The hypothesis was that the *P. pastoris* CBS7435 *Δdas1Δdas2* strain accumulates formaldehyde due to lack of the assimilatory pathway and hence induces the catalase promoter. To valuate this hypothesis a mutS strain was transformed with the eGFP-expression cassette harboring P_{CAT1}.
About 30 transformants of mutS and *Δdas1Δdas2* and about 20 transformants of the wild type were cultivated in in two same treated DWP with different clones from same transformations and RFU and OD_{600} were measured. RFU/OD_{600} and absolute RFU were plotted.
The three different strains showed no difference before induction with methanol, weak expression due to de-repression was observed as in the experiments before (compare Figure 38 C and Figure 39 A, B). After 24 hours of induction a distinct trend for higher expression of eGFP in *P. pastoris Δdas1Δdas2* transformants was observable, which continued through the whole induction phase. RFU/OD_{600} in mutS and wild type transformants didn't show significant differences during the whole induction time. Compared to the double knock-out, mutS and wild type showed 40-60 % less eGFP expression.

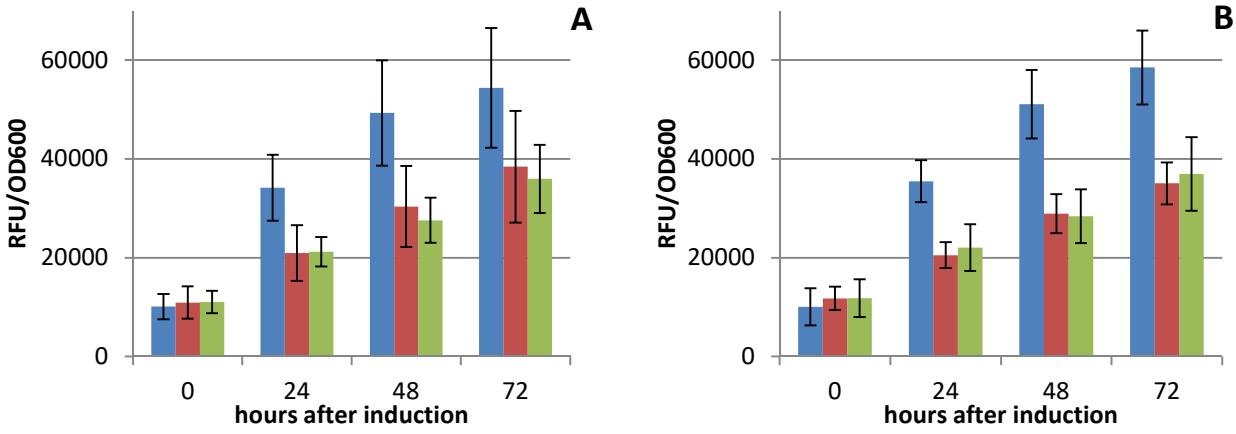


Figure 39 Comparison of *P. pastoris* CBS7435 *Δdas1Δdas2*, mutS and wild type expressing eGFP under the control of P_{CAT1} over a period of 72 hours. At 4 time points fluorescence and OD_{600} was measured. One before induction with methanol and 3 times after induction (after 24, 48 and 72 hours). Fluorescence was divided by OD_{600}, outliers were neglected, mean values and standard deviation were plotted
Diagrams **A** and **B** show the results of two independent DWP cultivations (different clones from the same transformation); blue: mean of *Δdas1Δdas2* clones at respective time point; red: mean of mutS clones; green: mean of wild type clones; black bars indicate standard deviation of mean values.

A different picture was observed when comparing absolute RFU values (Figure 40). Before induction no significant difference in the eGFP level as well as in the OD_{600} was observed (Table 25). The OD_{600} variation between the mutS and wild type strain was not significant during the complete cultivation. At some points the mutS strain showed even higher cell density, although methanol was the only carbon source. With longer induction time the mutS and wild type transformants had the highest absolute expression of eGFP due to more biomass and hence higher protein concentrations (Figure 40). 40-70% more protein/ higher GFP signal was observable on each of the screened plates compared to the double knock-out. Differences in eGFP expression levels between wild type and mutS strain were only between 5 and 10% after induction.

Table 25 Mean OD_{600} of *P. pastoris* CBS7435 *Δdas1Δdas2*, mutS and wild type expressing eGFP under the control of P_{CAT1}. The decreasing OD_{600} with time in the double knock-out strain arises from the growth limitation on methanol described in 3.2.1 and the consecutive addition of methanol due to the induction protocol.
KO: *P. pastoris* CBS7435 *Δdas1Δdas2*; mutS: of *P. pastoris* CBS7435 mutS; WT: *P. pastoris* CBS7435

	0 [h of induction]		**24 [h of induction]**		**48 [h of induction]**		**72 [h of induction]**	
strain	**Plate 1 [OD_{600}]**	**Plate 2 [OD_{600}]**	**Plate 1 [OD_{600}]**	**Plate 2 [OD_{600}]**	**Plate 1 [OD_{600}]**	**Plate 2 [OD_{600}]**	**Plate 1 [OD_{600}]**	**Plate 2 [OD_{600}]**
KO	4,77	3,00	1,28	1,48	1,02	1,07	0,83	0,68
mutS	5,10	2,78	4,07	5,34	3,38	4,69	3,20	3,56
WT	4,24	3,52	4,25	4,43	4,42	4,34	3,54	3,38

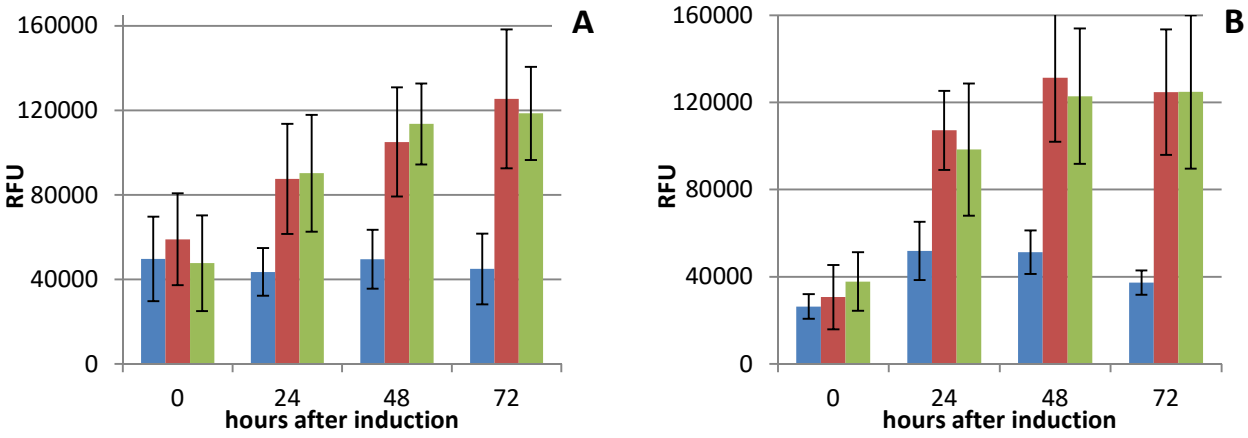


Figure 40 Comparison of *P. pastoris* CBS7435 *Δdas1Δdas2*, mutS and wild type expressing eGFP under the control of P_{CAT1} over a period of 72 hours. Same results as in Figure 39 were plotted, but OD_{600} correction was not performed.
Diagrams **A** and **B** show the results of two independent DWP cultivations (different clones from the same transformation); blue: mean of *Δdas1Δdas2* clones at respective time point; red: mean of mutS clones; green: mean of wild type clones; black bars indicate standard deviation of mean values.

Due to the fact that the assimilation pathway in the double knock-out is eliminated, the metabolic flux is re-directed towards the dissimilative pathways. Schroer K. et al. showed that the dissimilative pathway is limited by formaldehyde dehydrogenase (FLD) (27). This limitation of FLD could lead to an

accumulation of formaldehyde. As formaldehyde is a very toxic compound for the cell, it has to be detoxified in other ways, where probably further hydrogen peroxide is produced and P_{CAT1} was additionally induced.

Generally, no serious alteration in protein expression was observable for the single knock-out strains although the energy metabolism was severely altered. The constructed strains show nearly equal protein expression as the already established wildtype in terms of absolute RFU and RFU/OD_{600}. In the case of the double knock-out the relative and absolute expression levels are the same as the wild type levels when eGFP was produced under the control of the P_{GAP}. When it came to methanol induced production of the eGFP by P_{AOX1} and P_{CAT1} driven expression, the *P. pastoris Δdas1Δdas2* transformants reached a RFU/OD_{600} with the same level as for the wild type (*AOX1* promoter) or significantly higher RFU/OD_{600} (*CAT1* promoter), but absolute values were significantly lower for both constructs.

3.2.3. Activitiy assay and lipase B from *Candida Antarctica* (CalB)

Lipase B from *Candida Antarctica* (CalB) was chosen as alternative model protein to proof, if P_{CAT1} has similar effects on expression as for eGFP in the double knock-out, described in 3.2.2. CalB is a triacylglycerol lipase (EC 3.1.1.3, LC026014.1) commonly used in industry, such as detergent industry, oleochemistry or food industries (118). It shows no “interfacial activation” and higher activity towards carboxylic acid esters like ethyl octanoate or *p*-nitrophenyl butyrate, than on triglycerides (120) (121).

CalB was obtained in two different vectors either with P_{AOX1} or P_{CAT1} promoters (pPpT4-CalB+synPDI-pAOX1+pGAP-fwd and pPpT4-CalB+synPDI-pGAP+pCAT1-rev) from Lukas Sturmberger and Thomas Vogl (vector maps are pictured in 7.2, Figure 61, Figure 62). These vectors were linearized and used to transform *P. pastoris* wild type and *Δdas1Δdas2* as described in 2.10.10.

The gained wild type and *Δdas1Δdas2* transformants harbouring the *CalB* expression cassette under the control of P_{CAT1} (35 clones from each strain per DWP, two DWPs were cultivated in parallel with different clones from the same transformation) and *P. pastoris* wild type and *Δdas1Δdas2* transformed with the *CalB* expression cassette under the control of P_{AOX1} (seven of each background in each DWP). All these transformants were screened for their activity towards *p*-nitrophenol butyrate as described in 2.10.12.3. Empty strains from each background were used as negative control and established *P. pastoris* mutS strains expressing CalB under the control of P_{CAT1} (no. 643) and P_{AOX1} (no. 638) respectively were used as positive controls (strains are listed in Table 3).

Focus was set on the P_{CAT1} driven expression of CalB, because of the high GFP activity achieved with the double-knock-out strain harbouring the eGFP expression cassette under the control of P_{CAT1}, as discussed in 3.2.2.

To evaluate the expression levels of CalB a standard p-NPB assay was performed using the supernatant of the DWP cultivation. The activity level (U/mL/OD_{600}) of all tested CalB transformants (transformants of both plates are plotted in one graph: 1-35 plate 1 and 36-70 plate 2 in Figure 41; 1-7 plate 1 and 8-14 plate 2 in Figure 42) in different strain background rose with induction time as shown in Figure 41 and Figure 42 A, B and C. Highest activity levels were achieved after 76 h of induction with methanol. *CalB* under the control of the *CAT1* promoter showed best activity results in the tested strains. The wild type and the double-knock-out had comparable activity levels as the positive control (*P. pastoris* CBS7435 mutS pPpT4mutZeoMlyI-intArg4-bidi-CalB_VTU-synPDImutBmrI-GAP+pCAT1-rev K1 [short 8] (internal No. of Thomas Vogl's group: 643)) over the whole induction time, no significant difference were observable, only after 76 h of induction the activity in the *Δdas1Δdas2* was noticeable lower than in the wild type and the positive control. Transformants and positive control (*P. pastoris* CBS7435 mutS pPpT4mutZeoMlyI-intArg4-bidi-CalB_VTU-synPDImutBmrI-pAOX1+pGAP-fwd K1 [short 3] (internal No. of Thomas Vogl's group:638)) which expressed *CalB* under the control of the P_{AOX1} had an overall lower activity, but it raised with induction time to the same level as the P_{CAT1} construct in the positive control and the wild type transformants. The *Δdas1Δdas2* transformants showed after all induction times the lowest activity and also the lowest OD_{600} due to weak to no growth on methanol. As expected the empty strains showed no activity levels.

In contrary to the eGFP experiment, where the fluorescence was direct proportional to the gained amount of protein, for CalB only the volumetric activity was calculated. Therefore, protein concentration should be measured in next experiments.

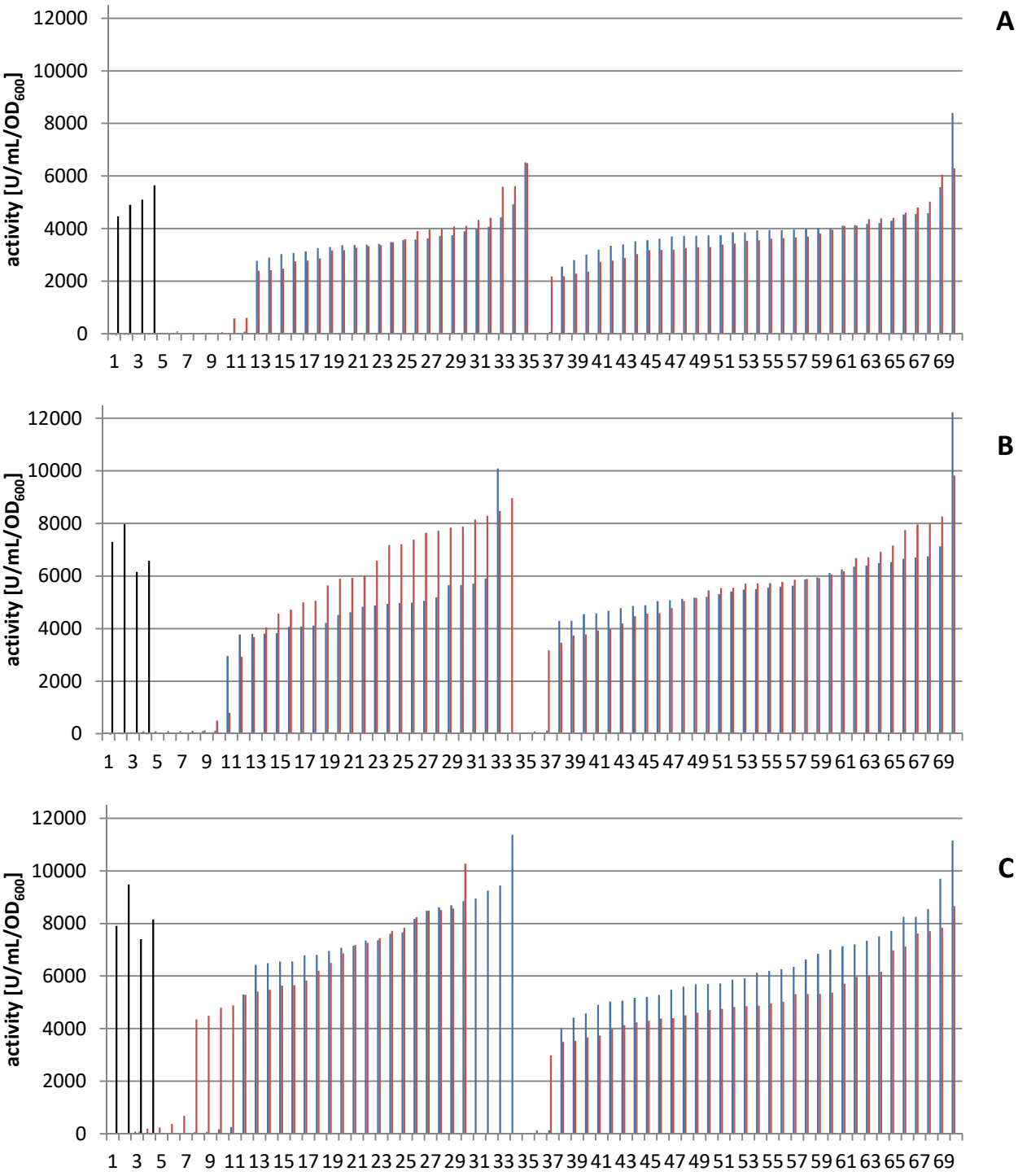


Figure 41 Landscape of the conversion of *p*-nitrophenylbutyrate to *p*-nitrophenol and butyric acid of *P. pastoris* wild type and *Δdas1Δdas2* expressing *CalB* und the control of the P_{CAT1}. Measurements were done after 24 h (**A**), 48 h (**B**) and 76 h (**C**) of induction with methanol.
blue: *P. pastoris* pPpT4-CalB+synPDI-pGAP+pCAT1-rev; red: *P. pastoris Δdas1Δdas2* pPpT4-CalB+synPDI-pGAP+pCAT1-rev; (1-35 transformants from plate 1; 36-70 transformants from plate 2)
black: positive pCAT1_CalB(643) (1 and 2 positive control from plate 1; 3 and 4 positive control from plate 2)

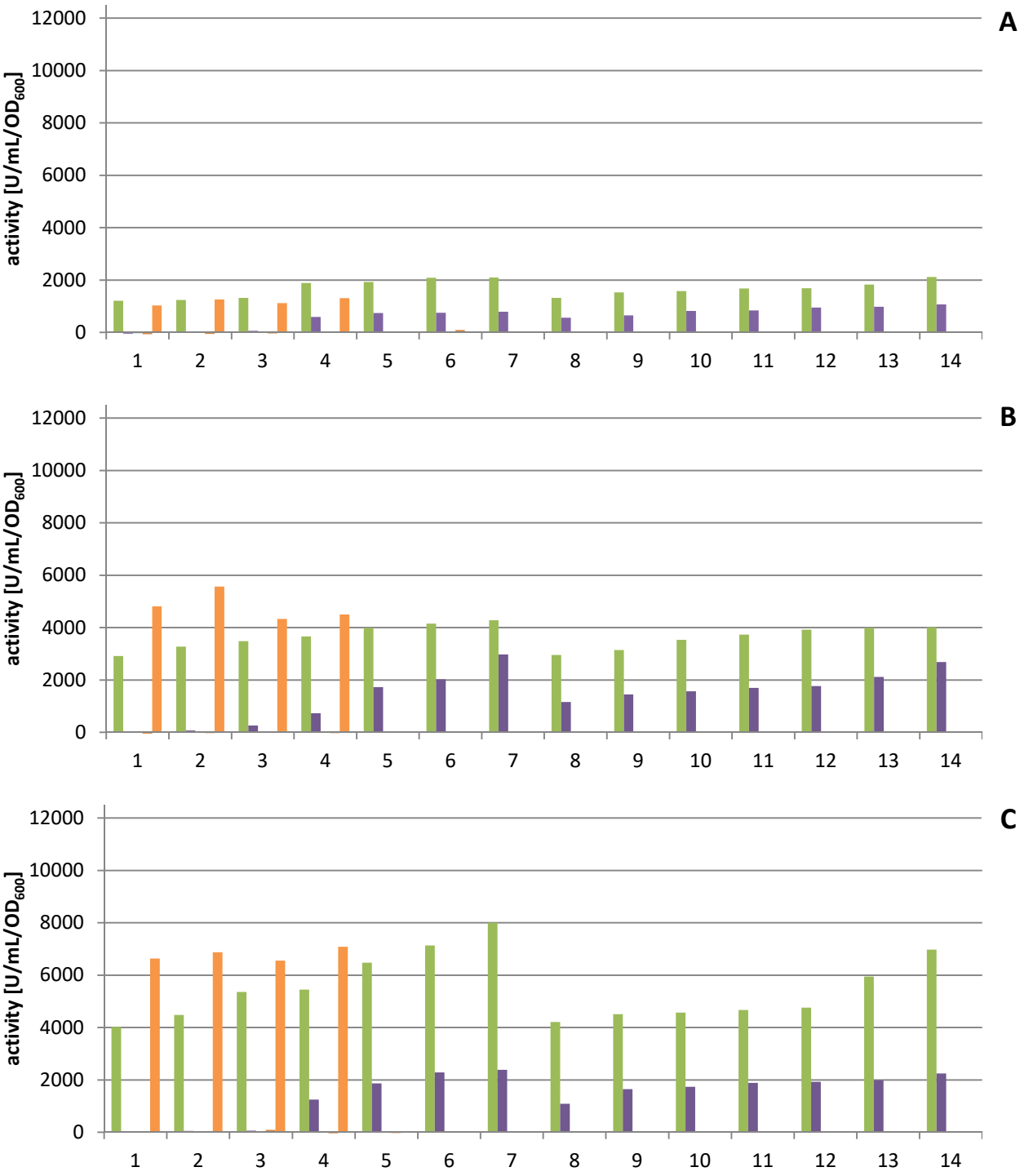


Figure 42 Landscape of the conversion of *p*-nitrophenylbutyrate to *p*-nitrophenol and butyric acid of *P. pastoris* wildtype and *Δdas1Δdas2* expressing *CalB* und the control of P_{AOX1} . Measurements were done after 24 h (**A**), 48 h (**B**) and 76 h (**C**) of induction with methanol.
green: *P. pastoris* pPpT4-CalB+synPDI-pAOX1+pGAP-fwd; purple: *P. pastoris Δdas1Δdas2* pPpT4-CalB+synPDI-pAOX1+pGAP-fwd (1-7 transformants from plate 1; 8-14 transformants from plate 2)
orange: positive control pAOX1_CalB (638) (1 and 2 positive control from plate 1; 3 and 4 positive control from plate 2)

3.2.4. Improvement of NADH regeneration in *P. pastoris* CBS7435

3.2.4.1. 2,3-butanediol dehydrogenase (BDH1) activity assay

To investigate NADH regeneration in the *P. pastoris* CBS7435 wild type, *Δdas1*, *Δdas2* and *Δdas1 Δdas2* strains 2, 3-butanediol dehydrogenase (GenBank entry: U12980, gene *YAL060W*) from *S. cerevisiae* strain BY4741 (ScBDH1) was used as a model protein in NADH-dependent whole-cell conversions.

BDH1 catalyses the stereospecific reduction of racematic acteoin ((*rac*)-acetoin; K_m = 4.5 mM) to (*2R,3R*) 2,3 butanediol and *meso*-butanediol, in the presence of NADH (87). Due to the high conversion rate (k_{cat} = 98,000 min^{-1} (87)) BDH1 is a good candidate to characterize the generated strains for improved NADH regeneration.

3.2.4.1.1. Standard curve

For determination of whole-cell conversion of acetoin to butanediol a standard curve was done. Therefore, different acetoin concentrations (7.66 mM, 3.06 mM, 1.53 mM and 0.77 mM) with the internal standard BuOH (8.60 mM) were determined by GC-analysis (2.10.12.2.1).

The retention times of S-acetoin, *R*-acetoin L-butanediol, D-butanediol and *n*-butanol (internal standard, IS) are listed in Table 26. Standard curves for the products were not established due to low product detection in pre-experiments. Conversion was calculated based on substrate declination.

Table 26 Retention times for compounds investigated with GC-analysis with FID.

Chemical	**Retention time [min]**
n-butanol	3.5
S-acetoin	4.1
R-acetoin	4.6
L-butanediol	11.3
D-butanediol	11.7

Standard curve (Figure 43) was calculated with the trend line function in "Excel 2010" and zero was set as cutting point. The resulting equation y = 0.068x with R^2=0.9955 was used for the calculation of acetoin concentration in the reaction, where x is the actual concentration of acetoin in mM and y as for the coefficient of A_x/A_{is} (A_x… peak area of acetoin; A_{is}…peak area of internal standard n-BuOH).

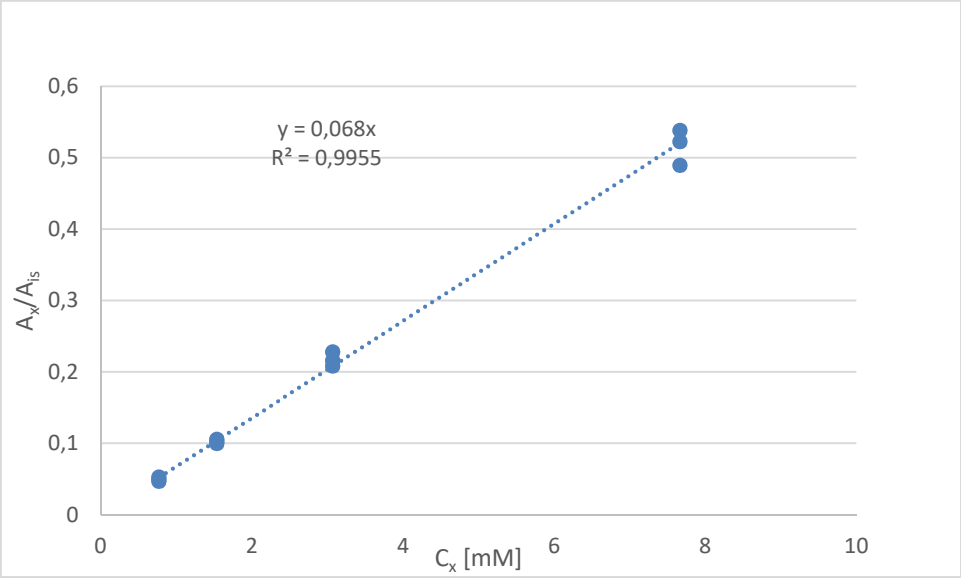


Figure 43 Acetoin standards after extraction. Trend line cutting point was set at zero.
A_x: peak area of acetion, A_{is}: peak area of n-BuOH, C_x: acetoin concentration.

3.2.4.1.2. Screening

Single colonies of *P. pastoris* CBS7435 wild type, *Δdas1*, *Δdas2* and *Δdas1 Δdas2* transformants harbouring the *BDH1* expression cassette were cultivated in DWP as described in 2.10.11.3.
In the first screening only a few transformants of each strain containing an integrated expression cassette were gained for acetoin conversion measurements. This was a pre-screening to evaluate if differences in acetoin conversion catalysed by BDH1 in the wild type and in the knock-out strain were observable. Due to serious problems with the GC-device, for the initial screening no valid values for a standard curve could be determined and no normalization to OD_{600} was performed. The relative conversion for each transformant was plotted. The ΔS (substrate declination) was calculated as the difference between A_x/A_{is} of the sterile control and A_x/A_{is} of the BDH1 expression transformant. ΔS was plotted as a landscape in Figure 44 A. In parallel the proportion of the summarized product peaks of D-butanediol and meso-butanediol (A_p) to the internal standard (A_{is}) were plotted referring to the transformants of the substrate declination graph (Figure 44 B).
As shown in Figure 44 A most of the double knock-out transformants had a measurable ΔS compared to the wild type clones where only about half of the clones obtained a ΔS. But, within these wild type transformants the highest substrate declination was observed for this experiment.
The single knock-out strain (*Δdas1* and *Δdas2*) transformants showed a substrate conversion as well. *Δdas2* strains had an overall higher conversion than the screened *Δdas1* ones. The ΔS of the best ones of each strain were comparable to each other, as shown in Figure 45 A.
As the substrate consumption was compared to product formation of the different transformants, shown in the landscape of Figure 44 B and for the 3 best clones of the landscape in Figure 45 B, it was

noticeable that the product building of the wild type transformants was not in line with the substrate declination. For example, the wild type clone with the highest substrate consumption (No. 36 in Figure 44 A) showed no detectable product formation (No. 36 in Figure 44 B). Overall the wild type transformants showed hardly any detectable product formations. Substrate declination and product formation for both single knock-out and the double knock-out transformants went best hand in hand. The landscape for these almost matched each other for ΔS and product building. (Figure 44 and Figure 45).

It seemed that there were other, not investigated causes for substrate conversion or other pathways and circumstances which further converted the products to not detectable molecules. But, if the high substrate declination was high, detectable amounts of product were present in most cases.

Conclusion of the pre-experiment was to focus on substrate declination as parameter for better NADH-regeneration, even if some maybe “false” positive clones were further investigated.

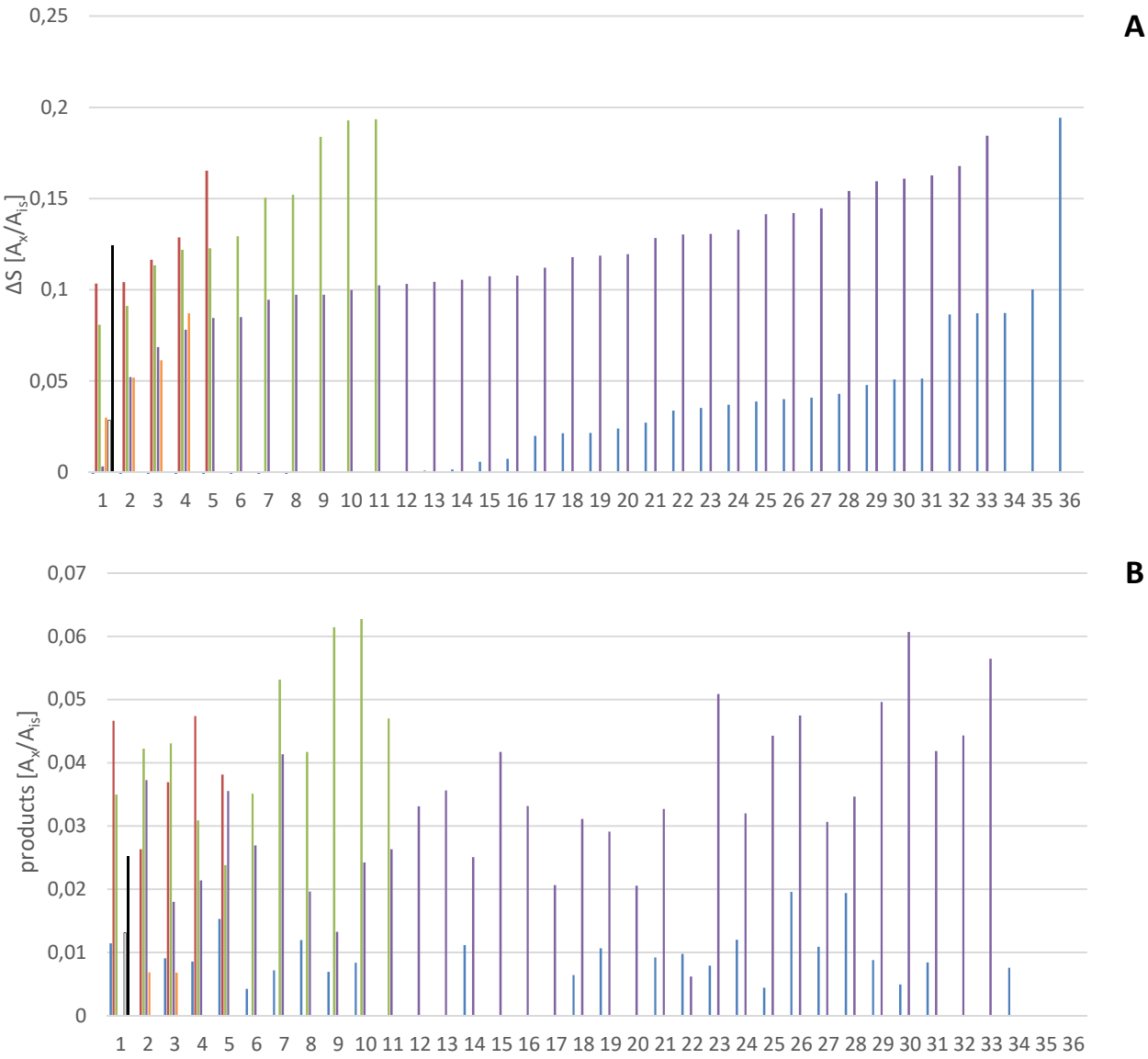


Figure 44 Landscape of the pre-screening of *P. pastoris* wild type, *Δdas1*, *Δdas2* and *Δdas1Δdas2* containing pPpT4GAP-S_ScBDH1. The results were not normalized to OD_{600}. For this experiment only empty strain controls of the wild type and the double knock-out were performed.
A: Substrate declination for each screened clone was plotted from lowest to highest difference.
B: Referring to the ΔS the product formation was plotted. The areas of the product peaks of D-butanediol and *meso*-butanediol were added.
Transformants: blue: wild type ScBDH1; red: *Δdas1* ScBDH1; green: *Δdas2* ScBDH1; purple: *Δdas1Δdas2* ScBDH1; orange: positive; white: wild type; black: *Δdas1Δdas2*

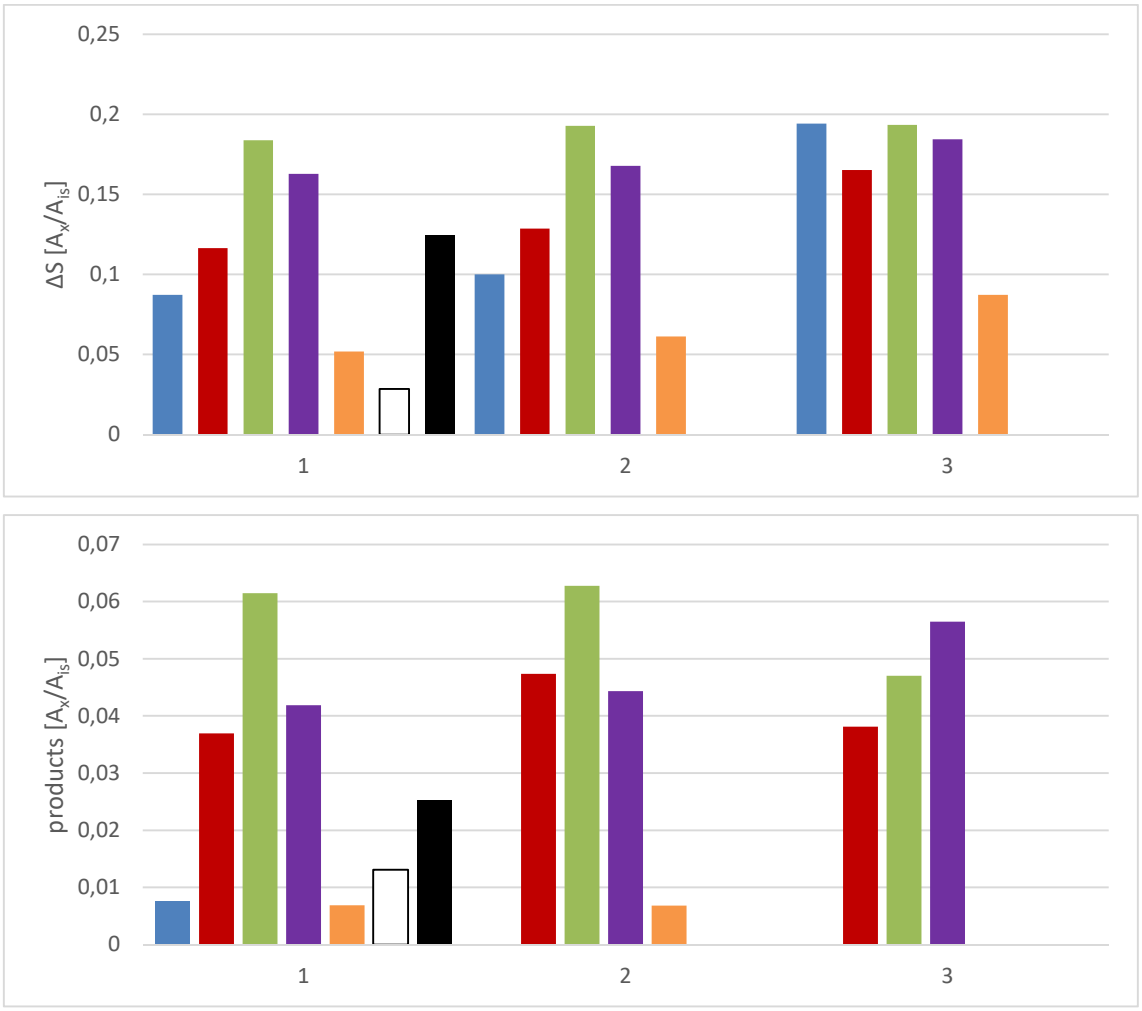


Figure 45 Comparison of the three best clones of the pre-screening of *P. pastoris* wild type, *Δdas1*, *Δdas2* and *Δdas1Δdas2* containing pPpT4GAP-S_ScBDH1 of the landscape in Figure 44. Three positive controls and empty strain controls of the wild type and *Δdas1Δdas2* are also shown. The results were not normalized to OD_{600}.
A: Substrate declination for the three best clones was plotted from lowest to highest difference.
B: Referring to the ΔS the product formation was plotted. The area of the product peaks of D-butanediol and meso-butanediol were added.
Transformants: blue: wild type ScBDH1; red: *Δdas1* ScBDH1; green: *Δdas2* ScBDH1; purple: *Δdas1Δdas2* ScBDH1; orange: positive; white: wild type; black: *Δdas1Δdas2*

For the further screening procedure 2 plates with *P. pastoris* CBS7435 wild type, *Δdas1*, *Δdas2* and *Δdas1Δdas2* clones containing pPpT4GAP-S_ScBDH1 were investigated. As controls 8 acetoin converting clones (positive control, +, *P. pastoris* CBS7435 harbouring pPICZ-BD1-BDH; strain collection no.: 3441), and 2-3 clones each of the strains (wild type, *Δdas1*, *Δdas2* and *Δdas1Δdas2*) without pPpT4GAP-S_ScBDH1 were cultivated in DWP (wild type and *Δdas2* transformants, confer Figure 46 A and B and *Δdas1* and *Δdas1Δdas2* confer Figure 47 A and B).
For the second screening each strain was newly transformed with the *BDH1* expression cassette. The standard curve from 3.2.4.1.1 was applied for calculation of substrate concentration of the reaction mixture. The OD_{600} was measured via a plate reader to calculate the $\Delta S/OD_{600}$ and product formation per OD_{600} $((A_x/A_{is})/OD_{600})$ for each screened clone.

In the second screening $\Delta S/OD_{600}$ showed a bigger difference in between the different strains compared to the pre-screening, where ΔS was not normalized to OD_{600}. Wild type, *Δdas1* and *Δdas2* strains harbouring pPpT4_GAP-S_ScBDH1 and empty ones grew to the 1.5-2 fold OD_{600} (Table 27) compared to the double-knock-out ones.

Substrate declination was in the best *Δdas1Δdas2* transformants 4-fold higher in the OD_{600} normalized calculation than in the other transformed strains (Figure 46 A and Figure 47 A). In absolute values, not considering the cell mass (OD_{600} values in Table 27), the conversion of the double-knock-out was as well clearly better. The product $(A_x/A_{is})/OD_{600}$ landscape linked to the substrate declination landscape showed that low substrate conversion led to low or no product formation, compare Figure 46 A and B and Figure 47 A and B.

The $\Delta S/OD_{600}$ values for the positive control, the transformants of *Δdas1*/*Δdas2* and the empty strains of the wild type, *Δdas2* and *Δdas1Δdas2* were all at about 1-2 mM/OD_{600}. The empty strain controls of *Δdas1* were not able to convert anything at all (Figure 47 A).

The best *Δdas1Δdas2* pPpT4GAP-S_ScBDH1 clones showed about 60% (absolute values) conversion of the substrate (substrate concentration for calculation of ΔS was 11.14 mM). Interestingly some of the empty strains showed nearly the same conversion as the positive control. This led to the assumption that the positive control could be wrong named and stored. Due to time limitations the genetic situation of *P. pastoris* CBS7435 harbouring pPICZ-BD1-BDH was not proved, but should be done in the future. It could have also been possible that something with the transformation plasmid has gone wrong and many of the transformants didn´t have a fully functional integrated vector.

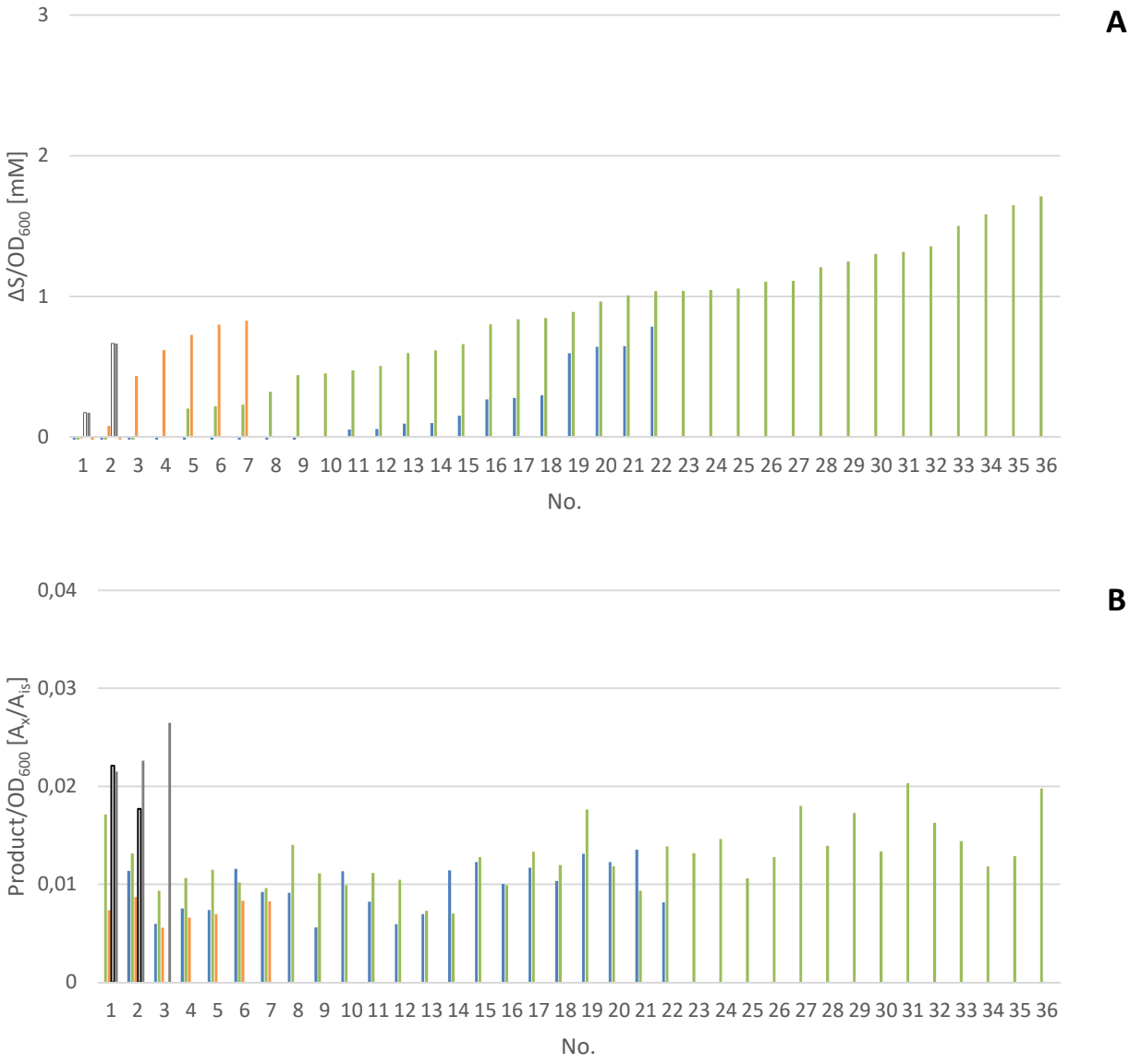


Figure 46 Landscape of the screening of transformants of *P. pastoris* wild type and *Δdas2* containing pPpT4GAP-S_ScBDH1 (copy number of vector not determined)
A: Substrate declination per OD_{600} for each screened clone was plotted from lowest to highest difference.
B: Referring to ΔS the product formation per OD_{600} was plotted. Areas of the product peaks of D-butanediol and meso-butanediol were added.
Blue: WT BDH; green: *Δdas2* BDH; orange: positive control; white: wild type; grey: *Δdas2*;

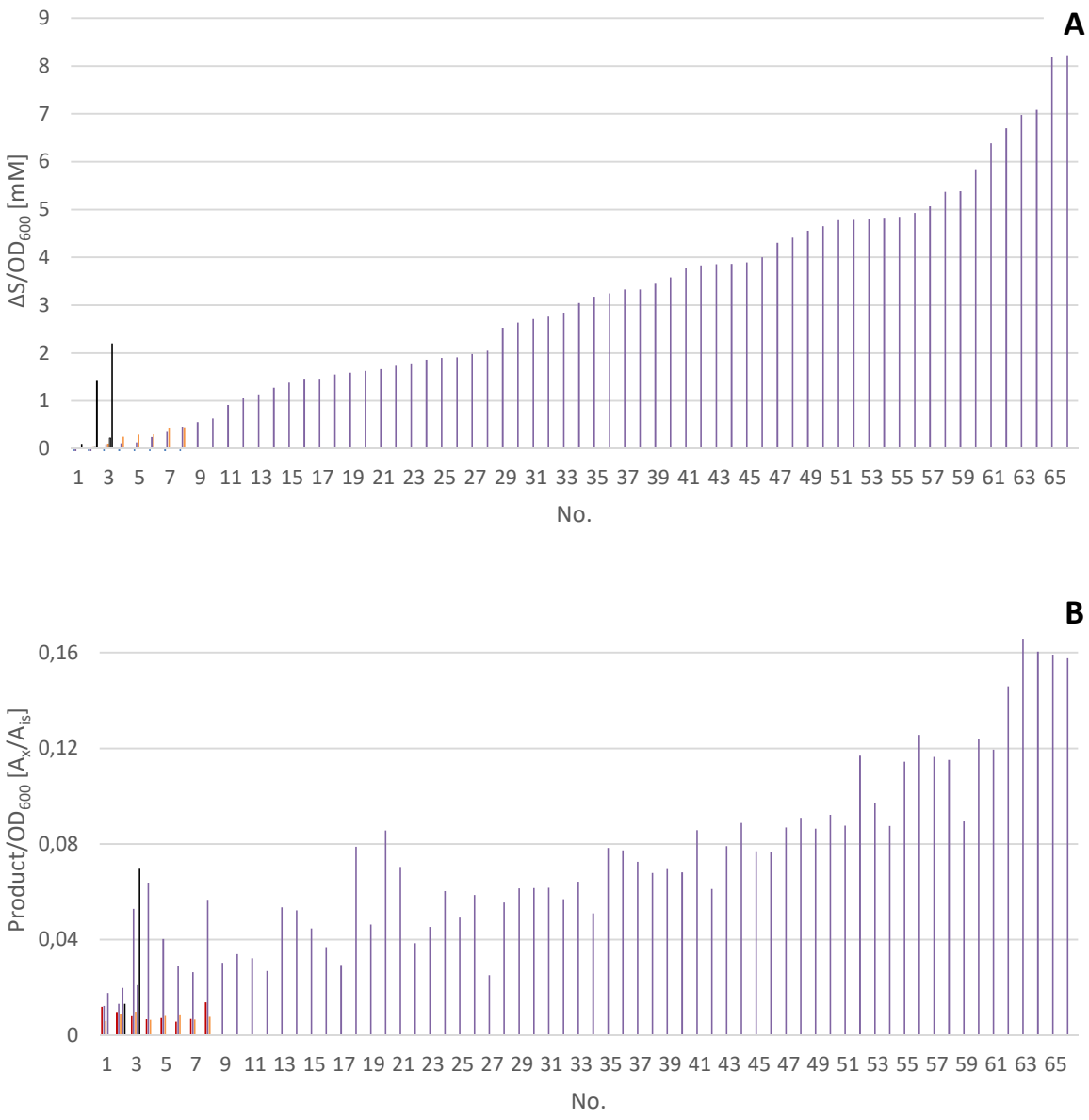


Figure 47 Landscape of the screening of transformants of *P. pastoris Δdas1* and *Δdas1Δdas2* containing pPpT4GAP-S_ScBDH1 (copy number of vector not determined)
A: Substrate declination per OD_{600} for each screened clone was plotted from lowest to highest difference.
B Referring to ΔS the product formation per OD_{600} was plotted. Areas of the product peaks of D-butanediol and meso-butanediol were added.
Red: *Δdas1* BDH; purple: *Δdas1Δdas2* BDH; orange: positive control; grey: *Δdas1*; black: *Δdas1Δdas2*; orange: sterile control

Table 27 Average OD_{600} of the screened transformants in Figure 46 A (plate 1) and Figure 47 A (plate 2). Measured with a plate reader at a dilution of 1:5

strain	OD_{600}	Plate no.
Wild type BDH	2.18	Plate 1
Wild type	1.78	
***Δdas2* BDH**	1.78	
Δdas2	1.49	
Positive control	1.78	
***Δdas1* BDH**	2.16	Plate 2
Δdas1	1.69	
***Δdas1Δdas2* BDH**	1.01	
Δdas1Δdas2	1.01	
Positive control	2.07	

The average conversion of acetoin to the two products (2*R*, 3*R*)-butane-2,3-diol and *meso*-butane-2,3-diol was at low levels for the wild type (3% conversion) and the *Δdas2* strain (8% conversion) indicating a not significantly improved NADH-regeneration in the *Δdas2* knock-out strain. As mentioned above the screened *Δdas1* transformants did not show any substrate declination in the reaction. In *P. pastoris Δdas1Δdas2* expressing the *BDH1* 31% conversion was reached, which means an 11-fold higher and about 4-fold higher conversion than the wildtype and the *Δdas2* strain, respectively. The double knock-out had a much higher conversion despite the lower number of cells within the reaction, which gave a first hint of an improvement in the regeneration of NADH due to the elimination of the assimilative pathway.

As result out of this screenings, as no proper candidate from both single knock-outs was able to be chosen, one of the best *P. pastoris* pPpT4GAP-S_ScBDH1 wildtype transformants and one of the double-knock-out transformants chosen for a time resolved conversion. For eliminating the discrepancy of growth in the DPW the best candidates were grown in shake flasks. The same cell amount was used for the biocatalytic reaction in the DWP to see which growth time is most favourable for the highest yields concerning conversion of substrate per OD_{600} culture. An empty strain control (wild type and *Δdas1Δdas2*) was chosen as control reference.

3.2.4.1.3. Re-screening – Time-resolved conversion

After the screening procedure one of the most promising candidates of the wild type transformants (clone 9 from the pre-screening) and one of the double-knock-out transformants (clone 29 from the screening) and an empty strain from each were chosen for a time-resolved conversion (see 2.10.12.2.2. and 2.10.12.2).

As seen in the pre-screening the OD_{600} was again about half for the double knock-out strains compared to the wild type (Table 28), which indicates again, that the *Δdas1Δdas2* knock-out, has limitation of growth on methanol, as discussed in 3.2.1.

Table 28 Mean value of the triple determination of the OD_{600} and absolute substrate declination ΔS (not normalized to OD_{600}) after 12 h reaction. The growth time refers to the inoculation time in the shake flasks

Clone	Vector	Growth [h]	OD_{600} mean	absolute ΔS mean [mM]
Wild type		24	1,468	0,88
Wild type		36	1,308	0,91
Wild type		48	1,361	1,02
Δdas1Δdas2		24	0,577	1,33
Δdas1Δdas2		36	0,520	0,43
Δdas1Δdas2		48	0,637	0,81
Wild type	pPpT4_GAP-S_ScBDH1	24	1,111	1,68
Wild type	pPpT4_GAP-S_ScBDH1	36	1,151	1,14
Wild type	pPpT4_GAP-S_ScBDH1	48	1,389	1,43
Δdas1Δdas2	pPpT4_GAP-S_ScBDH1	24	0,495	2,70
Δdas1Δdas2	pPpT4_GAP-S_ScBDH1	36	0,635	1,88
Δdas1Δdas2	pPpT4_GAP-S_ScBDH1	48	0,530	2,08

The highest $\Delta S/OD_{600}$ and also absolute conversion (ΔS) (Table 28) at any time point was achieved by the double knock-out clone expressing *BDH1*. Due to the fact that standard deviation was very high through the whole data, the experiment should be repeated for confirmation of the results, which might be attributed to improved NADH-regeneration.

In Figure 48 the relative conversions of the empty strains (*P. pastoris* wild type and Δdas1Δdas2) and the best double knock-out strain expressing *BDH1* under the control of the P_{AOX1} promoter are shown in comparison to the best wild type strain expressing *BDH1* (ΔS =100%). The strain expressing *BDH1* has at each measured time-point a higher substrate declination rate as its empty counterpart. This applies to the wild type strain and to the double knock-out strain as well. The conversion of the empty *P. pastoris Δdas1Δdas2* is after 24 h about 52% higher than the wild type producing BDH1. In later time of the experiment the conversion is at the nearly same level, 83% (36 h) and 124% (48 h), considering the standard deviation.

The high substrate declination in the empty double knock-out control led to the assumption, that eliminating *das1* and *das2* genes has an impact on the conversion of acetoin by site reactions, which are probably NADH dependent, within the cell. Deletion of the assimilative MUT-pathway may enhance the conversion of acetoin by this unknown endogen enzymes. Due to problems in product detection with the GC-device, it was not possible to elicit the proportion of the unwanted conversion

to the BDH1 driven one. The conversions of the empty wild type strain, which were between 39% (24 h), 71% (36 h) and 72% (48 h) and clearly below the rates of the settlement strain.

Schroer et al. underline this assumption, because they have also observed endogenous reduction reaction in their experiments with *BDH1* expressing *P. pastoris* strains and suggested to detect and eliminate *BDH1* homologs in this yeast (27). Reduction rate of acetoin was up to four-times higher by expressing *BDH1* in *P. pastoris Δdas1Δdas2* (confer Figure 48). This result confirms the assumption, that redirecting the carbon flux towards the dissimilative pathway improves NADH formation and consequently the reaction rate of BDH1 in the new generated chassis. The longer the cells grew in the pre-culture before substrate addition, the lower became the total conversion, but the empty wild type was constant at all-time points.

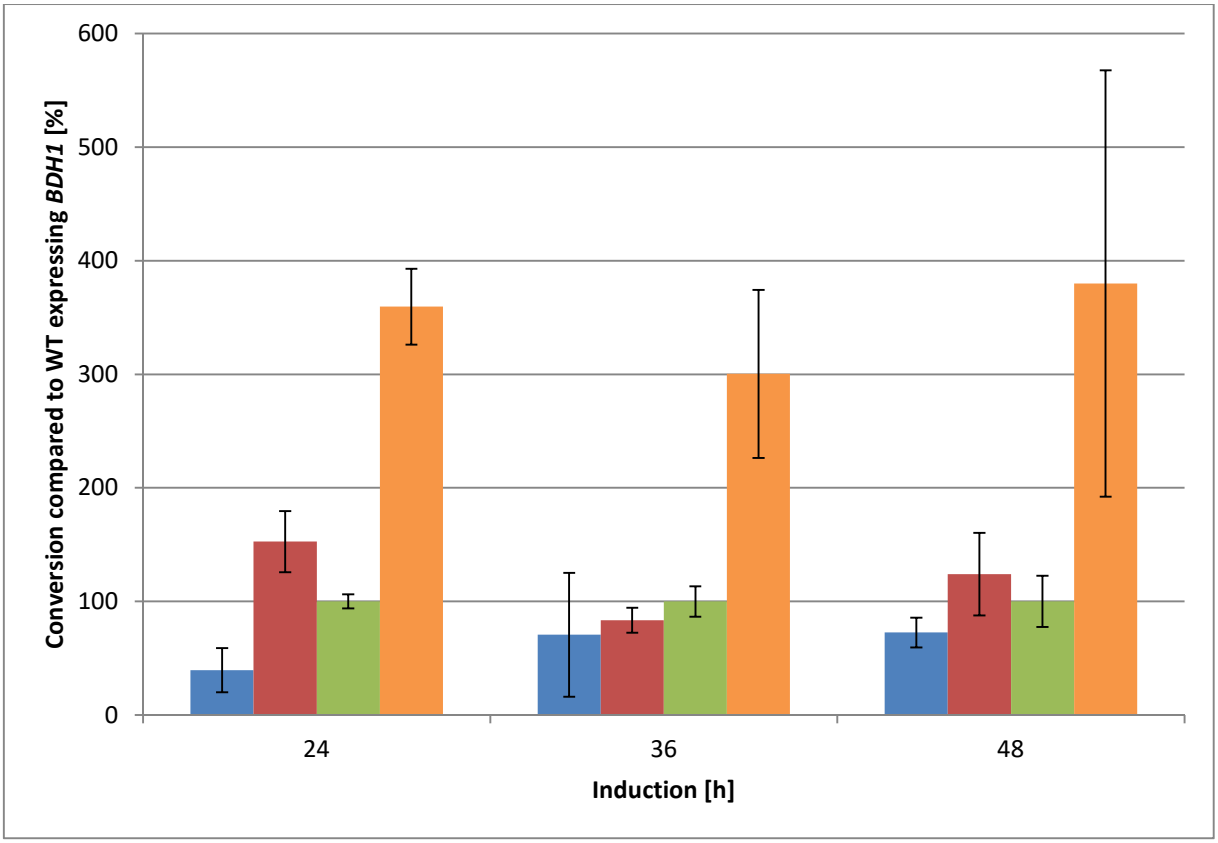


Figure 48 Time resolved conversion of acetoin to butanediol. The graph shows the substrate declination in comparison to the wild type strain expressing *BDH1*. Whereat the ΔS of the wild type expressing *BDH1* was set to 100% at each time-point. blue: wild type; red: *Δdas1Δdas2*; green: wild type pPpT4GAP-S_ScBDH1 clone 9 from pre-screening; orange: *Δdas1Δdas2* pPpT4GAP-S_ScBDH1

Summarizing NADH-regeneration was shown to be improved in the *Δdas1Δdas2* strain, because the empty strain as well as the *BDH1* expressing strain of the double knock-out had an overall better conversion than the wild type pendants. Causes for the unwanted reactions in the empty strain controls should definitely be further investigated, i.e. searching and eliminating of *BDH1* homologous genes.

4. CONCLUSION AND OUTLOOK

Efficient co-factor recycling is not only required for optimal enzyme performances in biocatalytic applications, but also to set up an ecological and sustainable process in further consequence. Performing biotransformation in whole cells where the cell metabolism can be exploited for co-factor supply is an elegant way to fulfill these requirements. In this thesis a new platform strain for whole-cell catalysis was generated based on the methylotrophic yeast *P. pastoris* by engineering its methanol utilization pathway. For this purpose, the assimilative pathway responsible for the biomass production was eliminated and the carbon flux was forced through the dissimilative pathway by oxidizing methanol to CO_2. Theoretically two molecules of NADH are formed per molecule methanol. The generated *P. pastoris Δdas1Δdas2* strain was tested for its performance in a dehydrogenase based whole cell biotransformation without external addition of a co-factor. The double knock-out strain displayed a better conversion than the wild type strain due to extended NADH supply by oxidation of methanol.

The cheap carbon source methanol serves as multi tool in the bioconversion of acetoin via BDH1: It acts as an inducer of the P_{AOX1} driven production of the protein as well as of the endogenous NADH regeneration system. Methanol itself is used as co-substrate for the NADH-regeneration. The oxidation of methanol is an irreversible process with a high thermodynamic driving force with CO_2 as the only side-product. Moreover, methanol has the ability of enhancing the solubility of organic substrates in the reaction mixture and *P. pastoris* is already adopted to elevated methanol concentrations.

In case of the newly developed *Pichia* strain no additional overexpression for improved NADH-regeneration is necessary compared to i.e. the often used formate dehydrogenase, which only yields one mole NADH per mole carbon.

The heterologous protein production with eGFP (driven by P_{AOX1} and P_{GAP}) in the generated knock-out strains showed same expression levels as the wild type. When eGFP was produced under the control of P_{CAT1} a higher yield of protein at lower cell density was observable for the *P. pastoris Δdas1Δdas2* strain. P_{CAT1} driven expression of *CalB* did not lead to higher activity in the double knock-out strain.

The generation of a new valuable host for whole-cell biotransformations, where NADH as co-factor is an issue, was demonstrated with the redesigned *P. pastoris* strain. A simple way to boost the efficiency of the endogenous NADH-regeneration rate, which leads to new opportunities of the whole-cell catalysis for pharmaceutical and chemical industries.

In the future the new *Pichia* chassis has to be tested with other NADH-dependent dehydrogenases to validate its applicability.

As next step to further enhance the NADH-regeneration of *P. pastoris Δdas1Δdas2* overexpressing of the formaldehyde dehydrogenase, which catalyzes the bottleneck reaction in the dissimilative pathway, would be possible.

5. ACKNOWLEDGMENTS

I would like to thank Univ.-Prof. Dipl.-Ing. Dr.techn. Anton Glieder and the ACIB GmbH to give me the opportunity to carry out my thesis in their department. A special thank-you I want to give Martina Geier for supervising my laboratory work and helping me with all the questions occurring during my thesis.

I gratefully acknowledge the help of Mudassar Ahmad in designing the knock-out vectors and providing the vector-system for generating the new *Pichia* strain. I further want to thank Thomas Vogl (TU Graz, Austria) for providing the plasmids harboring the sequence of eGFP under the control of P_{AOX1}, P_{CAT1}, and P_{GAP} respectively and the plasmids harboring the sequence of CalB under the control of P_{AOX1} and P_{CAT1} and Andrea Camattari for providing the *BDH1* harboring plasmid.

This research was funded by the Innovative Medicine Initiative Joint Undertaking project CHEM21 under grant agreement n°1153600, resources of which are composed of financial contribution from the European Union´s Seventh Framework Programme (FP7/2007-2013) and EFPIA companies´in kind contribution. Other supporters of this work have been the Federal Ministry of Science, Research and Economy (BMWFW), the Federal Ministry of Traffic, Innovation and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol, the Government of Lower Austria and ZIT – Technology Agency of the City of Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG.

Last but not least I thank my family and my friends for the understanding, the support, the nice words and everything else over the last years.

6. REFERENCES

1. **Liese A, Seel bach K, Wandrey C.** *Industrial biotransformations.* s.l. : Weinheim: Wiley-VCH, 2000.

2. **Buchholz K., Kasche V., Bornscheuer U. T.** *Biocatalysts and Enzyme Technology.* 2nd edn . s.l. : Wiley-VCH, 2012.

3. **Hilderbrandt G, Klavehn W.** *Verfahren zur Herstellung von 1-1-phenyl-2-methylaminopropan-l-ol. 548459* Germany, 24. March 1930.

4. **Sym, Ernest Alexander.** Lipase and its action. I. The synthetic action of pancreatic lipase in the system: oleic acid-glycerol-water-dissolved lipase. *Biochem J.* 1930, 24(5):1265–1281.

5. —. Action of Esterase in the Presence of Organic Solvents . *Biochemical Journal .* 1936, 609–617.

6. **Halling, P., Kvittingen, L.** Why did biocatalysis in organic media not take off in the 1930s? *Trends in Biotechnology.* 1999, 17 (9), pp. 343-344.

7. **Zaks A, Klibanov AM.** Enzyme-catalyzed processes in organic solvents. *Proc Natl Acad Sci U S A.* 1985, 82(10):3192-6.

8. **Zaks A, Klibanov AM.** Enzymatic catalysis in organic media at 100 degrees C. *Science.* 1984, 224(4654):1249-51.

9. **Panke S., Held M., Wubbolts M.G., Witholt B., Schmid A.** Pilot-scale production of (S)-styrene oxide from styrene by recombinant Escherichia coli synthesizing styrene monooxygenase. *Biotechnol Bioeng.* 2002, 80(1):33-41.

10. **Coleman M. H., Macrae A. R. (Unilever Ltd).** *Fat process and composition. GB1577933* 1980.

11. **Matsuo T., Sawamura N., Hashimoto Y., Hashida W.** *Producing a cacao butter substitute by transesterification of fats and oils . GB2035359* UK, 18. June 1980.

12. **K., Faber.** *Biotransformations in organic chemistry.* Berlin : Springer, 2004. Bd. 5th edn.

13. **Rozzell, J.D.** Commercial scale biocatalysis: Myths and realities. *Bioorganic and Medicinal Chemistry.* 1999, 7 (10), pp. 2253-2261.

14. **Onuffer, J.J., Ton, B.T., Klement, I., Kirsch, J.F.** The use of natural and unnatural amino acid substrates to define the substrate specificity differences of Escherichia coli aspartate and tyrosine aminotransferases. *Protein Science.* 1995, 4 (9), pp. 1743-1749.

15. **Yang, J., Yang, S., Yang, Y.-L., Zheng, H., Weng, L., Liu, L.** Microbial hydroxylation of 16α,17α-dimethyl-17β-(l-oxopropyl)androsta-l,4-dien-3-one to rimexolone by Curvularia lunata AS 3.4381. *Journal of Molecular Catalysis B: Enzymatic.* 2007, 47 (3-4), pp. 155-158.

16. **Motosugi, K., Esaki, N., Soda, K.** Enzymatic preparation of D- and L-lactic acid from racemic 2-chloropropionic acid. *Biotechnology and Bioengineering.* 1984, 26 (7), pp. 805-806.

17. **Mathew CD, Nagasawa T, Kobayashi M, Yamada H.** Nitrilase-Catalyzed Production of Nicotinic Acid from 3-Cyanopyridine in Rhodococcus rhodochrous J1. *Applied and Environmental Microbiology.* 1988, 54(4):1030-1032.

18. **Chuck, R.** A catalytic green process for the production of niacin. *Chimia.* 2000, 54 (9), pp. 508-513.

19. **Möller A., Friedrich H., Kuhn H., Winkler K. und Aktiengesellschaft, Degussa.** *Verfahren zur Herstellung grobkristalliner Nicotinsäure hoher Reinheit. EP0242535 A2* Deutschland, 28. Oct. 1987. Anmeldung.

20. **Nakamura K., Matsuda T.** Reduction of ketones. [Buchverf.] Waldmann H. Drauz K. *Enzyme catalysis in organic synthesis.* Weinheim : Wiley–VCH Verlag GmbH, 2002, Bde. vol. III, 2nd edn. , S. pp 991–1047.

21. **Ishige T., Honda K., Shimizu S.** Whole organism biocatalysis. *Current Opinion in Chemical Biology.* 2005, Vol. 9, Iss. 2, pp. 174-180.

22. **Iyer, P.V., Ananthanarayan, L.** Enzyme stability and stabilization-Aqueous and non-aqueous environment. *Process Biochemistry.* 2008, 43 (10), pp. 1019-1032.

23. **Van Beilen, J.B., Penninga, D., Witholt, B.** Topology of the membrane-bound alkane hydroxylase of Pseudomonas oleovorans. *Journal of Biological Chemistry.* 1992, 267 (13), pp. 9194-9201.

24. **Imaoka, S., Imai, Y., Shimada, T., Funae, Y.** Role of phospholipids in reconstituted cytochrome P450 3A form and mechanism of their activation of catalytic activity. *Biochemistry.* 1992, 31 (26), pp. 6063-6069.

25. **A.T. Deshmukh, P.J.T. Verheijen, R. Maleki Seifar, J.J. Heijnen, W.M. van Gulik.** In vivo kinetic analysis of the penicillin biosynthesis pathway using PAA stimulus response experiments. *Metabolic Engineering.* 2015, ISSN 1096-7176.

26. **Edegger, K., Stampfer, W., Seisser, B., Faber, K., Mayer, S.F., Oehrlein, R., Hafner, A., Kroutil, W.** Regio- and stereoselective reduction of diketones and oxidation of diols by biocatalytic hydrogen transfer. *European Journal of Organic Chemistry.* 2006, (8), pp. 1904-1909.

27. **Schroer, K., Peter Luef, K., Stefan Hartner, F., Glieder, A., Pscheidt, B.** Engineering the Pichia pastoris methanol oxidation pathway for improved NADH regeneration during whole-cell biotransformation. *Metabolic Engineering.* 2010, 12 (1), pp. 8-17.

28. **Kizaki, N., Yasohara, Y., Hasegawa, J., Wada, M., Kataoka, M., Shimizu, S.** Synthesis of optically pure ethyl (S)-4-chloro-3-hydroxybutanoate by Escherichia coli transformant cells coexpressing the carbonyl reductase and glucose dehydrogenase genes. *Applied Microbiology and Biotechnology.* 2001, 55 (5), pp. 590-595.

29. **Amidjojo, M., Weuster-Botz, D.** Asymmetric synthesis of the chiral synthon ethyl (S)-4-chloro-3-hydroxybutanoate using Lactobacillus kefir. *Tetrahedron Asymmetry.* 2005, 16 (4), pp. 899-901.

30. **Stewart, J.D.** Dehydrogenases and transaminases in asymmetric synthesis. *Current Opinion in Chemical Biology.* 2001, 5 (2), pp. 120-129.

31. **Weber, N., Gorwa-Grauslund, M., Carlquist, M.** Engineered baker's yeast as whole-cell biocatalyst for one-pot stereo-selective conversion of amines to alcohols. *Microbial Cell Factories.* 2014, 13 (1), art. no. 118.

32. **Li, Z., Van Beilen, J.B., Duetz, W.A., Schmid, A., De Raadt, A., Griengl, H., Witholt, B.** Oxidative biotransformations using oxygenases. *Current Opinion in Chemical Biology.* 2002, 6 (2), pp. 136-144.

33. **Chenault, H.K., Simon, E.S., Whitesides, G.M.** Cofactor regeneration for enzyme-catalysed synthesis. *Biotechnology and Genetic Engineering Reviews.* 1988, 6, pp. 221-270.

34. **Chenault, H.K., Whitesides, G.M.** Regeneration of nicotinamide cofactors for use in organic synthesis. *Applied Biochemistry and Biotechnology.* 1987, 14 (2), pp. 147-197.

35. **Boonstra, B., Rathbone, D.A., French, C.E., Walker, E.H., Bruce, N.C.** Cofactor regeneration by a soluble pyridine nucleotide transhydrogenase for biological production of hydromorphone. *Applied and Environmental Microbiology.* 2000, 66 (12), pp. 5161-5166.

36. **Duetz, W.A., Van Beilen, J.B., Witholt, B.** Using proteins in their natural environment: Potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis. *Current Opinion in Biotechnology.* 2001, 12 (4), pp. 419-425.

37. **Schubert, T., Hummel, W., Müller, M.** Highly enantioselective preparation of multifunctionalized propargylic building blocks. *Angewandte Chemie - International Edition.* 2002, 41 (4), pp. 634-637.

38. **Hao, J., Berry, A.** A thermostable variant of fructose bisphosphate aldolase constructed by directed evolution also shows increased stability in organic solvents. *Protein Engineering, Design and Selection.* 2004, 17 (9), pp. 689-697.

39. **Takahashi, H., Li, B., Sasaki, T., Miyazaki, C., Kajino, T., Inagaki, S.** Immobilized enzymes in ordered mesoporous silica materials and improvement of their stability and catalytic activity in an organic solvent. *Microporous and Mesoporous Materials.* 2001, 44-45, pp. 755-762.

40. **St. Clair, N., Wang, Y.-F., Margolin, A.L.** Cofactor-bound cross-linked enzyme crystals (CLEC) of alcohol dehydrogenase. *Angewandte Chemie - International Edition.* 2000, 39 (2), pp. 380-383.

41. **Liu W., Wang P.** Cofactor regeneration for sustainable enzymatic biosynthesis. *Biotechnology Advances.* 2007, 25 (4), pp. 369-384.

42. **Wichmann, R., Wandrey, C., Bückmann, A.F., Kula, M.-R.** Continuous enzymatic transformation in an enzyme membrane reactor with simultaneous NAD(H) regeneration. *Biotechnology and Bioengineering.* 1981, 23 (12), pp. 2789-2802.

43. **Katz, M., Frejd, T., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F.** Efficient Anaerobic Whole Cell Stereoselective Bioreduction With Recombinant Saccharomyces cerevisiae. *Biotechnology and Bioengineering.* 2003, 84 (5), pp. 573-582.

44. **Yamamoto, H., Mitsuhashi, K., Kimoto, N., Matsuyama, A., Esaki, N., Kobayashi, Y.** A novel NADH-dependent carbonyl reductase from Kluyveromyces aestuarii and comparison of NADH-regeneration system for the synthesis of ethyl (S)-4-chloro-3-hydroxybutanoate. *Bioscience, Biotechnology and Biochemistry.* 2004, 68 (3), pp. 638-649.

45. **Riebel B.R., Gibbs P.R., Wellborn W.B., Bommarius A.S.** Cofactor Regeneration of both NAD+ from NADH and NADP + from NADPH:NADH Oxidase from Lactobacillus sanfranciscensis. *Advanced Synthesis and Catalysis.* 2003, 345 (6-7), pp. 707-712.

46. **Sánchez, Ailen M., Andrews, Jared, Hussein, Insiya, Bennett, George N., San, Ka-Yiu.** Effect of Overexpression of a Soluble Pyridine Nucleotide Transhydrogenase (UdhA) on the Production of Poly(3-hydroxybutyrate) in Escherichia coli. *Biotechnology Progress.* 2006, 22, 420-425.

47. **Wu H., Tian C., Song X., Liu C., Yang D., Jiang Z.** Methods for the regeneration of nicotinamide coenzymes. *Green Chemistry.* 2013, 15 (7), pp. 1773-1789.

48. **Jones, J.B., Sneddon, D.W., Higgins, W., Lewis, A.J.** Preparative-scale reductions of cyclic ketones and aldehyde substrates of horse liver alcohol dehydrogenase with in situ sodium dithionite recycling of catalytic amounts of NAD. *Journal of the Chemical Society, Chemical Communications.* 1972, 0 (15), pp. 856-857.

49. **Raunio, R., Lilius, E.M.** Effect of dithionite on enzyme activities in vivo. *Enzymologia.* 1971, 40 (6), pp. 360-368.

50. **Devaux-Basseguy, R., Bergel, A., Comtat, M.** Potential applications of NAD(P)-dependent oxidoreductases in synthesis: A survey. *Enzyme and Microbial Technology.* 1997, 20 (4), pp. 248-258.

51. **Ali, I., McArthur, M., Hordy, N., Coulombe, S., Omanovic, S.** Electrochemical regeneration of the cofactor NADH employing a carbon nanofibers cathode. *International Journal of Electrochemical Science.* 2012, 7 (8), pp. 7675-7683.

52. **Lütz, S.** Transition Metal-Catalyzed Regeneration of Nicotinamide Cofactors. *The Handbook of Homogeneous Hydrogenation.* 2008, S. 1470-1482.

53. **Weckbecker, A., Gröger, H., Hummel, W.** Regeneration of nicotinamide coenzymes: Principles and applications for the synthesis of chiral compounds. *Advances in Biochemical Engineering/Biotechnology.* 2010, 120, pp. 195-242.

54. **DiCosimo, R., Wong, C.-H., Daniels, L., Whitesides, G.M.** Enzyme-catalyzed organic synthesis: Electrochemical regeneration of NAD(P)H from NAD(P) using methyl viologen and flavoenzymes. *Journal of Organic Chemistry.* 1981, 46 (22), pp. 4622-4623.

55. **Karyakin, A.A., Ivanova, Y.N., Karyakina, E.E.** Equilibrium (NAD+/NADH) potential on poly(Neutral Red) modified electrode. *Electrochemistry Communications.* 2003, 5 (8), pp. 677-680.

56. **Steckhan, E.** Electroenzymatic synthesis. *Top. Curr. Chem. .* 1994, 170, pp. 83–111.

57. **R. Wienkamp, E. Steckhan.** Indirect electrochemical regeneration of NADH by a bipyridinerhodium(I) complex as electron-transfer agent . *Angew. Chem. Int. Ed.* 1982, 21, pp. 782–783.

58. **S. Grammenudi, M. Franke, F. Vögtle, E. Steckhan.** The rhodium complex of a tris (bipyridine) ligand — its electrochemical behaviour and function as mediator for the regeneration of NADH from NAD+. *J. Incl. Phenom. .* 5, pp. 695–707.

59. **M. Franke, E. Steckhan.** Tris(2,2'-bipyridyl-5-sulfonic acid)rhodium(III), an improved redox catalyst for the light-induced and the electrochemically initiated enzymatic reduction of carbonyl compounds. *Angew. Chem. Int. Ed. .* 1988, 28, pp. 265–267.

60. **E. Steckhan, S. Herrmann, R. Ruppert, E. Dietz, M. Frede, E. Spika.** Analytical study of a series of substituted (2,2'-bipyridyl)(pentamethylcyclopentadienyl) rhodium and -iridium complexes with regard to their effectiveness as redox catalysts for the indirect electrochemical and chemical reduction of NAD(P)+. *Organometallics .* 1991, 10, pp. 1568–1577.

61. **M. Beley, J.-P. Collin.** Electrochemical regeneration of nicotinamide cofactor using a polypyrrole rhodium bis-terpyridine modified electrode. *Journal of Molecular Catalysis.* 1993, Volume 79, Issue 1-3, pp. 133-140.

62. **Damian, A., Maloo, Kh., Omanovic, S.** Direct electrochemical regeneration of NADH on Au, Cu and Pt-Au electrodes. *Chemical and Biochemical Engineering Quarterly.* 2007, 21 (1), pp. 21-32.

63. **Ali, I., Soomro, B., Omanovic, S.** Electrochemical regeneration of NADH on a glassy carbon electrode surface: The influence of electrolysis potential. *Electrochemistry Communications.* 2011, 13 (6), pp. 562-565.

64. **Ali, I., Gill, A., Omanovic, S.** Direct electrochemical regeneration of the enzymatic cofactor 1,4-NADH employing nano-patterned glassy carbon/Pt and glassy carbon/Ni electrodes. *Chemical Engineering Journal.* 2012, 188, pp. 173-180.

65. **Baddour, C.E., Fadlallah, F., Nasuhoglu, D., Mitra, R., Vandsburger, L., Meunier, J.-L.** A simple thermal CVD method for carbon nanotube synthesis on stainless steel 304 without the addition of an external catalyst. *Carbon.* 2009, 47 (1), pp. 313-318.

66. **Kim, J.H., Lee, S.H., Lee, J.S., Lee, M., Park, C.B.** Zn-containing porphyrin as a biomimetic light-harvesting molecule for biocatalyzed artificial photosynthesis. *Chemical Communications.* 2011, 47 (37), pp. 10227-10229.

67. **Kale, B.B., Baeg, J.-O., Lee, S.M., Chang, H., Moon, S.-J., Lee, C.W.** CdIn2S4 nanotubes and "marigold" nanostructures: A visible-light photocatalyst. *Advanced Functional Materials.* 2006, 16 (10), pp. 1349-1354.

68. **Kale, B.B., Baeg, J.-O., Apte, S.K., Sonawane, R.S., Naik, S.D., Patil, K.R.** Confinement of nano CdS in designated glass: A novel functionality of quantum dot-glass nanosystems in solar hydrogen production. *Journal of Materials Chemistry.* 2007, 17 (40), pp. 4297-4303.

69. **Park, C.B., Lee, S.H., Subramanian, E., Kale, B.B., Lee, S.M., Baeg, J.-O.** Solar energy in production of L-glutamate through visible light active photocatalyst - Redox enzyme coupled bioreactor. *Chemical Communications.* 2008, (42), pp. 5423-5425.

70. **Ahmad, M., Hirz, M., Pichler, H., Schwab, H.** Protein expression in Pichia pastoris: Recent achievements and perspectives for heterologous protein production. *Applied Microbiology and Biotechnology.* 2014, 98 (12), pp. 5301-5317.

71. **Hasslacher, M., Schall, M., Hayn, M., Bona, R., Rumbold, K., Lückl, J., Griengl, H., Kohlwein, S.D., Schwab, H.** High-level intracellular expression of hydroxynitrile lyase from the tropical rubber tree Hevea brasiliensis in microbial hosts. *Protein Expression and Purification.* 1997, 11 (1), pp. 61-71.

72. **Mellitzer, A., Ruth, C., Gustafsson, C., Welch, M., Birner-Grünberger, R., Weis, R., Purkarthofer, T., Glieder, A.** Synergistic modular promoter and gene optimization to push cellulase secretion by Pichia pastoris beyond existing benchmarks. *Journal of Biotechnology.* 2014, 191, pp. 187-195.

73. **Kang, H.A., Sohn, J.-H., Choi, E.-S., Chung, B.H., Yu, M.-H., Rhee, S.-K.** lycosylation of human α1-antitrypsin in Saccharomyces cerevisiae and methylotrophic yeasts. *Yeast.* 1998, 14 (4), pp. 371-381.

74. **Yasunori Chiba, Hiromi Akeboshi.** Glycan Engineering and Production of 'Humanized' Glycoprotein in Yeast Cells. *Biological and Pharmaceutical Bulletin.* 2009, Vol. 32,) No. 5, pp. 786-795.

75. **Smith, E.T., Perry, E.T., Sears, M.B., Johnson, D.A.** Expression of recombinant human mast cell chymase with Asn-linked glycans in glycoengineered Pichia pastoris. *Protein Expression and Purification.* 2014, 102, pp. 69-75.

76. **Krainer, F.W., Gmeiner, C., Neutsch, L., Windwarder, M., Pletzenauer, R., Herwig, C., Altmann, F., Glieder, A., Spadiut, O.** Knockout of an endogenous mannosyltransferase increases the homogeneity of glycoproteins produced in Pichia pastoris. *Scientific Reports.* 2013, 3, art. no. 3279.

77. **Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P., Stillman, C.A.** Functional characterization of the two alcohol oxidase genes from the yeast Pichia pastoris. *Molecular and Cellular Biology.* 1989, 9 (3), pp. 1316-1323.

78. **Cregg, J.M.** Introduction: Distinctions between Pichia pastoris and other expression systems. *Methods in Molecular Biology.* 2007, 389, pp. 1-10.

79. **Gleeson, M. A., Sudbery, P. E.** The methylotrophic yeasts. *Yeast.* 1988, Volume 4, Issue 1, pp. 1–15.

80. **Küberl, A., Schneider, J., Thallinger, G.G., Anderl, I., Wibberg, D., Hajek, T., Jaenicke, S., Brinkrolf, K., Goesmann, A., Szczepanowski, R., Pühler, A., Schwab, H., Glieder, A., Pichler, H.** High-

quality genome sequence of Pichia pastoris CBS7435. *Journal of Biotechnology.* 2011, 154 (4), pp. 312-320.

81. **Rußmayer, H., Buchetics, M., Gruber, C., Valli, M., Grillitsch, K., Modarres, G., Guerrasio, R., Klavins, K., Neubauer, S., Drexler, H., Steiger, M., Troyer, C., Al Chalabi, A., Krebiehl, G., Sonntag, D., Zellnig, G., Daum, G., Graf, A.B., et al.** Systems-level organization of yeast methylotrophic lifestyle. *BMC Biology.* 2015, 13 (1), art. no. 80.

82. **Geier, M., Brandner, C., Strohmeier, G.A., Hall, M., Hartner, F.S., Glieder, A.** Engineering Pichia pastoris for improved NADH regeneration: A novel chassis strain for whole-cell catalysis. *Beilstein Journal of Organic Chemistry.* 2015, 11, pp. 1741-1748.

83. **Trinh, L.B., Phue, J.N., Shiloach, J.** Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris. *Biotechnology and Bioengineering.* 2003, 82 (4), pp. 438-444.

84. **Chung, B.K.S., Selvarasu, S., Andrea, C., Ryu, J., Lee, H., Ahn, J., Lee, H., Lee, D.** Genome-scale metabolic reconstruction and in silico analysis of methylotrophic yeast Pichia pastoris for strain improvement. *Microbial Cell Factories.* 2010, 9, art. no. 50.

85. **Solà, A., Jouhten, P., Maaheimo, H., Sánchez-Ferrando, F., Szyperski, T., Ferrer, P.** Metabolic flux profiling of Pichia pastoris grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates. *Microbiology.* 2007, 153 (1), pp. 281-290.

86. **Abad, S., Nahalka, J., Bergler, G., Arnold, S.A., Speight, R., Fotheringham, I., Nidetzky, B., Glieder, A.** Stepwise engineering of a Pichia pastoris D-amino acid oxidase whole cell catalyst. *Microbial Cell Factories.* 2010, 9, art. no. 24.

87. **Gonzalez, E., Fernandez, M.R., Larroy, C., Sola, L., Pericas, M.A., Pares, X., Biosca, J.A.** Characterization of a (2R,3R)-2,3-butanediol dehydrogenase as the Saccharomyces cerevisiae YAL060W gene product: Disruption and induction of the gene. *Journal of Biological Chemistry.* 2000, 275 (46), pp. 35876-35885.

88. **Sakai, Y., Nakagawa, T., Shimase, M., Kato, N.** Regulation and physiological role of the DAS1 gene, encoding dihydroxyacetone synthase, in the methylotrophic yeast Candida boidinii. *Journal of Bacteriology.* 1998, 180 (22), pp. 5885-5890.

89. **Janowicz, Z.A., Eckart, M.R., Drewke, C., Roggenkamp, R.O., Hollenberg, C.P., Maat, J., Ledeboer, A.M., Visser, C., Verrips, C.T.** Cloning and charactesization of theDAS gene encoding the major methanol assimilatory enzyme from the methylotrophic yeast Hansenula polymorpha. *Nucleic Acids Research.* 1985, 13 (9), pp. 3043-3062.

90. **CLC bio, a QUIAGEN company.** [Online] http://www.clcbio.com/products/clc-main-workbench/.

91. **National Center for Biotechnolgy Information.** NCBI. [Online] 2014. http://blast.ncbi.nlm.nih.gov/Blast.cgi.

92. **Higgins D.R., Cregg J.M.** Gene replacement. *Pichia Protocols.* Totowa, New Jersey : Humana Press, 1998.

93. **Wu, S., Letchworth, G.J.** High efficiency transformation by electroporation of Pichia pastoris pretreated with lithium acetate and dithiothreitol. *BioTechniques.* 2004, 36 (1), pp. 152-154.

94. **Higgins, D.R., Cregg, J.M.** Introduction to Pichia pastoris. *Methods in Molecular Biology - Pichia protocols.* 103. Totowa, New jersey : Human Press, 1998, S. 1-15.

95. **Harju, Susanna, Fedosyuk, Halyna and Peterson, Kenneth.** Rapid isolation of yeast genomic DNA: Bust n' Grab. *BMC Biotechnology.* 2004, Vol. 4, 8, pp. 10.1186/1472-6750-4-8.

96. **Charles S. Hoffman, Fred Winston.** A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformaion of Escherichia coli. *Gene.* 1987, Volume 57, Issues 2–3, pp. 267-272.

97. **Lin-Cereghino, J., Wong, W.W., Xiong, S., Giang, W., Luong, L.T., Vu, J., Johnson, S.D., Lin-Cereghino, G.P.** Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris. *BioTechniques.* 2005, 38 (1), pp. 44-48.

98. **Weis, R., Luiten, R., Skranc, W., Schwab, H., Wubbolts, M., Glieder, A.** Reliable high-throughput screening with Pichia pastoris by limiting yeast cell death phenomena. *FEMS Yeast Research.* 2004, 5 (2), pp. 179-189.

99. **Costa, L., Brissos, V., Lemos, F., Ribeiro, F.R., Cabral, J.M.S.** Comparing the effect of immobilization methods on the activity of lipase biocatalysts in ester hydrolysis. *Bioprocess and Biosystems Engineering.* 31 (4), 2008, pp. 323-327.

100. **Näätsaari, L., Mistlberger, B., Ruth, C., Hajek, T., Hartner, F.S., Glieder, A.** Deletion of the pichia pastoris ku70 homologue facilitates platform strain generation for gene expression and synthetic biology. *PLoS ONE.* 2012, 7 (6), art. no. e39720.

101. **Critchlow, S.E., Jackson, S.P.** DNA end-joining: From yeast to man. *Trends in Biochemical Sciences.* 1998, 23 (10), pp. 394-398.

102. **Dudášová, Z., Dudáš, A., Chovanec, M.** Non-homologous end-joining factors of Saccharomyces cerevisiae. *FEMS Microbiology Reviews.* 2004, 28 (5), pp. 581-601.

103. **Franz S. Hartner, Anton Glieder.** Regulation of methanol utilisation pathway genes in yeasts. *Microbial Cell Factories.* 2006, 5:39.

104. **Thermo Fisher Scientific Inc.** www.thermofisher.com. [Online] 2013. [Zitat vom: 25. 01 2016.] https://tools.thermofisher.com/content/sfs/manuals/MAN0012035_DreamTaqDNAPolymerase_200U_UG.pdf.

105. **Schaaff-Gerstenschlager, I., Mannhaupt, G., Vetter, I., Zimmermann, F.K., Feldmann, H.** TKL2, a second transketolase gene of Saccharomyces cerevisiae. Cloning, sequence and deletion analysis of the gene. *European Journal of Biochemistry.* 1993, 217 (1), pp. 487-492. .

106. **Schaaff-Gerstenschläger, I., Zimmermann, F.K.** Pentose-phosphate pathway in Saccharomyces cerevisiae: analysis of deletion mutants for transketolase, transaldolase, and glucose 6-phosphate dehydrogenase. *Current Genetics.* 1993, 24 (5), pp. 373-376.

107. **Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M.** Heterologous protein production using the Pichia pastoris expression system. *Yeast.* 2005, 22 (4), pp. 249-270.

108. **M. Jahic, J.C. Rotticci-Mulder, M. Martinelle, K. Hult, S-O. Enfors.** Modeling of growth and energy metabolism of Pichia pastoris producing a fusion protein. *Bioprocess Biosyst Eng .* 2002, 24, 385–393.

109. **Krainer, F.W., Dietzsch, C., Hajek, T., Herwig, C., Spadiut, O., Glieder, A.** Recombinant protein expression in Pichia pastoris strains with an engineered methanol utilization pathway. *Microbial Cell Factories.* 2012, 11, art. no. 22.

110. **Waterham H, Digan M, Koutz P, Lair S, Cregg J.** Isolation of the Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene.* 1997, 186:37-44.

111. **Inan M, Meagher MM.** Non-repressing carbon sources for alcoholoxidase (AOX1) promoter of Pichia pastoris. *J Biosci Bioeng.* 2001, 92:585–589.

112. **Lazarow, P. E. Purdue and P. B.** Peroxisome biogenesis. *Annual Review of Cell and Developmental Biology.* 2001, vol. 17, pp. 701–752.

113. **I. J. van der Klei, H. Yurimoto, Y. Sakai, and M. Veenhuis.** The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochimica et Biophysica Acta.* 2006, vol. 1763, no. 12, pp. 1453–1462.

114. **Horiguchi, H., Yurimoto, Y., Goh, T.-K., Nakagawa, T., Kato, N., Sakai, Y.** Peroxisomal catalase in the methylotrophic yeast Candida boidinii: Transport efficiency and metabolic significance. *Journal of Bacteriology.* 2001, 183 (21), pp. 6372-6383.

115. **Subramani, Suresh.** Components Involved in Peroxisome Import, Biogenesis, Proliferation, Turnover, and Movement. *Physiological Reviews.* 1998, Vol. 78 no. 1, 171-188.

116. **Vogl, T., Sturmberger, L., Kickenweiz, T., Wasmayer, R., Schmid, C., Hatzl, A.-M., Gerstmann, M.A., Pitzer, J., Wagner, M., Thallinger, G.G., Geier, M., Glieder, A.** A Toolbox of Diverse Promoters Related to Methanol Utilization: Functionally Verified Parts for Heterologous Pathway Expression in Pichia pastoris. *ACS Synthetic Biology.* 2016, 5 (2), pp. 172-186.

117. **Cregg JM, Tschopp JF, Stillman C, et al.** High-level expression and efficient assembly of hepatitis B surface antigen in Pichia pastoris. *BioTechnology .* 1987, 5: 479–485.

118. **Kupcsulik, B., Sevella, B.** Effect of methanol concentration on the recombinant Pichia pastoris MutS fermentation. *Periodica Polytechnica: Chemical Engineering.* 2004, 48 (2), pp. 73-87.

119. **Schmid, R.D., Verger, R.** Lipases: Interfacial enzymes with attractive applications. *Angewandte Chemie - International Edition.* 1998, 37 (12), pp. 1609-1633.

120. **Martinelle, M., Holmquist, M., Hult, K.** On the interfacial activation of Candida antarctica lipase A and B as compared with Humicola lanuginosa lipase. *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism.* 1995, 1258 (3), pp. 272-276.

121. **Rotticci-Mulder, J.C., Gustavsson, M., Holmquist, M., Hult, K., Martinelle, M.** Expression in Pichia pastoris of Candida antarctica lipase B and lipase B fused to a cellulose-binding domain. *Protein Expression and Purification.* 2001, 21 (3), pp. 386-392.

122. **Yves Durocher, Michael Butler.** Expression systems for therapeutic glycoprotein production. *Current Opinion in Biotechnology.* 2009, Volume 20, Issue 6, pp. 700-707.

7. APPENDIX

7.1. Sequences

Table 29 Sequences used in this work.

Name	Sequence
pPpKC1	gaagttcctatactttctagagaataggaacttcggccgatcaggcccaacttactccaaccttctacgataactcttgtcctaat gtgtccaacatcgttagagacaccattgtcaatgaattgagatcagatccacgtattgctgcatctatcttgagacttcactttcat gactgcttcgtcaacggttgtgatgcttccatcttgctggacaacactacctctttcagaactgagaaggacgctttcggtaatgc caactctgctagaggatttccagtcattgacagaatgaaggctgccgttgaatctgcatgtcctagaactgtgtcatgtgctgacc ttctgactattgccgctcagcaatctgttaccttagctggtggaccatcctggagagttccattgggtcgtagagactcccttcaa gcctttctggaccttgcaaatgctaacttgcctgctccattctttaccttacctcaattgaaagactctttcagaaacgttggtctta acagatcatccgacttggttgccttatctggaggtcacacctttggtaagaaccaatgtagattcatcatggatcgtctgtacaac ttctctaacaccggtttgccagatcctactctgaacaccacttacttgcaaaccttaagaggtttgtgcccacttaacggaaatct gtctgctctggttgacttcgatttgcgtactcctaccatcttcgacaacaagtactatgtcaacttggaggaacagaagggtctta tccaatctgaccaggagttgttctcctctcctaacgctactgataccattccattggtgagatccttcgcaaactccactcaaacct tctttaacgctttcgtcgaggcaatggacagaatgggtaacattactcctttgaccggtactcaaggacagattagattgaactg ccgtgttgtcaactctaactcggccactagggccgaagttcctatactttctagagaataggaacttcagatctaacatccaaag acgaaaggttgaatgaaaccttttgccatccgacatccacaggtccattctcacacataagtgccaaacgcaacaggagggg atacactagcagcagaccgttgcaaacgcaggacctccactcctcttctcctcaacacccacttttgccatcgaaaaaccagccc agttattgggcttgattggagctcgctcattccaattccttctattaggctactaacaccatgactttattagcctgtctatcctggc ccccctggcgaggttcatgtttgtttatttccgaatgcaacaagctccgcattacacccgaacatcactccagatgagggctttct gagtgtggggtcaaatagtttcatgttccccaaatggcccaaaactgacagtttaaacgctgtcttggaacctaatatgacaaaa gcgtgatctcatccaagatgaactaagtttggttcgttgaaatgctaacggccagttggtcaaaaagaaacttccaaaagtcgg cataccgtttgtcttgtttggtattgattgacgaatgctcaaaaataatctcattaatgcttagcgcagtctctctatcgcttctgaa ccccggtgcacctgtgccgaaacgcaaatggggaaacacccgctttttggatgattatgcattgtctccacattgtatgcttccaa gattctggtgggaatactgctgatagcctaacgttcatgatcaaaatttaactgttctaacccctacttgacagcaatatataaac agaaggaagctgccctgtcttaaacctttttttttatcatcattattagcttactttcataattgcgactggttccaattgacaagct tttgattttaacgacttttaacgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgccacaatttgatatattat gtaaaacaccacctaaggtgcttgttcgtcagtttgtggaaaggtttgaaagaccttcaggtgagaaaatagcattatgtgctgc tgaactaacctatttatgttggatgattacacataacggaacagcaatcaagagagccacattcatgagctataatactatcata agcaattcgctgagtttcgatattgtcaataaatcactccagtttaaatacaagacgcaaaaagcaacaattctggaagcctcat taaagaaattgattcctgcttgggaatttacaattattccttactatggacaaaaacatcaatctgatatcactgatattgtaagt agtttgcaattacagttcgaatcatcggaagaagcagataagggaaatagccacagtaaaaaaatgcttaaagcacttctaag tgagggtgaaagcatctgggagatcactgagaaaatactaaattcgtttgagtatacttcgagatttacaaaaacaaaaacttt

	ataccaattcctcttcctagctactttcatcaattgtggaagattcagcgatattaagaacgttgatccgaaatcatttaagttagt ccaaaataagtatttgggagtaataatccagtgtttagtgacagagacaaagacaagcgttagtaggcacatatacttctttag cgcaaggggtaggatcgatccacttgtatatttggatgaatttttgaggaactctgaaccagtcctaaaacgagtaaataggac cggcaattcttcaagcaataaacaggaataccaattattaaaagataacttagtcagatcgtacaataaagctttgaagaaaa atgcgccttattcaatctttgctataaaaaatggcccaaaatctcacattggaagacatttgatgacctcatttctttcaatgaag ggcctaacggagttgactaatgttgtgggaaattggagcgataagcgtgcttctgccgtggccaggacaacgtatactcatcag ataacagcaatacctgatcactacttcgcactagtttctcggtactatgcatacgatccaatatcaaaggaaatgatagcattga aggatgagactaatccaattgaggagtggcagcatatagaacagctaaagggtagtgctgaaggaagcatacgataccccgc atggaatgggataatatcacaggaggtactagactacctttcatcctacataaatagacgcatataagttttagccttagacatg actgttcctcagttcaagttgggcacttacgagaagaccggtcttgctagattctaatcaagaggatgtcagaatgccatttgcct gagagatgcaggcttcatttttgatacttttttatttgtaacctatatagtataggatttttttttgtcattttgtttcttctcgtacgagc ttgctcctgatcagcctatctcgcagctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggta tttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgtgcggaacggaacgtatcttagcatggttgtgcgaca gattcactgtgaaagactgttcattatacccacgtttcactgggagatgtaagccttaggtgttttaccctgattagataatacaa taaccaacagaaatacgagaatctaaactaatttcgatgattcatttttcttttttaccgcgctgcctcttttggcaattctttcacct atattctaccttctctttccttttgttctaaacttattaccagctagtgttgttactttatacttccggctcgtataatacgacaaggtg taaggaggactaaaccatggctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttgagttctgga ctgataggctcggtttctcccgtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcatctccgcag ttcaggaccaggttgtgccagacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtggtctgaggt cgtgtctaccaacttccgtgatgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtttgcactgcgt gatccagctggtaactgcgtgcatttcgtcgcagaagagcaggactaatatactgagtttgttaatgatacaataaactgttata gtacatacaattgaaactctcttatctatactgggggaccttctcgcagaatggtataaatatctactaactgactgtcgtacggc ctaggggtctcttcttcgattatttgcaggtcggaacatccttcgtctgatgcggatctcctgagacaaagttcacgggtatctagt attctatcagcataaatggaggacctttctaaactaaactttgaatcgtctccagcagcatcctcgcattggtaccactgagcgtc agaccccgtagaaaagatcaaaggatcttcttgagatccttttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccg ctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaa atactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgt taccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggt cgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagct atgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgca cgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgat gctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgctggccttttgctcac atgt
pPpKC1_DAS1	gaagttcctatactttctagagaataggaacttcggccgatcaggccacgggaagtctttacagttttagttaggagcccttatat atgacagtaatgctagtacgttttgttttgtttaattaataacttagtttatgttagcctagtatagactccatcaatttttttttgttat tacgtaagccgcgatgataatatctgatgaaaaattcctatcagaaaataatttatcaaaagtttcatgcgatatgagactaagt agaatagggactcccaaagtgtcagtcacaagggtcattcccgttcgtaatgtggtgatagcgaggagaaaacctgtcagagc aagtaacaccgacgcaaagacatggctaatgaaagaagagcagagaagaataagacagaaggagcaggagatgaaacaa

	aggctagaggaactagaaaggttcaaaacaaaagtacagaaatcatatataaggaaagaggataggcatttggcacaagag atagaaaaggatcttgacataatcactgatgattacaatttggacagcgatgtagacctggtctttggagaactcatgcaagca gaagagcagcccaaatcgttgaagagtttaccaggagccagtgatgcaagtgataacacagataaatcggtggaactgttttc ctttccatctccgaatctgacgctacctgaaaaggtgatacaccatattgggccactggtgaagcacatcagtaatcctgaaaac attcaatgggggagacttttactggatttggaaaaaaatcaggggtttaacggtctatctgccgtagatgttaccagactgattc aaaatatacccaaagaggaaaaatatcagcatatgtctttgattcatgaaatgatgtttaacagtgggatcagtcctgatcggta cttgacagatttgatgatgactgccttctctgaaaggagctactatgaaccattggttgaggctcttttccaagactatgacatca atggctgggctccaacagattatacttttggagcattgatcaaagtatattccaagggaaagaagctcgataagatcaataatc ttctgaatcaaatgaaactaaaataccataaggaacccaataaaaagatatataccatggtcttacaaacctgtatgaagattg atgattataaaaagacttctgaggtctttgatgccatgaagtttaattctgtcgccactaatccagatacgacggcttatggttcg atgatattaatgcatgtgctcaatgataatgtggaagcagcattggatctgtatgacaatttaaattggactacgatatgctcca atccaaattgtcaaaattgaccaccgaaaaagaacaattggaatttgacaagaggaacaactcactagattctcaaacggagc gtcacctagagtcagtttccaagtcaattacagaaagtttggaaacagaagaggagtatctacaattgaattccaaacttaaag tcgagctgtccgaattcatgtcgctaaggctttcttacttggaccccatttttgaaagtttcattaaagttcagtcaaaaattttcat ggacatttatgacacattaaagagcggactaccttatgttgattctctatccaaagaggattatcagtccaagatcttggactcta gaatagataacattctgtcgaaaatggaagcgctgaaccttcaagcttacattgatgattagagcaatgatataaacaacaatt gagtgacaggtctactttgttctcaaaaggccataaccatctgtttgcatctcttatcaccacaccatcctcctcatctggccttca attgtggggaacaactagcatcccaacaccagactaactccacccagatgaaaccagttgtcgcttaccagtcaatgaatgttg agctaacgttccttgaaactcgaatgatcccagccttgctgcgtatcatccctccgctattccgccgcttgctccaaccatgtttcc gccttttttcgaacaagttcaaatacctatctttggcaggacttttcctcctgccttttttagcctcaggtctcggttagcctctaggc aaattctggtcttcatacctatatcaacttttcatcagatagcctttgggttcaaaaaagaactaaagcaggatgcctgatatata aatcccagatgatctgcttttgaaactattttcagtatcttgattcgtttacttacaaacaacggccactagggccgaagttcctat actttctagagaataggaacttcagatctaacatccaaagacgaaaggttgaatgaaacctttttgccatccgacatccacagg tccattctcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgttgcaaacgcaggacctccactcct cttctcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttgattggagctcgctcattccaattccttctatta ggctactaacaccatgactttattagcctgtctatcctggcccccctggcgaggttcatgtttgtttatttccgaatgcaacaagct ccgcattacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagtttcatgttccccaaatggcccaaaac tgacagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatgaactaagtttggttcgttgaaatgc taacggccagttggtcaaaaagaaacttccaaaagtcggcataccgtttgtcttgtttggtattgattgacgaatgctcaaaaat aatctcattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccgaaacgcaaatggggaaacacccgc tttttggatgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgctgatagcctaacgttcatgatcaa aatttaactgttctaacccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacctttttttttatcatcattat tagcttactttcataattgcgactggttccaattgacaagcttttgattttaacgacttttaacgacaacttgagaagatcaaaaa acaactaattattcgaaacgatgccacaatttgatatattatgtaaaacaccacctaaggtgcttgttcgtcagtttgtggaaag gtttgaaagaccttcaggtgagaaaatagcattatgtgctgctgaactaacctatttatgttggatgattacacataacggaaca gcaatcaagagagccacattcatgagctataatactatcataagcaattcgctgagtttcgatattgtcaataaatcactccagt ttaaatacaagacgcaaaaagcaacaattctggaagcctcattaaagaaattgattcctgcttgggaatttacaattattcctta ctatggacaaaaacatcaatctgatatcactgatattgtaagtagtttgcaattacagttcgaatcatcggaagaagcagataa

	gggaaatagccacagtaaaaaaatgcttaaagcacttctaagtgagggtgaaagcatctgggagatcactgagaaaatacta aattcgtttgagtatacttcgagatttacaaaaacaaaaactttataccaattcctcttcctagctactttcatcaattgtggaaga ttcagcgatattaagaacgttgatccgaaatcatttaagttagtccaaaataagtatttgggagtaataatccagtgtttagtgac agagacaaagacaagcgttagtaggcacatatacttctttagcgcaaggggtaggatcgatccacttgtatatttggatgaattt ttgaggaactctgaaccagtcctaaaacgagtaaataggaccggcaattcttcaagcaataaacaggaataccaattattaaa agataacttagtcagatcgtacaataaagctttgaagaaaaatgcgccttattcaatctttgctataaaaaatggcccaaaatct cacattggaagacatttgatgacctcatttctttcaatgaagggcctaacggagttgactaatgttgtgggaaattggagcgata agcgtgcttctgccgtggccaggacaacgtatactcatcagataacagcaatacctgatcactacttcgcactagtttctcggta ctatgcatacgatccaatatcaaaggaaatgatagcattgaaggatgagactaatccaattgaggagtggcagcatatagaac agctaaagggtagtgctgaaggaagcatacgataccccgcatggaatgggataatatcacaggaggtactagactacctttca tcctacataaatagacgcatataagttttagccttagacatgactgttcctcagttcaagttgggcacttacgagaagaccggtct tgctagattctaatcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcatttttgatacttttttatttgtaacctat atagtataggatttttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatctcgcagctgatgaatatcttgtggt aggggtttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagtacagaagattaagtgagaccttc gtttgtgcggaacggaacgtatcttagcatggttgtgcgacagattcactgtgaaagactgttcattatacccacgtttcactggg agatgtaagccttaggtgttttaccctgattagataatacaataaccaacagaaatacgagaatctaaactaatttcgatgattc atttttctttttaccgcgctgcctcttttggcaattctttcacctatattctaccttctctttccttttgttctaaacttattaccagctag tgttgttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatggctaaactcacctctgctgttcca gtcctgactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctcccgtgacttcgtagaggacgacttt gccggtgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgccagacaacactctggcatgggtat gggttcgtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtgatgcatctggtccagctatgacc gagatcggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgtgcatttcgtcgcagaagagcag gactaatatactgagtttgttaatgatacaataaactgttatagtacatacaattgaaactctcttatctatactgggggaccttct cgcagaatggtataaatatctactaactgactgtcgtacggcctaggggtctcttcttcgattatttgcaggtcggaacatccttc gtctgatgcggatctcctgagacaaagttcacgggtatctagtattctatcagcataaatggaggacctttctaaactaaactttg aatcgtctccagcagcatcctcgcattggtaccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttt ttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaact ctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaa gaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccg ggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggag cgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggac aggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtc ctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaa cgcggccttttacggttcctggccttttgctggccttttgctcacatgt
pPpKC1_DAS2	gaagttcctatactttctagagaataggaacttcggccgatcaggccgtagatttggccactaacgggttagtagttgtgtaagt ctattaaatttgatttttgtttatggatgatcatcgtagtggctatctgtttacctgtaggacatcctagggtgggatggtgatgtac acccccctcaatcttcagatgcaacactatgtggtaggtcattgacataaggtttaggaaagacctgttttttgaccaataaatgg aacaggaaggaaaggaggaaccagtttacgaaccccgtcggacatacaaaaccaaaaaatgagagcatgtagctcctcttga

	tggctttgcaggaaacactgctgcactcagaaatggcaacgcccggatattgaagggacgaagattgtgggggagaaacctgt cactgcagatatgaggagtttgatttataggaaacatttttcaatgtgggtgaatcctctgcgacctacatactatgtgcataata cgtgcataaaaagacaacaagataaatatagtaaaatatggggtaatagagagggctcaaccgaacgttagtggtaggattg gggaaacagtattggagagagtggaatagatacacagctagtgttggattgtatagtagtttcgtgtttataggtcgctgattct agtactgcgtacagctgaagagagaaaaaaaaatctgaaaactggggtaaagaactccttaaaaatttttcatatgtatgatg aaagactgctgcaggagacgttcacacttgaaaaatttgatcgaagcgagctacaagtccatatcgtgttggaacagaatctgg aggtgtaaacaccgtcattccctaatctaagtttctgtccagccgtttagaaagttcctcttcgtcaataaagtttaactttgaacg tgagagttgagctaatcggaaatactcccgagagcacacccgagcagtgaagcggggcatcatcaaaagaattaaggggtgc tgggtgcggattgcgcgcagtagctggcatcaatgccaaacgtagaactcatacattctcctatcagaatcatacgacatcgag agatacaccagccagcaccacttgaacactcccaatgagggtgcggtggtgggggaataagcagaactgtagatttaaatcaa actcttcatccagactctcatcaagatggtttgtcttcataaggacctgcgttccagctctattgacggctttcttgaaccctgacc aggacataatgatatttgagggtgttagttacttcgtctccagagtagcttatagggagaaaaaccgagacaacgatggaactc ccatgtagattccaccgccccaattactgttttgggcaatcctgttgataagacgcattctagagttgtttcatgaaagggttacg ggtgttgattggtttgagatatgccagaggacagatcaatctgtggtttgctaaactggaagtctggtaaggactctagcaagtc cgttactcaaaaagtcataccaagtaagattacgtaacacctgggcatgactttctaagttagcaagtcaccaagagggtccta tttaacgtttggcggtatctgaaacacaagacttgcctatcccatagtacatcatattacctgtcaagctatgctacccccacagaa ataccccaaaagttgaagtgaaaaaatgaaaattactggtaacttcaccccataacaaacttaataatttctgtagccaatgaa agtaaaccccattcaatgttccgagatttagtatacttgcccctataagaaacgaaggatttcagcttccttaccccatgaacag aaatcttccatttacccccccactggagagatccgcccaaacgaacagataatagaaaaaagaaattcggacaaatagaacac tttctcagccaattaaagtcattccatgcactccctttagctgccgttccatccctttgttgagcaacaccatcgttagccagtacg aaagaggaaacttaaccgataccttggagaaatctaaggcgcgaatgagtttagcctagatatccttagtgaagggttgttccg atacttctccacattcagtcatagatgggcagctttgttatcatgaagagacggaaacgggcattaagggttaaccgccaaatta tataaagacaacatgtccccagtttaaagtttttctttcctattcttgtatcctgagtgaccgttgtgtttaatataacaagttcgttt taacttaagaccaaaaccagttacaacaaattataacccctctaaacactaaagttcactcttatcaaactatcaaacatcaaa aggccactagggccgaagttcctatactttctagagaataggaacttcagatctaacatccaaagacgaaaggttgaatgaaa ccttttgccatccgacatccacaggtccattctcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccg ttgcaaacgcaggacctccactcctcttctcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttgattgga gctcgctcattccaattccttctattaggctactaacaccatgactttattagcctgtctatcctggccccccctggcgaggttcatgt ttgtttatttccgaatgcaacaagctccgcattacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagtt tcatgttccccaaatggcccaaaactgacagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatg aactaagtttggttcgttgaaatgctaacggccagttggtcaaaaagaaacttccaaaagtcggcataccgtttgtcttgtttggt attgattgacgaatgctcaaaaataatctcattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccga aacgcaaatggggaaacacccgctttttggatgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgc tgatagcctaacgttcatgatcaaaatttaactgttctaacccctacttgacagcaatatataaacagaaggaagctgccctgtc ttaaacctttttttttatcatcattattagcttactttcataattgcgactggttccaattgacaagcttttgattttaacgacttttaa cgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgccacaatttgatatattatgtaaaacaccacctaaggt gcttgttcgtcagtttgtggaaaggtttgaaagaccttcaggtgagaaaatagcattatgtgctgctgaactaacctatttatgtt ggatgattacacataacggaacagcaatcaagagagccacattcatgagctataatactatcataagcaattcgctgagtttcg

	atattgtcaataaatcactccagtttaaatacaagacgcaaaaagcaacaattctggaagcctcattaaagaaattgattcctg cttgggaatttacaattattccttactatggacaaaaacatcaatctgatatcactgatattgtaagtagtttgcaattacagttcg aatcatcggaagaagcagataagggaaatagccacagtaaaaaaatgcttaaagcacttctaagtgagggtgaaagcatctg ggagatcactgagaaaatactaaattcgtttgagtatacttcgagatttacaaaaacaaaaactttataccaattcctcttccta gctactttcatcaattgtggaagattcagcgatattaagaacgttgatccgaaatcatttaagttagtccaaaataagtatttggg agtaataatccagtgtttagtgacagagacaaagacaagcgttagtaggcacatatacttctttagcgcaaggggtaggatcga tccacttgtatatttggatgaatttttgaggaactctgaaccagtcctaaaacgagtaaataggaccggcaattcttcaagcaat aaacaggaataccaattattaaaagataacttagtcagatcgtacaataaagctttgaagaaaaatgcgccttattcaatctttg ctataaaaaatggcccaaaatctcacattggaagacatttgatgacctcatttctttcaatgaagggcctaacggagttgactaa tgttgtgggaaattggagcgataagcgtgcttctgccgtggccaggacaacgtatactcatcagataacagcaatacctgatca ctacttcgcactagtttctcggtactatgcatacgatccaatatcaaaggaaatgatagcattgaaggatgagactaatccaatt gaggagtggcagcatatagaacagctaaagggtagtgctgaaggaagcatacgataccccgcatggaatgggataatatcac aggaggtactagactacctttcatcctacataaatagacgcatataagttttagccttagacatgactgttcctcagttcaagttg ggcacttacgagaagaccggtcttgctagattctaatcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcattt ttgatacttttttatttgtaacctatatagtataggatttttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatct cgcagctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagt acagaagattaagtgagaccttcgtttgtgcggaacggaacgtatcttagcatggttgtgcgacagattcactgtgaaagactgt tcattatacccacgtttcactgggagatgtaagccttaggtgttttaccctgattagataatacaataaccaacagaaatacgag aatctaaactaatttcgatgattcatttttcttttttaccgcgctgcctcttttggcaattctttcacctatattctaccttctctttccttt tgttctaaacttattaccagctagtgttgttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatg gctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctccc gtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgcca gacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtg atgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgt gcatttcgtcgcagaagagcaggactaatatactgagtttgttaatgatacaataaactgttatagtacatacaattgaaactct cttatctatactgggggaccttctcgcagaatggtataaatatctactaactgactgtcgtacggcctaggggtctcttcttcgatt atttgcaggtcggaacatccttcgtctgatgcggatctcctgagacaaagttcacgggtatctagtattctatcagcataaatgga ggacctttctaaactaaactttgaatcgtctccagcagcatcctcgcattggtaccactgagcgtcagaccccgtagaaaagatc aaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttg ccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagc cgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagt ggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttc gtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttc ccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggga aacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagc ctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggccttttgctcacatgt
pPpKC1_DAS1& 2	gaagttcctatactttctagagaataggaacttcggccgatcaggccgtgacgagttagtaaagaactggaaaaagttagttga tagctatgtcaaagagtaccctcgtgagggacaagagttcctttctcgtgttagaggtgagcttccaaagaactggagaacttac

	attcctcaagacaagcctaccgaaccaaccgccaccagaacctctgctagagaaattgttagggcccttggaaagaaccttcct caagttattgccggttccggtgacttatctgtctcaattcttttgaactgggacggagtgaagtacttcttcaaccctaagttacag actttctgtggattaggtggtgactactctggtagatatattgagtttggtatcagagaacactctatgtgtgctattgccaacggt ttggctgcatacaacaagggtactttcttgcctattacctctaccttctacatgttctacctgtatgcagcacctgccttgcgtatgg ctgctcttcaagagttgaaagcgattcacattgctacacacgactctattggagctggtgaagatggtccaacccaccagcctat tgctttgtcttcattattcagagctatgcccaacttctactacatgagaccagccgatgctaccgaagttgcagctctgtttgaagt ggctgttgagcttgaacactccacattgctttctctgtccagacacgaggttgaccaatacccaggtaagacttctgcccaagga gccaaaagaggtggttacgttgttgaagactgcgaaggaaagccagatgtgcaactgatcggaactggttccgagttggaatt cgctattaagactgctcgtttgctaagacaacagaagggatggaaggtcagagttctgtcattcccatgtcagagattgtttgac gagcagtctattacttacagacgttccgtccttagaagaggagaagttccaactgtcgttgttgaggcctatgtcgcatacggat gggagagatacgccactgctggttacaccatgaacaccttcggtaagtctcttcctgttgaggatgtctacaaatacttcggata cactcctgagaagattggtgagagagtggttcaatatgtcaactctatcaaggctagtcctcaaatcctttacgaattccacgac ttgaagggaaaaccaaagcatgacaagttgtaaacgggaagtctttacagttttagttaggagcccttatatatgacagtaatg ctagtacgttttgttttgtttaattaataacttagtttatgttagcctagtatagactccatcaatttaaatctacttatagtttgtcgt gctttggttttcccttcaaatcggtgaattcgtaaaggatttgaggactagccttaatagagttgacgtatgcagcaaccttctca ccaatcttctcaggagtgtatccgaagtatttgtagacatcctcaacaggaagagacttaccaaaggtgttcatggtgtaaccag cagtggcgtatctctcccatccgtatgcgacataggcctcaacaacgacagttggaacctctcctcttctaaggacagaacgtct gtatgccagggattgttggtcaaacagtctctgacatgggaatgacagaactctgaccttccatcccttctgttgtcttagcaaac gagcagttttgacggcaaactccaattcggaaccagcaccaattaattggacgtctggcttaccctcacagtcttcaacgacgta accacctcttttggctccctcagccgaagtcttacctgggtattgctcaacctcgtgtctggacagagagaacaaggtggagtgc tcgagctcaacagctacttcaaacagagctgcaacctcggtagcatcggctggtctaatgtagtagaagttgggcatagctctg aataatgaagacaaagcaataggctggtgcgttggaccatcttcaccagctccgatggagtcgtgtgtagcaatgtgaattgct ttcaactcttgaagtgcagccatacgcaaggcaggtgctgcatacaggtagaacatgtagaaagttgaggtaataggcaagaa agtacccttgttgtatgcagccaaaccattggcaatagcacacatagagtgttctctgataccaaactcaatatatctaccagag tagtcaccacccaatccacagaaagtttgtaacttagggttgaagaagtacttaactcctccccagttcaaaagaatggacacg gacaagtcaccagaaccagcaataacctgaggaaggttttgtcccagagatctaacaatttctctagcagaggttctggtagca gttggctcggttggtttgtcctgtggaatgaaactcttccagttctttggaagttcacctctaatacgggcctttaattcctcacctt cttgaggatagttcttaacgtactcatccaaaagtttcttccagttagcaactagttgatcaccctcggctggtttttcagcaaaga aatcatagacctcctgtgggaaccagaactttctagcgacatcgaaaccgtacttggccttcaactcccggataccttcctcacc aagagcagaaccgtgagcagcgggccactagggccgaagttcctatactttctagagaataggaacttcagatctaacatcca aagacgaaaggttgaatgaaacctttttgccatccgacatccacaggtccattctcacacataagtgccaaacgcaacaggag gggatacactagcagcagaccgttgcaaacgcaggacctccactcctcttctcctcaacacccactttgccatcgaaaaacca gcccagttattgggcttgattggagctcgctcattccaattccttctattaggctactaacaccatgactttattagcctgtctatcc tggcccccctggcgaggttcatgtttgtttatttccgaatgcaacaagctccgcattacacccgaacatcactccagatgagggct ttctgagtgtggggtcaaatagtttcatgttccccaaatggcccaaaactgacagtttaaacgctgtcttggaacctaatatgaca aaagcgtgatctcatccaagatgaactaagtttggttcgttgaaatgctaacggccagttggtcaaaaagaaacttccaaaagt cggcataccgtttgtcttgtttggtattgattgacgaatgctcaaaaataatctcattaatgcttagcgcagtctctctatcgcttct gaaccccggtgcacctgtgccgaaacgcaaatggggaaacacccgctttttggatgattatgcattgtctccacattgtatgcttc

	caagattctggtgggaatactgctgatagcctaacgttcatgatcaaaatttaactgttctaacccctacttgacagcaatatata aacagaaggaagctgccctgtcttaaaccttttttttttatcatcattattagcttactttcataattgcgactggttccaattgacaa gcttttgattttaacgacttttaacgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgccacaatttgatatat tatgtaaaacaccacctaaggtgcttgttcgtcagtttgtggaaaggtttgaaagaccttcaggtgagaaaatagcattatgtgc tgctgaactaacctatttatgttggatgattacacataacggaacagcaatcaagagagccacattcatgagctataatactatc ataagcaattcgctgagtttcgatattgtcaataaatcactccagtttaaatacaagacgcaaaaagcaacaattctggaagcc tcattaaagaaattgattcctgcttgggaatttacaattattccttactatggacaaaaacatcaatctgatatcactgatattgta agtagtttgcaattacagttcgaatcatcggaagaagcagataagggaaatagccacagtaaaaaaatgcttaaagcacttct aagtgagggtgaaagcatctgggagatcactgagaaaatactaaattcgtttgagtatacttcgagatttacaaaaacaaaaa ctttataccaattcctcttcctagctactttcatcaattgtggaagattcagcgatattaagaacgttgatccgaaatcatttaagtt agtccaaaataagtatttgggagtaataatccagtgtttagtgacagagacaaagacaagcgttagtaggcacatatacttcttt agcgcaaggggtaggatcgatccacttgtatatttggatgaatttttgaggaactctgaaccagtcctaaaacgagtaaatagg accggcaattcttcaagcaataaacaggaataccaattattaaaagataacttagtcagatcgtacaataaagctttgaagaa aaatgcgccttattcaatctttgctataaaaaatggcccaaaatctcacattggaagacatttgatgacctcatttctttcaatga agggcctaacggagttgactaatgttgtgggaaattggagcgataagcgtgcttctgccgtggccaggacaacgtatactcatc agataacagcaatacctgatcactacttcgcactagtttctcggtactatgcatacgatccaatatcaaaggaaatgatagcatt gaaggatgagactaatccaattgaggagtggcagcatatagaacagctaaagggtagtgctgaaggaagcatacgatacccc gcatggaatgggataatatcacaggaggtactagactacctttcatcctacataaatagacgcatataagttttagccttagaca tgactgttcctcagttcaagttgggcacttacgagaagaccggtcttgctagattctaatcaagaggatgtcagaatgccatttgc ctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagtataggatttttttgtcattttgtttcttctcgtacga gcttgctcctgatcagcctatctcgcagctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttgg tatttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgtgcggaacggaacgtatcttagcatggttgtgcga cagattcactgtgaaagactgttcattatacccacgtttcactgggagatgtaagccttaggtgttttaccctgattagataatac aataaccaacagaaatacgagaatctaaactaatttcgatgattcatttttcttttttaccgcgctgcctcttttggcaattctttcac ctatattctaccttctctttccttttgttctaaacttattaccagctagtgttgttactttatacttccggctcgtataatacgacaagg tgtaaggaggactaaaccatggctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttgagttctg gactgataggctcggtttctcccgtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcatctccgc agttcaggaccaggttgtgccagacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtggtctga ggtcgtgtctaccaacttccgtgatgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtttgcactg cgtgatccagctggtaactgcgtgcatttcgtcgcagaagagcaggactaatatactgagtttgttaatgatacaataaactgtt atagtacatacaattgaaactctcttatctatactgggggaccttctcgcagaatggtataaatatctactaactgactgtcgtac ggcctaggggtctcttcttcgattatttgcaggtcggaacatccttcgtctgatgcggatctcctgagacaaagttcacgggtatct agtattctatcagcataaatggaggacctttctaaactaaactttgaatcgtctccagcagcatcctcgcattggtaccactgagc gtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttttctgcgcgtaatctgctgcttgcaaacaaaaaaacca ccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagatac caaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatc ctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcag cggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtg

	agctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagc gcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttg tgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgctggccttttgc tcacatgt
pPpKC1_DAS1& 2_clean	gaagttcctatactttctagagaataggaacttcggccgatcaggccacgggaagtctttacagttttagttaggagcccttatat atgacagtaatgctagtacgttttgttttgtttaattaataacttagtttatgttagcctagtatagactccatcaatttttttttgttat tacgtaagccgcgatgataatatctgatgaaaaattcctatcagaaaataatttatcaaaagtttcatgcgatatgagactaagt agaatagggactcccaaagtgtcagtcacaagggtcattcccgttcgtaatgtggtgatagcgaggagaaaacctgtcagagc aagtaacaccgacgcaaagacatggctaatgaaagaagagcagagaagaataagacagaaggagcaggagatgaaacaa aggctagaggaactagaaaggttcaaaacaaaagtacagaaatcatatataaggaaagaggataggcatttggcacaagag atagaaaaggatcttgacataatcactgatgattacaatttggacagcgatgtagacctggtctttggagaactcatgcaagca gaagagcagcccaaatcgttgaagagtttaccaggagccagtgatgcaagtgataacacagataaatcggtggaactgttttc ctttccatctccgaatctgacgctacctgaaaaggtgatacaccatattgggccactggtgaagcacatcagtaatcctgaaaac attcaatgggggagacttttactggatttggaaaaaaatcaggggtttaacggtctatctgccgtagatgttaccagactgattc aaaatatacccaaagaggaaaaatatcagcatatgtctttgattcatgaaatgatgtttaacagtgggatcagtcctgatcggta cttgacagatttgatgatgactgccttctctgaaaggagctactatgaaccattggttgaggctcttttccaagactatgacatca atggctgggctccaacagattatacttttggagcattgatcaaagtatattccaagggaaagaagctcgataagatcaataatc ttctgaatcaaatgaaactaaaataccataaggaacccaataaaaagatatataccatggtcttacaaacctgtatgaagattg atgattataaaaagacttctgaggtctttgatgccatgaagtttaattctgtcgccactaatccagatacgacggcttatggttcg atgatattaatgcatgtgctcaatgataatgtggaagcagcattggatctgtatgacaatttaaatctacagttctgcttattcccc caccaccgcaccctcattgggagtgttcaagtggtgctggctggtgtatctctcgatgtcgtatgattctgataggagaatgtatg agttctacgtttggcattgatgccagctactgcgcgcaatccgcacccagcacccctttaattcttttgatgatgccccgcttcactg ctcgggtgtgctctcgggagtatttccgattagctcaactctcacgttcaaagttaaactttattgacgaagaggaactttctaaa cggctggacagaaacttagattagggaatgacggtgtttacacctccagattctgttccaacacgatatggacttgtagctcgct tcgatcaaatttttcaagtgtgaacgtctcctgcagcagtctttcatcatacatatgaaaaatttttaaggagttctttaccccagtt ttcagatttttttttctctcttcagctgtacgcagtactagaatcagcgacctataaacacgaaactactatacaatccaacacta gctgtgtatctattccactctctccaatactgtttccccaatcctaccactaacgttcggttgagccctctctattaccccatatttta ctatatttatcttgttgtctttttatgcacgtattatgcacatagtatgtaggtcgcagaggattcacccacattgaaaaatgtttcc tataaatcaaactcctcatatctgcagtgacaggtttctcccccacaatcttcgtcccttcaatatccgggcgttgccatttctgag tgcagcagtgtttcctgcaaagccatcaagaggagctacatgctctcatttttttggttttgtatgtccgacggggttcgtaaactgg ttcctcctttccttcctgttccatttattggtcaaaaaacaggtctttcctaaaccttatgtcaatgacctaccacatagtgttgcatc tgaagattgaggggtgtacatcaccatcccaccctaggatgtcctacaggtaaacagatagccactacgatgatcatccataa acaaaaatcaaatttaatagacttacacaactactaacccgttagtggccaaatctacggccactagggccgaagttcctatac tttctagagaataggaacttcagatctaacatccaaagacgaaaggttgaatgaaaccttttgccatccgacatccacaggtcc attctcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgttgcaaacgcaggacctccactcctctt ctcctcaacacccactttgccatcgaaaaaccagcccagttattgggcttgattggagctcgctcattccaattccttctattagg ctactaacaccatgactttattagcctgtctatcctggcccccctggcgaggttcatgtttgtttatttccgaatgcaacaagctcc gcattacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagtttcatgttccccaaatggcccaaaactg

	acagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatgaactaagtttggttcgttgaaatgcta acggccagttggtcaaaaagaaacttccaaaagtcggcataccgtttgtcttgtttggtattgattgacgaatgctcaaaaataa tctcattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccgaaacgcaaatggggaaacacccgcttt ttggatgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgctgatagcctaacgttcatgatcaaaat ttaactgttctaacccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacctttttttttatcatcattattag cttactttcataattgcgactggttccaattgacaagcttttgattttaacgacttttaacgacaacttgagaagatcaaaaaaca actaattattcgaaacgatgccacaatttgatatattatgtaaaacaccacctaaggtgcttgttcgtcagtttgtggaaaggttt gaaagaccttcaggtgagaaaatagcattatgtgctgctgaactaacctatttatgttggatgattacacataacggaacagca atcaagagagccacattcatgagctataatactatcataagcaattcgctgagtttcgatattgtcaataaatcactccagtttaa atacaagacgcaaaaagcaacaattctggaagcctcattaaagaaattgattcctgcttgggaatttacaattattccttactat ggacaaaaacatcaatctgatatcactgatattgtaagtagtttgcaattacagttcgaatcatcggaagaagcagataaggga aatagccacagtaaaaaaatgcttaaagcacttctaagtgagggtgaaagcatctgggagatcactgagaaaatactaaattc gtttgagtatacttcgagatttacaaaaacaaaaactttataccaattcctcttcctagctactttcatcaattgtggaagattcag cgatattaagaacgttgatccgaaatcatttaagttagtccaaaataagtatttgggagtaataatccagtgtttagtgacagag acaaagacaagcgttagtaggcacatatacttctttagcgcaaggggtaggatcgatccacttgtatatttggatgaatttttga ggaactctgaaccagtcctaaaacgagtaaataggaccggcaattcttcaagcaataaacaggaataccaattattaaaagat aacttagtcagatcgtacaataaagctttgaagaaaaatgcgccttattcaatctttgctataaaaaatggcccaaaatctcaca ttggaagacatttgatgacctcatttctttcaatgaagggcctaacggagttgactaatgttgtgggaaattggagcgataagcg tgcttctgccgtggccaggacaacgtatactcatcagataacagcaatacctgatcactacttcgcactagtttctcggtactatg catacgatccaatatcaaaggaaatgatagcattgaaggatgagactaatccaattgaggagtggcagcatatagaacagcta aagggtagtgctgaaggaagcatacgataccccgcatggaatgggataatatcacaggaggtactagactacctttcatccta cataaatagacgcatataagttttagccttagacatgactgttcctcagttcaagttgggcacttacgagaagaccggtcttgcta gattctaatcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagt ataggatttttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatctcgcagctgatgaatatcttgtggtaggg gtttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagtacagaagattaagtgagaccttcgtttg tgcggaacggaacgtatcttagcatggttgtgcgacagattcactgtgaaagactgttcattatacccacgtttcactgggagat gtaagccttaggtgttttaccctgattagataatacaataaccaacagaaatacgagaatctaaactaatttcgatgattcatttt tctttttaccgcgctgcctcttttggcaattctttcacctatattctaccttctctttccttttgttctaaacttattaccagctagtgttg ttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatggctaaactcacctctgctgttccagtcct gactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctcccgtgacttcgtagaggacgactttgccgg tgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgccagacaacactctggcatgggtatgggttc gtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtgatgcatctggtccagctatgaccgagat cggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgtgcatttcgtcgcagaagagcaggacta atatactgagtttgttaatgatacaataaactgttatagtacatacaattgaaactctcttatctatactgggggaccttctcgcag aatggtataaatatctactaactgactgtcgtacggcctaggggtctcttcttcgattatttgcaggtcggaacatccttcgtctga tgcggatctcctgagacaaagttcacgggtatctagtattctatcagcataaatggaggacctttctaaactaaactttgaatcgt ctccagcagcatcctcgcattggtaccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctg cgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttc

	cgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactc tgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttgg actcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaac gacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtat ccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgg gtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggc ctttttacggttcctggccttttgctggccttttgctcacatgt
pPpKC1_TKL1	gaagttcctatactttctagagaataggaacttcggccgatcaggcctttgtgcgcctttaggtagttagttaattagtaagttcat agtgcgggaagaataggctgtcttagaatgctcgacaaagttaccaggattgcgaggattatttattggagtgaatacttgtaat ttattttgcttccttcttactctcctcttccttcgactcattatttgccaccctcaaagatggccccgatatccgttgcgtttgtatgtct aggaaacatttgccgaagccccatggctgaggcggtgttcacccacttagtcaaagaaaaaggactcgactctaagttcaaaa agattgactcctttggaacttcaggataccatatcgacgatactcctgatcaccgttccgtggcgtgttgcaagaaacatggagtt cctgtgaaccatagaggtcagcagatctcgccggacgacttctaccactttgactacatactggccatggatgaatcaaatctct ccaatctcaaacgcatacagcccaaagactccaatgccgtagtcaggctctttggagactacagaacggacaagtccttccaca ggattgtggaggatccttactacggcgggtcttctggatttgagagaaatttcaaacagattacgcatttcagtgaagaattctta aacaaagagttatagattatagattatcaacttttcaaagatacaacgtttccaacttgtccttctcaaaagtaaaatcgtcttttc tcctgggaagccatgtctcctggtaatcaaagtatttccagttgctcttttgttgaacgtgctcttcaataggcggaggagtttgat tagcttcgtcattattgagcaggaaccaggtcttgagctctttgtggtacttccagccccttctatgcaactccttcgccgtattaat ttgtaggaaactgcctgagttgtcgaataccttatgttcatcagctgcattctcgtagctgtgataatagaaacagtagaataaa gtctccaaatcaaagttctgtaaaatctttgccgagttcataatctccaacaaaggttcctgagggaacgaaatatgggtagggt gtggttgtctagggatatacatgtttggcacatctgcatcaaatgagtctggacaatttaaatatgcattcaactttgatattgtgt atcgacacttcttgacgtagtatgtcactcaatgatcgcaatgctgccttcattggggcatactgaccgtatccaatgaacgaata gaatgccagtacagatgaaaaaaacaccaagtgccttttgtagggcttttctacctttgtcattaccttgagcgcaacatgtgag aagtacaacgctgtcttgtaattgatatcgattccactgctcagagtcttttcatcaagctctaagaaaagtccggggatggagg agccagcgcaacagaaaactatgtcgggactaaaaccaacttcctctaaaacacgctcacactggtcatatttggagatatctg ctgccacgtaagaaattgtgttgcccttctcggtcccgtgaatatcaatagcgtcctttactacctgctttagtaaggattctgttct agccacaatcacaacggaacatccttttccatacaaaagtttagcaaactcggctccaacaccctgggatgcacccgtaataat tgccttttgttggtgacatcaaaattatcaaacattccagttccctctacactttgtatgagaatgatagctgaaattgtgcacca gatgttagaagataaggtcgtgtcatgaactaatatcatgaattccgagggtggctcaacaactattcacgtgacttggacgttg gaagttgaggtggttggtggatgttgcacggagtatcatttgtaagcatgaaatcagtctaaaaaacttgcagaatagcagagc ggttcggaaattcattcaaaaccacctcctcagattggatctgccctactctgtttagctctgggagattttctcggtcgtgttcttt cgctggtctacccacgctataggaatcgctgtgaacgctaccttcttcccaacttctcggtgactattataagccattcccactttg ttttcaagcaccaacaacccacccccaccttatctactccatcttgggtgtccccgcgcctgttgcaaagtccgaaccatagaacc cccgacctttgtcccactaaccctcagacacccctcggaagtcagggagaaaccactccgaagtacattaatcatccctcgtatt ctcgacggtgcccatttttctttataaaaagggagacacaggttgcttcactaactctagacttgtattctacatccactctacaca ggccactagggccgaagttcctatactttctagagaataggaacttcagatctaacatccaaagacgaaaggttgaatgaaac ctttttgccatccgacatccacaggtccattctcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgt tgcaaacgcaggacctccactcctcttctcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttgattggag

	ctcgctcattccaattccttctattaggctactaacaccatgactttattagcctgtctatcctggccccctggcgaggttcatgtt tgtttatttccgaatgcaacaagctccgcattacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagttt catgttccccaaatggcccaaaactgacagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatg aactaagtttggttcgttgaaatgctaacggccagttggtcaaaaagaaacttccaaaagtcggcataccgtttgtcttgtttggt attgattgacgaatgctcaaaaataatctcattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccga aacgcaaatggggaaacacccgctttttggatgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgc tgatagcctaacgttcatgatcaaaatttaactgttctaacccctacttgacagcaatatataaacagaaggaagctgccctgtc ttaaacctttttttttatcatcattattagcttactttcataattgcgactggttccaattgacaagcttttgattttaacgacttttaa cgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgccacaatttgatatattatgtaaaacaccacctaaggt gcttgttcgtcagtttgtggaaaggtttgaaagaccttcaggtgagaaaatagcattatgtgctgctgaactaacctatttatgtt ggatgattacacataacggaacagcaatcaagagagccacattcatgagctataatactatcataagcaattcgctgagtttcg atattgtcaataaatcactccagtttaaatacaagacgcaaaaagcaacaattctggaagcctcattaaagaaattgattcctg cttgggaatttacaattattccttactatggacaaaaacatcaatctgatatcactgatattgtaagtagtttgcaattacagttcg aatcatcggaagaagcagataagggaaatagccacagtaaaaaaatgcttaaagcacttctaagtgagggtgaaagcatctg ggagatcactgagaaaatactaaattcgtttgagtatacttcgagatttacaaaaacaaaaactttataccaattcctcttccta gctactttcatcaattgtggaagattcagcgatattaagaacgttgatccgaaatcatttaagttagtccaaaataagtatttggg agtaataatccagtgtttagtgacagagacaaagacaagcgttagtaggcacatatacttctttagcgcaaggggtaggatcga tccacttgtatatttggatgaatttttgaggaactctgaaccagtcctaaaacgagtaaataggaccggcaattcttcaagcaat aaacaggaataccaattattaaaagataacttagtcagatcgtacaataaagctttgaagaaaaatgcgccttattcaatctttg ctataaaaaatggcccaaaatctcacattggaagacatttgatgacctcatttctttcaatgaagggcctaacggagttgactaa tgttgtgggaaattggagcgataagcgtgcttctgccgtggccaggacaacgtatactcatcagataacagcaatacctgatca ctacttcgcactagtttctcggtactatgcatacgatccaatatcaaaggaaatgatagcattgaaggatgagactaatccaatt gaggagtggcagcatatagaacagctaaagggtagtgctgaaggaagcatacgataccccgcatggaatgggataatatcac aggaggtactagactacctttcatcctacataaatagacgcatataagttttagccttagacatgactgttcctcagttcaagttg ggcacttacgagaagaccggtcttgctagattctaatcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcattt ttgatacttttttatttgtaacctatatagtataggatttttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatct cgcagctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagt acagaagattaagtgagaccttcgtttgtgcggaacggaacgtatcttagcatggttgtgcgacagattcactgtgaaagactgt tcattatacccacgtttcactgggagatgtaagccttaggtgttttaccctgattagataatacaataaccaacagaaatacgag aatctaaactaatttcgatgattcatttttcttttttaccgcgctgcctcttttggcaattctttcacctatattctaccttctctttccttt tgttctaaacttattaccagctagtgttgttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatg gctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctccc gtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgcca gacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtg atgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgt gcatttcgtcgcagaagagcaggactaatatactgagtttgttaatgatacaataaactgttatagtacatacaattgaaactct cttatctatactgggggaccttctcgcagaatggtataaatatctactaactgactgtcgtacggcctaggggtctcttcttcgatt atttgcaggtcggaacatccttcgtctgatgcggatctcctgagacaaagttcacgggtatctagtattctatcagcataaatgga

	ggacctttctaaactaaactttgaatcgtctccagcagcatcctcgcattggtaccactgagcgtcagaccccgtagaaaagatc aaaggatcttcttgagatccttttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttg ccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagc cgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagt ggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttc gtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttc ccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggga aacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagc ctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgctggccttttgctcacatgt
pPpKC1_TKL1_dis	gaagttcctatactttctagagaataggaacttcggccgatcaggccactctcacgagcaatttggtatcaacagatttggtatttt ctggaccaggccctgagatttacaagttctttgagttcactgctgaggggttgctgacagggcttctaaagtcgtccaattctac aagggaaaggaacttctatcgccacttaataaggcttttgagtctgttcatgcgtagtttgtgcgcctttaggtagttagttaatta gtaagttcatagtgcgggaagaataggctgtcttagaatgctcgacaaagttaccaggattgcgaggattatttattggagtga atacttgtaatttattttgcttccttcttactctcctcttccttcgactcattatttgccaccctcaaagatggccccgatatccgttgc gtttgtatgtctaggaaacatttgccgaagccccatggctgaggcggtgttcacccacttagtcaaagaaaaaggactcgactct aagttcaaaaagattgactcctttggaacttcaggataccatatcgacgatactcctgatcaccgttccgtggcgtgttgcaaga aacatggagttcctgtgaaccatagaggtcagcagatctcgccggacgacttctaccactttgactacatactggccatggatga atcaaatctctccaatctcaaacgcatacagcccaaagactccaatgccgtagtcaggctctttggagactacagaacggacaa gtccttccacaggattgtggaggatccttactacggcgggtcttctggatttgagagaaatttcaaacagattacgcatttcagtg aagaattcttaaacaaagagttatagattatagattatcaacttttcaaagatacaacgtttccaacttgtccttctcaaaagtaa aatcgtcttttctcctgggaagccatgtctcctggtaatcaaagtatttccagttgctcttttgttgaacgtgctcttcaataggcgg aggagtttgattagcttcgtcattattgagcaggaaccaggtcttgagctctttgtggtacttccagccccttctatgcaactccttc gccgtattaatttaaatatgcattcaactttgatattgtgtatcgacacttcttgacgtagtatgtcactcaatgatcgcaatgctg ccttcattggggcatactgaccgtatccaatgaacgaatagaatgccagtacagatgaaaaaaacaccaagtgccttttgtagg gcttttctacctttgtcattaccttgagcgcaacatgtgagaagtacaacgctgtcttgtaattgatatcgattccactgctcagag tcttttcatcaagctctaagaaaagtccggggatggaggagccagcgcaacagaaaactatgtcgggactaaaaccaacttcc tctaaaacacgctcacactggtcatatttggagatatctgctgccacgtaagaaattgtgttgcccttctcggtcccgtgaatatc aatagcgtcctttactacctgctttagtaaggattctgttctagccacaatcacaacggaacatccttttccatacaaaagtttagc aaactcggctccaacaccctgggatgcacccgtaataattgccttttttgttggtgacatcaaaattatcaaacattccagttccct ctacactttgtatgagaatgatagctgaaattgtgcaccagatgttagaagataaggtcgtgtcatgaactaatatcatgaattc cgagggtggctcaacaactattcacgtgacttggacgttggaagttgaggtggttggtggatgttgcacggagtatcatttgtaa gcatgaaatcagtctaaaaaacttgcagaatagcagagcggttcggaaattcattcaaaaccacctcctcagattggatctgcc ctactctgtttagctctgggagattttctcggtcgtgttctttcgctggtctacccacgctataggaatcgctgtgaacgctaccttc ttcccaacttctcggtgactattataagccattcccactttgttttcaagcaccaacaacccacccccaccttatctactccatcttg ggtgtccccgcgcctgttgcaaagtccgaaccatagaaccccgacctttgtcccactaaccctcagacacccctcggaagtca gggagaaaccactccgaagtacattaatcatccctcgtattctcgacggtgcccatttttctttataaaaagggagacacaggttg cttcactaactctagacttgtattctacatccactctacacaggccactagggccgaagttcctatactttctagagaataggaac ttcagatctaacatccaaagacgaaaggttgaatgaaacctttttgccatccgacatccacaggtccattctcacacataagtgc

	caaacgcaacaggaggggatacactagcagcagaccgttgcaaacgcaggacctccactcctcttctcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttgattggagctcgctcattccaattccttctattaggctactaacaccatgacttttattagcctgtctatcctggcccccctggcgaggttcatgtttgtttatttccgaatgcaacaagctccgcattacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagtttcatgttccccaaatggcccaaaactgacagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatgaactaagtttggttcgttgaaatgctaacggccagttggtcaaaaagaaacttccaaaagtcggcataccgtttgtcttgtttggtattgattgacgaatgctcaaaaataatctcattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccgaaacgcaaatggggaaacacccgctttttggatgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgctgatagcctaacgttcatgatcaaaatttaactgttctaaccccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacctttttttttatcatcattattagcttactttcataattgcgactggttccaattgacaagcttttgattttaacgacttttaacgacaacttgagaagatcaaaaaacaactaattattcgaaacgatgccacaatttgatatattatgtaaaacaccacctaaggtgcttgttcgtcagtttgtggaaaggtttgaaagaccttcaggtgagaaaatagcattatgtgctgctgaactaacctatttatgttggatgattacacataacggaacagcaatcaagagagccacattcatgagctataatactatcataagcaattcgctgagtttcgatattgtcaataaatcactccagtttaaatacaagacgcaaaaagcaacaattctggaagcctcattaaagaaattgattcctgcttgggaatttacaattattccttactatggacaaaaacatcaatctgatatcactgatattgtaagtagtttgcaattacagttcgaatcatcggaagaagcagataagggaaatagccacagtaaaaaaatgcttaaagcacttctaagtgagggtgaaagcatctgggagatcactgagaaaatactaaattcgtttgagtatacttcgagatttacaaaaacaaaaactttataccaattcctcttcctagctactttcatcaattgtggaagattcagcgatattaagaacgttgatccgaaatcatttaagttagtccaaaataagtatttgggagtaataatccagtgtttagtgacagagacaaagacaagcgttagtaggcacatatacttctttagcgcaaggggtaggatcgatccacttgtatatttggatgaatttttgaggaactctgaaccagtcctaaaacgagtaaataggaccggcaattcttcaagcaataaacaggaataccaattattaaaagataacttagtcagatcgtacaataaagctttgaagaaaaatgcgccttattcaatctttgctataaaaaatggcccaaaatctcacattggaagacatttgatgacctcatttctttcaatgaagggcctaacggagttgactaatgttgtgggaaattggagcgataagcgtgcttctgccgtggccaggacaacgtatactcatcagataacagcaatacctgatcactacttcgcactagtttctcggtactatgcatacgatccaatatcaaaggaaatgatagcattgaaggatgagactaatccaattgaggagtggcagcatatagaacagctaaagggtagtgctgaaggaagcatacgataccccgcatggaatgggataatatcacaggaggtactagactacctttcatcctacataaatagacgcatataagttttagccttagacatgactgttcctcagttcaagttgggcacttacgagaagaccggtcttgctagattctaatcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagtataggatttttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatctcgcagctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgtgcggaacggaacgtatcttagcatggttgtgcgacagattcactgtgaaagactgttcattatacccacgtttcactgggagatgtaagccttaggtgtttaccctgattagataatacaataaccaacagaaatacgagaatctaaactaatttcgatgattcatttttcttttttaccgcgctgcctcttttggcaattctttcacctatattctaccttctctttccttttgttctaaacttattaccagctagtgttgttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatggctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctcccgtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgccagacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtgatgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgtgcatttcgtcgcagaagagcaggactaatatactgagtttgttaatgatacaataaactgttatagtacatacaattgaaactctcttatctatactgggggaccttctcgcagaatggtataaatat

	ctactaactgactgtcgtacggcctaggggtctcttcttcgattatttgcaggtcggaacatccttcgtctgatgcggatctcctga gacaaagttcacgggtatctagtattctatcagcataaatggaggacctttctaaactaaactttgaatcgtctccagcagcatc ctcgcattggtaccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttttctgcgcgtaatctgct gcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactgg cttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcct acatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgata gttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaa ctgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggca gggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctct gacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcct ggccttttgctggccttttgctcacatgt
pPpT4mutZeoM lyI-intArg4- EGFP- pGAPshort	tggccttttgctcacatgtatttaaattgccagtgtatgtgcacttatagaggatgtacaaatggtcagagtttccccaaagagca gatggaaaagcaattagattttcgttactgaatcaggcattcaaaaagaaacaataagtcgcaacaatgatgttaatctcgaat attgaagtaaatgcttccagcaataatgtgacagttgctcgaatctagaacggtattaacagaatagaccgagactctcctattt cagcgtcctaaactctatcttgggaccaaggaagaaagctacgcacttaagaataggaccatgcataccaaaacaatagacct gacctgatacgctatgcactgcaaatcgaagaaaaaagcaattcattcggaacaaaacgtcagagcgggttttctaatagaat ttgcgtaatatataaacctaaataccataattgaggtgtttctttttctctgataaaagtacaacgaagtgctcttgtcataccgca acaaagatgttgaacacttcgtatcagtttcccaaagatgttgcaagatacgacgtggcagatgctgtcaatgagcaacttcaa gctacctttttgaattcaaaggttcaacatgagcccggttcaagatatcaacaccaagagggaatcaactcatcaccagagaga aaaaattcagtcttttcatctgctgatatatctaccacatcgtcacctacttctccgaagtctaaagccaattctcaatttgctaga gactctgtcgacagttctatctacccgaggaaaccgatacataggagatctaatacagaggtatcatccaatgattccgaaaaa gatttcagtttggactccctttttaaagcacagtatcgggatacaaagctgaaaccctccccatttcaagcttctagtggtaggaa ttctgtaccggtttacagaaggacgactcttgatgcgccaaccacagtgacaatagacatagaggaaatgacaaaaggattat gcgaggatgctgctggagacgattcaaagtttagtttagaaaggtcctccatttatgctgatagaatactagatacccgtgaact ttgtctcaggagatccgcatcagacgaaggatgttccgacctgcaaataatcgaagaagagacccctaggccgtacgacagtc agttagtagatatttataccattctgcgagaaggtccttttttgtagaaatgtcttggtgtcctcgtccaatcaggtagccatctctg aaatatctggctccgttgcaactccgaacgacctgctggcaacgtaaaattctccggggtaaaacttaaatgtggagtaatgga accagaaacgtctcttcccttctctcctccttccaccgcccgttaccgtccctaggaaattttactctgctggagagcttcttctacgg ccccccttgcagcaatgctcttcccagcattacgttgcgggtaaaacggaggtcgtgtacccgacctagcagcccagggatggaa aagtcccggccgtcgctggcaataatagcgggcggacgcatgtcatgagattattggaaaccaccagaatcgaatataaaagg cgaacacctttcccaattttggtttctcctgacccaaagactttaaatttaatttatttgtccctatttcaatcaattgaacaactatc aaaacacaatggctagcaaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggca caaattttctgtcagtggagagggtgaaggtgatgctacatacggaaagcttacccttaaatttatttgcactactggaaaacta cctgttccatggccaacacttgtcactactttgacttatggtgttcaatgcttttcccgttatccggatcatatgaaacggcatgact ttttcaagagtgccatgcccgaaggttatgtacaggaacgcactatatctttcaaagatgacgggaactacaagacgcgtgctg aagtcaagtttgaaggtgataccttgttaatcgtatcgagttaaaaggtattgattttaaagaagatggaaacattctcggaca caaacttgagtacaactataactcacacaatgtatacatcacggcagacaaacaaaagaatggaatcaaagctaacttcaaa attcgtcacaacattgaagatggttccgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtcctttt

	accagacaaccattacctgtcgacacaatctgccctttcgaaagatcccaacgaaaagcgtgaccacatggtccttcttgagttt gtaactgctgctgggattacacatggcatggatgaattgtacaagtaagcggccgctcaagaggatgtcagaatgccatttgcc tgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagtataggatttttttgtcattttgtttcttctcgtacgag cttgctcctgatcagcctatctcgcagcagatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggt atttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgtgcggatccttcagtaatgtcttgtttcttttgttgca gtggtgagccattttgacttcgtgaaagtttctttagaatagttgtttccagaggccaaacattccacccgtagtaaagtgcaagc gtaggaagaccaagactggcataaatcaggtataagtgtcgagcactggcaggtgatcttctgaaagtttctactagcagataa gatccagtagtcatgcatatggcaacaatgtaccgtgtggatctaagaacgcgtcctactaaccttcgcattcgttggtccagttt gttgttatcgatcaacgtgacaaggttgtcgattccgcgtaagcatgcatacccaaggacgcctgttgcaattccaagtgagcca gttccaacaatctttgtaatattagagcacttcattgtgttgcgcttgaaagtaaaatgcgaacaaattaagagataatctcgaa accgcgacttcaaacgccaatatgatgtgcggcacacaataagcgttcatatccgctgggtgactttctcgctttaaaaaattat ccgaaaaaattttctagagtgttgttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatggcta aactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctcccgtga cttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgccagaca acactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtgatgca tctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgtgcatt tcgtcgcagaagagcaggactaacaattgacaccttacgattatttagagagtatttattagttttattgtatgtatacggatgttt tattatctatttatgcccttatattctgtaactatccaaaagtcctatcttatcaagccagcaatctatgtccgcgaacgtcaacta aaaataagctttttatgctcttctctctttttttcccttcggtataattataccttgcatccacagattctcctgccaaattttgcataa tcctttacaacatggctatatgggagcacttagcgccctccaaaacccatattgcctacgcatgtataggtgtttttttccacaatat tttctctgtgctctctttttattaaagagaagctctatatcggagaagcttctgtggccgttatattcggccttatcgtgggaccaca ttgcctgaattggtttgccccggaagattggggaaacttggatctgattaccttagctgcaggtaccactgagcgtcagaccccg tagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagc ggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttc ttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtg gctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctga acgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaa gcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggag cttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtca gggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgc
pPpT4mutZeoMlyI-intArg4-EGFP-pAOX1BglII	tggccttttgctcacatgtatttaaattgccagtgtatgtgcacttatagaggatgtacaaatggtcagagtttccccaaagagca gatggaaaagcaattagattttcgttactgaatcaggcattcaaaaagaaacaataagtcgcaacaatgatgttaatctcgaat attgaagtaaatgcttccagcaataatgtgacagttgctcgaatctagaacggtattaacagaatagaccgagactctcctattt cagcgtcctaaactctatcttgggaccaaggaagaaagctacgcacttaagaataggaccatgcataccaaaacaatagacct gacctgatacgctatgcactgcaaatcgaagaaaaaagcaattcattcggaacaaaacgtcagagcgggttttctaatagaat ttgcgtaatatataaacctaaataccataattgaggtgtttctttttctctgataaaagtacaacgaagtgctcttgtcataccgca acaaagatgttgaacacttcgtatcagtttcccaaagatgttgcaagatacgacgtggcagatgctgtcaatgagcaacttcaa gctacctttttgaattcaaaggttcaacatgagcccggttcaagatatcaacaccaagagggaatcaactcatcaccagagaga

	aaaaattcagtcttttcatctgctgatatatctaccacatcgtcacctacttctccgaagtctaaagccaattctcaatttgctaga gactctgtcgacagttctatctacccgaggaaaccgatacataggagatctaatacagaggtatcatccaatgattccgaaaaa gatttcagtttggactccctttttaaagcacagtatcgggatacaaagctgaaaccctccccatttcaagcttctagtggtaggaa ttctgtaccggtttacagaaggacgactcttgatgcgccaaccacagtgacaatagacatagaggaaatgacaaaaggattat gcgaggatgctgctggagacgattcaaagtttagtttagaaaggtcctccatttatgctgatagaatactagatacccgtgaact ttgtctcaggagatccgcatcagacgaaggatgttccgacctgcaaataatcgaagaagagacccctaggccgtacgacagtc agttagtagatatttataccattctgcgagaaggtcctagatctaacatccaaagacgaaaggttgaatgaaacctttttgccat ccgacatccacaggtccattctcacacataagtgccaaacgcaacaggaggggatacactagcagcagaccgttgcaaacgc aggacctccactcctcttctcctcaacacccacttttgccatcgaaaaaccagcccagttattgggcttgattggagctcgctcatt ccaattccttctattaggctactaacaccatgactttattagcctgtctatcctggcccccctggcgaggttcatgtttgtttatttcc gaatgcaacaagctccgcattacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagtttcatgttcccc aaatggcccaaaactgacagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatgaactaagttt ggttcgttgaaatgctaacggccagttggtcaaaaagaaacttccaaaagtcggcataccgtttgtcttgtttggtattgattgac gaatgctcaaaaataatctcattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccgaaacgcaaat ggggaaacacccgctttttggatgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgctgatagcct aacgttcatgatcaaaatttaactgttctaacccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacctttt ttttttatcatcattattagcttactttcataattgcgactggttccaattgacaagcttttgattttaacgacttttaacgacaactt gagaagatcaaaaaacaactaattattcgaaacgatggctagcaaaggagaagaacttttcactggagttgtcccaattcttgt tgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgctacatacggaaagcttacccctt aaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttgacttatggtgttcaatgcttttcccgtt atccggatcatatgaaacggcatgactttttcaagagtgccatgcccgaaggttatgtacaggaacgcactatatctttcaaaga tgacgggaactacaagacgcgtgctgaagtcaagtttgaaggtgataccttgttaatcgtatcgagttaaaaggtattgatttt aaagaagatggaaacattctcggacacaaacttgagtacaactataactcacacaatgtatacatcacggcagacaaacaaa agaatggaatcaaagctaacttcaaaattcgtcacaacattgaagatggttccgttcaactagcagaccattatcaacaaaata ctccaattggcgatggccctgtccttttaccagacaaccattacctgtcgacacaatctgccctttcgaaagatcccaacgaaaa gcgtgaccacatggtccttcttgagtttgtaactgctgctgggattacacatggcatggatgaattgtacaagtaagcggccgct caagaggatgtcagaatgccatttgcctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagtataggattt tttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatctcgcagcagatgaatatcttgtggtaggggtttgggaa aatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgtgcggatcc ttcagtaatgtcttgtttcttttgttgcagtggtgagccattttgacttcgtgaaagtttctttagaatagttgtttccagaggccaaa cattccacccgtagtaaagtgcaagcgtaggaagaccaagactggcataaatcaggtataagtgtcgagcactggcaggtgat cttctgaaagtttctactagcagataagatccagtagtcatgcatatggcaacaatgtaccgtgtggatctaagaacgcgtccta ctaaccttcgcattcgttggtccagtttgttgttatcgatcaacgtgacaaggttgtcgattccgcgtaagcatgcatacccaagg acgcctgttgcaattccaagtgagccagttccaacaatctttgtaatattagagcacttcattgtgttgcgcttgaaagtaaaatg cgaacaaattaagagataatctcgaaaccgcgacttcaaacgccaatatgatgtgcggcacacaataagcgttcatatccgct gggtgactttctcgctttaaaaaattatccgaaaaaattttctagagtgttgttactttatacttccggctcgtataatacgacaag gtgtaaggaggactaaaccatggctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttgagttct ggactgataggctcggtttctcccgtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcatctccg

	cagttcaggaccaggttgtgccagacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtggtctga ggtcgtgtctaccaacttccgtgatgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtttgcactg cgtgatccagctggtaactgcgtgcatttcgtcgcagaagagcaggactaacaattgacaccttacgattatttagagagtattt attagttttattgtatgtatacggatgttttattatctatttatgcccttatattctgtaactatccaaaagtcctatcttatcaagcca gcaatctatgtccgcgaacgtcaactaaaaataagctttttatgctcttctctctttttttcccttcggtataattataccttgcatcc acagattctcctgccaaattttgcataatcctttacaacatggctatatgggagcacttagcgccctccaaaacccatattgccta cgcatgtataggtgtttttttccacaatattttctctgtgctctctttttattaaagagaagctctatatcggagaagcttctgtggcc gttatattcggccttatcgtgggaccacattgcctgaattggtttgccccggaagattggggaaacttggatctgattaccttagct gcaggtaccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatccttttttttctgcgcgtaatctgctgcttg caaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttca gcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacata cctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttac cggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgag atacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtc ggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgactt gagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggcct tttgc
pPpT4mutZeoMlyI-intArg4-EGFP-pCAT1	tggccttttgctcacatgtatttaaattgccagtgtatgtgcacttatagaggatgtacaaatggtcagagtttccccaaagagca gatggaaaagcaattagattttcgttactgaatcaggcattcaaaaagaaacaataagtcgcaacaatgatgttaatctcgaat attgaagtaaatgcttccagcaataatgtgacagttgctcgaatctagaacggtattaacagaatagaccgagactctcctattt cagcgtcctaaactctatcttgggaccaaggaagaaagctacgcacttaagaataggaccatgcataccaaaacaatagacct gacctgatacgctatgcactgcaaatcgaagaaaaaagcaattcattcggaacaaaacgtcagagcgggttttctaatagaat ttgcgtaatatataaacctaaataccataattgaggtgtttcttttctctgataaaagtacaacgaagtgctcttgtcataccgca acaaagatgttgaacacttcgtatcagtttcccaaagatgttgcaagatacgacgtggcagatgctgtcaatgagcaacttcaa gctacctttttgaattcaaaggttcaacatgagcccggttcaagatatcaacaccaagagggaatcaactcatcaccagagaga aaaaattcagtcttttcatctgctgatatatctaccacatcgtcacctacttctccgaagtctaaagccaattctcaatttgctaga gactctgtcgacagttctatctacccgaggaaaccgatacataggagatctaatacagaggtatcatccaatgattccgaaaaa gatttcagtttggactccctttttaaagcacagtatcgggatacaaagctgaaaccctccccatttcaagcttctagtggtaggaa ttctgtaccggtttacagaaggacgactcttgatgcgccaaccacagtgacaatagacatagaggaaatgacaaaaggattat gcgaggatgctgctggagacgattcaaagtttagtttagaaaggtcctccatttatgctgatagaatactagatacccgtgaact ttgtctcaggagatccgcatcagacgaaggatgttccgacctgcaaataatcgaagaagagacccctaggccgtacgacagtc agttagtagatatttataccattctgcgagaaggtcctagtgtgtaatcatatatataataaatgaggaataataattgaataga gatttaacgagtcgaagtttctgaaatatacgcacagtttatatttatgattttgatatctaactacagtcttctccatatatttaac tataaataataaagtatataactcttatgaaactgtttcaccacatttttttctacgtaatcgaactccgaatgcggttctcctgta accttaattgtagcatagatcacttaaataaactcatggcctgacatctgtacacgttcttattggtcttttagcaatcttgaagtct ttctattgttccggtcggcattacctaataaattcgaatcgagattgctagtacctgatatcatatgaagtaatcatcacatgcaa gttccatgataccctctactaatggaattgaacaaagtttaagcttctcgcacgagaccgaatccatactatgcacccctcaaag ttgggattagtcaggaaagctgagcaattaacttccctcgattggcctggacttttcgcttagcctgccgcaatcggtaagtttca

	ttatcccagcggggtgatagcctctgttgctcatcaggccaaaatcatatataagctgtagacccagcacttcaattacttgaaat tcaccataacacttgctctagtcaagacttacaattaaaatggctagcaaaggagaagaacttttcactggagttgtcccaattc ttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgctacatacggaaagcttac ccttaaatttatttgcactactggaaaactacctgttccatggccaacacttgtcactactttgacttatggtgttcaatgcttttcc cgttatccggatcatatgaaacggcatgactttttcaagagtgccatgcccgaaggttatgtacaggaacgcactatatctttca aagatgacgggaactacaagacgcgtgctgaagtcaagtttgaaggtgataccttgttaatcgtatcgagttaaaaggtattg attttaaagaagatggaaacattctcggacacaaacttgagtacaactataactcacacaatgtatacatcacggcagacaaa caaaagaatggaatcaaagctaacttcaaaattcgtcacaacattgaagatggttccgttcaactagcagaccattatcaacaa aatactccaattggcgatggccctgtccttttaccagacaaccattacctgtcgacacaatctgccctttcgaaagatcccaacg aaaagcgtgaccacatggtccttcttgagtttgtaactgctgctgggattacacatggcatggatgaattgtacaagtaagcggc cgctcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagtatag gatttttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatctcgcagcagatgaatatcttgtggtaggggttt gggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgtgc ggatccttcagtaatgtcttgtttcttttgttgcagtggtgagccattttgacttcgtgaaagtttctttagaatagttgtttccagag gccaaacattccacccgtagtaaagtgcaagcgtaggaagaccaagactggcataaatcaggtataagtgtcgagcactggc aggtgatcttctgaaagtttctactagcagataagatccagtagtcatgcatatggcaacaatgtaccgtgtggatctaagaacg cgtcctactaaccttcgcattcgttggtccagtttgttgttatcgatcaacgtgacaaggttgtcgattccgcgtaagcatgcatac ccaaggacgcctgttgcaattccaagtgagccagttccaacaatctttgtaatattagagcacttcattgtgttgcgcttgaaagt aaaatgcgaacaaattaagagataatctcgaaaccgcgacttcaaacgccaatatgatgtgcggcacacaataagcgttcata tccgctgggtgactttctcgctttaaaaaattatccgaaaaaattttctagagtgttgttactttatacttccggctcgtataatacg acaaggtgtaaggaggactaaaccatggctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgttga gttctggactgataggctcggtttctcccgtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttcat ctccgcagttcaggaccaggttgtgccagacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagtg gtctgaggtcgtgtctaccaacttccgtgatgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagttt gcactgcgtgatccagctggtaactgcgtgcatttcgtcgcagaagagcaggactaacaattgacaccttacgattatttagaga gtatttattagttttattgtatgtatacggatgttttattatctatttatgcccttatattctgtaactatccaaaagtcctatcttatca agccagcaatctatgtccgcgaacgtcaactaaaaataagctttttatgctcttctctctttttttcccttcggtataattataccttg catccacagattctcctgccaaattttgcataatcctttacaacatggctatatgggagcacttagcgccctccaaaacccatatt gcctacgcatgtataggtgtttttttccacaatattttctctgtgctctctttttattaaagagaagctctatatcggagaagcttctgt ggccgttatattcggccttatcgtgggaccacattgcctgaattggtttgccccggaagattggggaaacttggatctgattacct tagctgcaggtaccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgct gcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactgg cttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcct acatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgata gttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaa ctgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggca gggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctct

	gacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcct ggccttttgc
pPICZBd1-BDH1	agatctaacatccaaagacgaaaggttgaatgaaacctttttgccatccgacatccacaggtccattctcacacataagtgcca aacgcaacaggaggggatacactagcagcagaccgttgcaaacgcaggacctccactcctcttctcctcaacacccactttagg ctactaacaccatgactttattagcctgtctatcctggcccccctggcgaggttcatgtttgtttatttccgaatgcaacaagctcc gcattacacccgaacatcactccagatgagggctttctgagtgtggggtcaaatagtttcatgttccccaaatggcccaaaactg acagtttaaacgctgtcttggaacctaatatgacaaaagcgtgatctcatccaagatgaactaagtttggttcgttgaaatgcta acggccagttggtcaaaaagaaacttccaaaagtcggcataccgtttgtcttgtttggtattgattgacgaatgctcaaaaataa tctcattaatgcttagcgcagtctctctatcgcttctgaaccccggtgcacctgtgccgaaacgcaaatggggaaacacccgcttt ttggatgattatgcattgtctccacattgtatgcttccaagattctggtgggaatactgctgatagcctaacgttcatgatcaaaat ttaactgttctaacccctacttgacagcaatatataaacagaaggaagctgccctgtcttaaacctttttttttatcatcattattag cttactttcataattgcgactggttccaattgacaagcttttgattttaacgacttttaacgacaacttgagaagatcaaaaaaca actaattattcgaaagaattcgaaacgatgagagctttggcatatttcaagaagggtgatattcacttcactaatgatatcccta ggccagaaatccaaaccgacgatgaggttattatcgacgtctcttggtgtgggatttgtggctcggatcttcacgagtacttggat ggtccaatcttcatgcctaaagatggagagtgccataaattatccaacgctgctttacctctggcaatgggccatgagatgtcag gaattgtttccaaggttggtcctaaagtgacaaaggtgaaggttggcgaccacgtggtcgttgatgctgccagcagttgtgcgg acctgcattgctggccacactccaaattttacaattccaaaccatgtgatgcttgtcagaggggcagtgaaaatctatgtaccca cgccggttttgtaggactaggtgtgatcagtggtggctttgctgaacaagtcgtagtctctcaacatcacattatcccggttccaa aggaaattcctctagatgtggctgctttagttgagcctctttctgtcacctggcatgctgttaagatttctggtttcaaaaaaggca gttcagccttggttcttggtgcaggtcccattgggttgtgtaccattttggtacttaagggaatgggggctagtaaaattgtagtgt ctgaaattgcagagagaagaatagaaatggccaagaaactgggcgttgaggtgttcaatccctccaagcacggtcataaatct atagagatactacgtggtttgaccaagagccatgatgggtttgattacagttatgattgttctggtattcaagttactttcgaaac ctctttgaaggcattaacattcaaggggacagccaccaacattgcagtttggggtccaaaacctgtcccattccaaccaatggat gtgactctccaagagaaagttatgactggttcgatcggctatgttgtcgaagccttcgaagaagttgttcgtgccatccacaacg gagacatcgccatggaagattgtaagcaactaatcactggtaagcaaaggattgaggacggttgggaaaagggattccaaga gttgatggatcacaaggaatccaacgttaagattctattgacgcctaacaatcacggtgaaatgaagtaagcggccgccagctt tctagaacaaaaactcatctcagaagaggatctgaatagcgccgtcgaccatcatcatcatcattgagtttgtagccttaga catgactgttcctcagttcaagttgggcacttacgagaagaccggtcttgctagattctaatcaagaggatgtcagaatgccattt gcctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagtataggatttttttgtcattttgtttcttctcgtac gagcttgctcctgatcagcctatctcgcagctgatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttctt ggtatttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgtgcggatccccccacacaccatagcttcaaaatg tttctactccttttttactcttccagattttctcggactccgcgcatcgccgtaccacttcaaaacacccaagcacagcatactaaa ttttccctctttcttcctctagggtgtcgttaattacccgtactaaaggtttggaaaagaaaaaagagaccgcctcgtttctttttct tcgtcgaaaaaggcaataaaaatttttatcacgtttctttttcttgaaatttttttttttagttttttttctctttcagtgacctccattga tatttaagttaataaacggtcttcaatttctcaagtttcagtttcatttttcttgttctattacaacttttttttacttcttgttcattagaa agaaagcatagcaatctaatctaaggggcggtgttgacaattaatcatcggcatagtatatcggcatagtataatacgacaag gtgaggaactaaaccatggccaagttgaccagtgccgttccggtgctcaccgcgcgcgacgtcgccggagcggtcgagttctgg accgaccggctcgggttctcccgggacttcgtggaggacgacttcgccggtgtggtccgggacgacgtgaccctgttcatcagc

	gcggtccaggaccaggtggtgccggacaacaccctggcctgggtgtgggtgcgcggcctggacgagctgtacgccgagtggtc ggaggtcgtgtccacgaacttccgggacgcctccgggccggccatgaccgagatcggcgagcagccgtgggggcgggagttc gccctgcgcgacccggccggcaactgcgtgcacttcgtggccgaggagcaggactgacacgtccgacggcggcccacgggtcc caggcctcggagatccgtccccctttccttttgtcgatatcatgtaattagttatgtcacgcttacattcacgccctcccccacatc cgctctaaccgaaaaggaaggagttagacaacctgaagtctaggtccctatttatttttttatagttatgttagtattaagaacgt tatttatatttcaaatttttctttttttttctgtacagacgcgtgtacgcatgtaacattatactgaaaaccttgcttgagaaggttttg gacgctcgaaggctttaatttgcaagctggagaccaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaag gccgcgttgctggcgtttttccataggctccgccccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaac ccgacaggactataaagataccaggcgtttcccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggat acctgtccgcctttctcccttcgggaagcgtggcgctttctcaatgctcacgctgtaggtatctcagttcggtgtaggtcgttcgctc caagctgggctgtgtgcacgaacccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggta agacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttctt gaagtggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaag agttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggttttttttgtttgcaagcagcagattacgcgcagaaaa aaaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtca tgagatc
pPpT4_GAP_S_ ScBDH	agatccaattcccgctttgactgcctgaaatctccatcgcctacaatgatgacatttggatttggttgactcatgttggtattgtga aatagacgcagatcgggaacactgaaaaatacacagttattattcatttaaatttttttgtagaaatgtcttggtgtcctcgtccaa tcaggtagccatctctgaaatatctggctccgttgcaactccgaacgacctgctggcaacgtaaaattctccggggtaaaactta aatgtggagtaatggaaccagaaacgtctcttcccttctctctccttccaccgcccgttaccgtccctaggaaatttttactctgctg gagagcttcttctacggccccccttgcagcaatgctcttcccagcattacgttgcgggtaaaacggaggtcgtgtacccgacctag cagcccagggatggaaaagtcccggccgtcgctggcaataatagcgggcggacgcatgtcatgagattattggaaaccacca gaatcgaatataaaaggcgaacacctttcccaattttggtttctcctgacccaaagactttaaatttaatttatttgtccctatttca atcaattgaacaactatcaaaacacagaattcgaaacgatgagagctttggcatatttcaagaagggtgatattcacttcacta atgatatccctaggccagaaatccaaaccgacgatgaggttattatcgacgtctcttggtgtgggatttgtggctcggatcttcac gagtacttggatggtccaatcttcatgcctaaagatggagagtgccataaattatccaacgctgctttacctctggcaatgggcc atgagatgtcaggaattgtttccaaggttggtcctaaagtgacaaaggtgaaggttggcgaccacgtggtcgttgatgctgcca gcagttgtgcggacctgcattgctggccacactccaaatttacaattccaaaccatgtgatgcttgtcagaggggcagtgaaaa tctatgtacccacgccggttttgtaggactaggtgtgatcagtggtggctttgctgaacaagtcgtagtctctcaacatcacattat cccggttccaaaggaaattcctctagatgtggctgctttagttgagcctctttctgtcacctggcatgctgttaagatttctggtttc aaaaaaggcagttcagccttggttcttggtgcaggtcccattgggttgtgtaccattttggtacttaagggaatgggggctagta aaattgtagtgtctgaaattgcagagagaagaatagaaatggccaagaaactgggcgttgaggtgttcaatccctccaagcac ggtcataaatctatagagatactacgtggtttgaccaagagccatgatgggtttgattacagttatgattgttctggtattcaagtt actttcgaaacctctttgaaggcattaacattcaaggggacagccaccaacattgcagtttggggtccaaaacctgtcccattcc aaccaatggatgtgactctccaagagaaagttatgactggttcgatcggctatgttgtcgaagccttcgaagaagttgttcgtgc catccacaacggagacatcgccatggaagattgtaagcaactaatcactggtaagcaaaggattgaggacggttgggaaaag ggattccaagagttgatggatcacaaggaatccaacgttaagattctattgacgcctaacaatcacggtgaaatgaagtaagc ggccgctcaagaggatgtcagaatgccatttgcctgagagatgcaggcttcatttttgatacttttttatttgtaacctatatagta

	taggatttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcctatctcgcagcagatgaatatcttgtggtagggg tttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttcagagtacagaagattaagtgagaccttcgtttgt gcggatccttcagtaatgtcttgtttcttttgttgcagtggtgagccattttgacttcgtgaaagtttctttagaatagttgtttccag aggccaaacattccacccgtagtaaagtgcaagcgtaggaagaccaagactggcataaatcaggtataagtgtcgagcactg gcaggtgatcttctgaaagtttctactagcagataagatccagtagtcatgcatatggcaacaatgtaccgtgtggatctaagaa cgcgtcctactaaccttcgcattcgttggtccagtttgttgttatcgatcaacgtgacaaggttgtcgattccgcgtaagcatgcat acccaaggacgcctgttgcaattccaagtgagccagttccaacaatctttgtaatattagagcacttcattgtgttgcgcttgaaa gtaaaatgcgaacaaattaagagataatctcgaaaccgcgacttcaaacgccaatatgatgtgcggcacacaataagcgttca tatccgctgggtgactttctcgctttaaaaaattatccgaaaaaattttctagagtgttgttactttatacttccggctcgtataata cgacaaggtgtaaggaggactaaaccatggctaaactcacctctgctgttccagtcctgactgctcgtgatgttgctggtgctgtt gagttctggactgatagactcggtttctcccgtgacttcgtagaggacgactttgccggtgttgtacgtgacgacgttaccctgttc atctccgcagttcaggaccaggttgtgccagacaacactctggcatgggtatgggttcgtggtctggacgaactgtacgctgagt ggtctgaggtcgtgtctaccaacttccgtgatgcatctggtccagctatgaccgagatcggtgaacagccctggggtcgtgagtt tgcactgcgtgatccagctggtaactgcgtgcatttcgtcgcagaagagcaggactaacaattgacaccttacgattatttagag agtatttattagttttattgtatgtatacggatgttttattatctatttatgcccttatattctgtaactatccaaaagtcctatcttatc aagccagcaatctatgtccgcgaacgtcaactaaaaataagctttttatgctcttctctctttttttcccttcggtataattatacctt gcatccacagattctcctgccaaattttgcataatcctttacaacatggctatatgggagcacttagcgccctccaaaacccatat tgcctacgcatgtataggtgtttttttccacaatattttctctgtgctctctttttattaaagagaagctctatatcggagaagcttctg tggccgttatattcggccttatcgtgggaccacattgcctgaattggtttgccccggaagattggggaaacttggatctgattacc ttagctgcaggtaccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgc tgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgtcggatcaagagctaccaactctttttccgaaggtaactg gcttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcc tacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgat agttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccga actgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggc agggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctc tgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacggttcc tggccttttgctggccttttgctcacatgttctttcctgcggtaccc
pPpT4-CalB+synPDI-pAOX1+pGAP-fwd	tggccttttgctcacatgtatttaaattgccagtgtatgtgcacttatagaggatgtacaaatggtcagagtttccccaaagagca gatggaaaagcaattagattttcgttactgaatcaggcattcaaaaagaaacaataagtcgcaacaatgatgttaatctcgaat attgaagtaaatgcttccagcaataatgtgacagttgctcgaatctagaacggtattaacagaatagaccgagactctcctattt cagcgtcctaaactctatcttgggaccaaggaagaaagctacgcacttaagaataggaccatgcataccaaaacaatagacct gacctgatacgctatgcactgcaaatcgaagaaaaaagcaattcattcggaacaaaacgtcagagcgggttttctaatagaat ttgcgtaatatataaacctaaataccataattgaggtgtttctttttctctgataaaagtacaacgaagtgctcttgtcataccgca acaaagatgttgaacacttcgtatcagtttcccaaagatgttgcaagatacgacgtggcagatgctgtcaatgagcaacttcaa gctacctttttgaattcaaaggttcaacatgagcccggttcaagatatcaacaccaagagggaatcaactcatcaccagagaga aaaaattcagtcttttcatctgctgatatatctaccacatcgtcacctacttctccgaagtctaaagccaattctcaatttgctaga gactctgtcgacagttctatctacccgaggaaaccgatacataggagatctaatacagaggtatcatccaatgattccgaaaaa

	gatttcagtttggactccctttttaaagcacagtatcgggatacaaagctgaaaccctccccatttcaagcttctagtggtaggaa ttctgtaccggtttacagaaggacgactcttgatgcgccaaccacagtgacaatagacatagaggaaatgacaaaaggattat gcgaggatgctgctggagacgattcaaagtttagtttagaaaggtcctccatttatgctgatagaatactagatacccgtgaact ttgtctcaggagatccgcatcagacgaaggatgttccgacctgcaaataatcgaagaagagacccctaggccgtacgacagtc agttagtagatatttataccattctgcgagaaggtcccctgcagggcacaaacgaaggtctcacttaatcttctgtactctgaag aggagtgggaaataccaagaaaaacatcaaactcgaatgattttcccaaacccctaccacaagatattcatctgctgcgagat aggctgatcaggagcaagctcgtacgagaagaaacaaaatgacaaaaaaaatcctatactatataggttacaaataaaaaa gtatcaaaaatgaagcctgcatctctcaggcaaatggcattctgacatcctcttgagcggccgcttatggggtcacgataccgga acaagttctcttaccaacagcgaaaggtctagcatatggcatcaaatctggttcacagttttgcttaggaccagcaacaatagct gcggcagcaggagccaacaatgcagcggcagcgacctttttgctctggagtaaggtcgtttgctggtaaagggttacagtcagtg ataccgtaatcagcagaacgagcttgaccagtggtggatctaagtgcggatctaccaacgacgtaagagaattgggaggtaag agaaccggcgtggtcaataacgaacaaaggaccacaaacagcttgtgcttggacgttctttccgttgaacaagtaagaggagt ccaatggggagttagacacttgaggctgaacgatctcgtcagtggcagagtacaggttagtggtagggacaatttgggtcaaa cctcctgcgtttctcaaggcggtagtcaaagcagaaccggtggtttgttgccacacagaaggagcggaaacagccaatgcgtcc aatggtccagcaagaacagttcccttgtagtcaggtgcgaaggccatcaatctgtcaaccttggaacggatagatgggaagaa ggtcaaaccccattgagcaaccaatccaccttgagaccaagtaaggactggcaacttgttgttaccagaaccagcgtaaagag cggtgattgcgttgaccatgtattcggtgttaacttgggtgtcgttcaacatgaatggaggtggggaaatccagcatggagtgta acccaactgagtagacagaggaatccagttggagtcgaaagattgtggtccagtggtaccagtacctggaaccaacaaaattg gcttagagacagaagatggagaagcaccttgacaggtcaaacctgcgtcaaggacggactttggctgggagaaagcaggatc agaaccagatggaagagcttcggcctctctcttctcgagagagacaccctcttccttagcagcaatggaagcgatagtggtgtt aatgaacaacaaaccgttgttagtggagttagagaaaggcaaaacagcgacgtcgaaatcaccctcaaggtcagagtaaccg ataactgcttcagctggaatttgagcagtctcgtcttcagtggtagtgttaacaggggcagccaatgcagaggaggcagcgaac aagacagcggtgaaaatagatgggaatctcatcgtttcgaataattagttgtttttgatcttctcaagttgtcgttaaaagtcgtt aaaatcaaaagcttgtcaattggaaccagtcgcaattatgaaagtaagctaataatgatgataaaaaaaaggtttaagacagg gcagcttccttctgtttatatattgctgtcaagtaggggttagaacagttaaattttgatcatgaacgttaggctatcagcagtatt cccaccagaatcttggaagcatacaatgtggagacaatgcataatcatccaaaaagcgggtgtttccccatttgcgtttcggcac aggtgcaccggggttcagaagcgatagagagactgcgctaagcattaatgagattatttttgagcattcgtcaatcaataccaa acaagacaaacggtatgccgacttttggaagtttcttttgaccaactggccgttagcatttcaacgaaccaaacttagttcatct tggatgagatcacgcttttgtcatattaggttccaagacagcgtttaaactgtcagttttgggccatttggggaacatgaaactat ttgaccccacactcagaaagccctcatctggagtgatgttcgggtgtaatgcggagcttgttgcattcggaaataaacaaacatg aacctcgccaggggggccaggatagacaggctaataaagtcatggtgttagtagcctaatagaaggaattggaatgagcgag ctccaatcaagcccaataactgggctggtttttcgatggcaaaagtgggtgttgaggagaagaggagtggaggtcctgcgtttg caacggtctgctgctagtgtatcccctcctgttgcgtttggcacttatgtgtgagaatggacctgtggatgtcggatggcaaaaag gtttcattcaacctttcgtctttggatgttagatctttttgtagaaatgtcttggtgtcctcgtccaatcaggtagccatctctgaaa tatctggctccgttgcaactccgaacgacctgctggcaacgtaaaattctccggggtaaaacttaaatgtggagtaatggaacc agaaacgtctcttcccttctctctccttccaccgcccgttaccgtccctaggaaattttactctgctggagagcttcttctacggccc ccttgcagcaatgctcttcccagcattacgttgcgggtaaaacggaggtcgtgtacccgacctagcagcccagggatggaaaag tcccggccgtcgctggcaataatagcgggcggacgcatgtcatgagattattggaaaccaccagaatcgaatataaaaggcga

	acacctttcccaattttggtttctcctgacccaaagactttaaatttaatttatttgtccctatttcaatcaattgaacaactatcaa aacacaatgcaattcaattgggacatcaagacagttgcatccatccttttctgccttgactcttgcacaagcttctgaccaagaag ctattgctccagaagactctcacgttgtgaagttgactgaggctactttcgagtccttcatcacctctaaccctcatgtattggcag agtttttcgcaccttggtgtggtcactgcaagaagttaggtcctgagttggtatctgctgctgagattttgaaggacaatgagcaa gtcaagattgcccagattgactgcactgaggaaaaggagttgtgtcaaggttacgagattaagggttaccctaccttgaaggtg tttcacggtgaagttgaggttccatctgactaccaaggacaaagacagtcccaatccatcgtttcctacatgctgaagcaatctc ttccacctgtatccgagatcaacgcaactaaggacttggatgacaccattgcagaggctaaagagccagttatcgtccaagtcc ttccagaagatgcatccaacttggagtccaatacgacctttacggagttgcaggtacattgcgtgagaagttcacctttgtctct accaagtctactgactacgctaagaagtacacctctgactctactccagcttacctgttggttagacctggagaagagccatccg tctactctggtgaggaattggatgagactcaccttgttcactggatagacatcgagtccaaacctctgtttggagacattgatggt tccaccttcaagtcttatgctgaggctaacattcctcttgcctactatttctacgagaacgaagagcaaagagcagcagctgctg acatcattaagccatttgctaaggagcaaagaggcaagattaacttcgttggtttggatgcagtcaagttcggcaaacatgcca agaaccttaacatggatgaggagaagttgccactgttcgttatccatgacttggtttccaacaagaaattcggtgttcctcaaga ccaagaactgaccaacaaggacgttacagagctgattgagaagttcattgcaggtgaagcagaacccattgtcaagtctgagc ctattccagagatacaagaggagaaggtcttcaagttggtaggtaaagctcacgatgaagtcgttttcgacgaatccaaggac gttttggtcaagtactatgctccttggtgtggtcactgtaagagaatggcaccagcttacgaagagcttgctactttgtacgccaa tgatgaggatgcatcttccaaggtggttattgccaagcttgatcacactcttaacgacgttgataacgtggacatacagggatac ccaaccttgatcctttatcctgctggtgacaagtctaacccacagttatacgacggttccagagaccttgagtctttggctgagttt gtcaaggaaagaggtactcacaaggttgacgctttagctttgagacctgttgaagaggagaaagaagctgaggaagaagctg agtctgaagctgatgctcacgacgaattgtaagcggccgctcaagaggatgtcagaatgccatttgcctgagagatgcaggctt catttttgatacttttttatttgtaacctatatagtataggatttttttttgtcattttgtttcttctcgtacgagcttgctcctgatcagcc tatctcgcagcagatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcctcttc agagtacagaagattaagtgagaccttcgtttgtgcggatccttcagtaatgtcttgtttcttttgttgcagtggtgagccattttg acttcgtgaaagtttctttagaatagttgtttccagaggccaaacattccacccgtagtaaagtgcaagcgtaggaagaccaag actggcataaatcaggtataagtgtcgagcactggcaggtgatcttctgaaagtttctactagcagataagatccagtagtcatg catatggcaacaatgtaccgtgtggatctaagaacgcgtcctactaaccttcgcattcgttggtccagtttgttgttatcgatcaa cgtgacaaggttgtcgattccgcgtaagcatgcatacccaaggacgcctgttgcaattccaagtgagccagttccaacaatctttt gtaatattagagcacttcattgtgttgcgcttgaaagtaaaatgcgaacaaattaagagataatctcgaaaccgcgacttcaaa cgccaatatgatgtgcggcacacaataagcgttcatatccgctgggtgactttctcgctttaaaaaattatccgaaaaaatttttct agagtgttgttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatggctaaactcacctctgctgt tccagtcctgactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctcccgtgacttcgtagaggacga ctttgccggtgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgccagacaacactctggcatggg tatgggttcgtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtgatgcatctggtccagctatg accgagatcggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgtgcatttcgtcgcagaagag caggactaacaattgacaccttacgattatttagagagtatttattagttttattgtatgtatacggatgttttattatctatttatgc ccttatattctgtaactatccaaaagtcctatcttatcaagccagcaatctatgtccgcgaacgtcaactaaaaataagctttttat gctcttctctctttttttcccttcggtataattataccttgcatccacagattctcctgccaaattttgcataatcctttacaacatggc tatatgggagcacttagcgccctccaaaacccatattgcctacgcatgtataggtgtttttccacaatattttctctgtgctctctt

	tttattaaagagaagctctatatcggagaagcttctgtggccgttatattcggccttatcgtgggaccacattgcctgaattggttt gccccggaagattggggaaacttggatctgattaccttagctgcaggtaccactgagcgtcagaccccgtagaaaagatcaaa ggatcttcttgagatccttttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccg gatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagccgt agttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggc gataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttcgtgc acacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccg aagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaac gcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagccta tggaaaaacgccagcaacgcggccttttacggttcctggccttttgc
pPpT4-CalB+synPDI-pGAP+pCAT1-rev	tggccttttgctcacatgtatttaaattgccagtgtatgtgcacttatagaggatgtacaaatggtcagagtttccccaaagagca gatggaaaagcaattagattttcgttactgaatcaggcattcaaaaagaaacaataagtcgcaacaatgatgttaatctcgaat attgaagtaaatgcttccagcaataatgtgacagttgctcgaatctagaacggtattaacagaatagaccgagactctcctattt cagcgtcctaaactctatcttgggaccaaggaagaaagctacgcacttaagaataggaccatgcataccaaaacaatagacct gacctgatacgctatgcactgcaaatcgaagaaaaaagcaattcattcggaacaaaacgtcagagcgggttttctaatagaat ttgcgtaatatataaacctaaataccataattgaggtgtttcttttctctgataaaagtacaacgaagtgctcttgtcataccgca acaaagatgttgaacacttcgtatcagtttcccaaagatgttgcaagatacgacgtggcagatgctgtcaatgagcaacttcaa gctacctttttgaattcaaaggttcaacatgagcccggttcaagatatcaacaccaagagggaatcaactcatcaccagagaga aaaaattcagtcttttcatctgctgatatatctaccacatcgtcacctacttctccgaagtctaaagccaattctcaatttgctaga gactctgtcgacagttctatctacccgaggaaaccgatacataggagatctaatacagaggtatcatccaatgattccgaaaaa gatttcagtttggactccctttttaaagcacagtatcgggatacaaagctgaaaccctccccatttcaagcttctagtggtaggaa ttctgtaccggtttacagaaggacgactcttgatgcgccaaccacagtgacaatagacatagaggaaatgacaaaaggattat gcgaggatgctgctggagacgattcaaagtttagtttagaaaggtcctccatttatgctgatagaatactagatacccgtgaact ttgtctcaggagatccgcatcagacgaaggatgttccgacctgcaaataatcgaagaagagacccctaggccgtacgacagtc agttagtagatatttataccattctgcgagaaggtcccctgcagggcacaaacgaaggtctcacttaatcttctgtactctgaag aggagtgggaaataccaagaaaaacatcaaactcgaatgattttcccaaacccctaccacaagatattcatctgctgcgagat aggctgatcaggagcaagctcgtacgagaagaaacaaaatgacaaaaaaaatcctatactatataggttacaaataaaaaa gtatcaaaaatgaagcctgcatctctcaggcaaatggcattctgacatcctcttgagcggccgcttatggggtcacgataccgga acaagttctcttaccaacagcgaaaggtctagcatatggcatcaaatctggttcacagttttgcttaggaccagcaacaatagct gcggcagcaggagccaacaatgcagcggcagcgacctttgctctggagtaaggtcgtttgctggtaaagggttacagtcagtg ataccgtaatcagcagaacgagcttgaccagtggtggatctaagtgcggatctaccaacgacgtaagagaattgggaggtaag agaaccggcgtggtcaataacgaacaaaggaccacaaacagcttgtgcttggacgttctttccgttgaacaagtaagaggagt ccaatggggagttagacacttgaggctgaacgatctcgtcagtggcagagtacaggttagtggtagggacaatttgggtcaaa cctcctgcgtttctcaaggcggtagtcaaagcagaaccggtggtttgttgccacacagaaggagcggaaacagccaatgcgtcc aatggtccagcaagaacagttcccttgtagtcaggtgcgaaggccatcaatctgtcaaccttggaacggatagatgggaagaa ggtcaaaccccattgagcaaccaatccaccttgagaccaagtaaggactggcaacttgttgttaccagaaccagcgtaaagag cggtgattgcgttgaccatgtattcggtgttaacttgggtgtcgttcaacatgaatggaggtggggaaatccagcatggagtgta acccaactgagtagacagaggaatccagttggagtcgaaagattgtggtccagtggtaccagtacctggaaccaacaaaattg

	gcttagagacagaagatggagaagcaccttgacaggtcaaacctgcgtcaaggacggactttggctgggagaaagcaggatc agaaccagatggaagagcttcggcctctctcttctcgagagagacaccctcttccttagcagcaatggaagcgatagtggtgtt aatgaacaacaaaccgttgttagtggagttagagaaaggcaaaacagcgacgtcgaaatcaccctcaaggtcagagtaaccg ataactgcttcagctggaatttgagcagtctcgtcttcagtggtagtgttaacaggggcagccaatgcagaggaggcagcgaac aagacagcggtgaaaatagatgggaatctcattttaattgtaagtcttgactagagcaagtgttatggtgaatttcaagtaattg aagtgctgggtctacagcttatatatgattttggcctgatgagcaacagaggctatcaccccgctgggataatgaaacttaccga ttgcggcaggctaagcgaaaagtccaggccaatcgagggaagttaattgctcagctttcctgactaatcccaactttgaggggt gcatagtatggattcggtctcgtgcgagaagcttaaactttgttcaattccattagtagagggtatcatggaacttgcatgtgatg attacttcatatgatatcaggtactagcaatctcgattcgaatttattaggtaatgccgaccggaacaatagaaagacttcaaga ttgctaaaagaccaataagaacgtgtacagatgtcaggccatgagtttatttaagtgatctatgctacaattaaggttacaggag aaccgcattcggagttcgattacgtagaaaaaaatgtggtgaaacagtttcataagagttatatactttattatttatagttaaat atatggagaagactgtagttagatatcaaaatcataaatataaactgtgcgtatatttcagaaacttcgactcgttaaatctctat tcaattattattcctcatttattatatatatgattacacacttttttgtagaaatgtcttggtgtcctcgtccaatcaggtagccatct ctgaaatatctggctccgttgcaactccgaacgacctgctggcaacgtaaaattctccggggtaaaacttaaatgtggagtaat ggaaccagaaacgtctcttcccttctctctccttccaccgcccgttaccgtccctaggaaattttactctgctggagagcttcttcta cggccccccttgcagcaatgctcttcccagcattacgttgcgggtaaaacggaggtcgtgtacccgacctagcagcccagggatg gaaaagtcccggccgtcgctggcaataatagcgggcggacgcatgtcatgagattattggaaaccaccagaatcgaatataaa aggcgaacacctttcccaattttggtttctcctgacccaaagactttaaatttaatttatttgtccctatttcaatcaattgaacaac tatcaaaacacaatgcaattcaattgggacatcaagacagttgcatccatcctttctgccttgactcttgcacaagcttctgacca agaagctattgctccagaagactctcacgttgtgaagttgactgaggctactttcgagtccttcatcacctctaaccctcatgtatt ggcagagtttttcgcaccttggtgtggtcactgcaagaagttaggtcctgagttggtatctgctgctgagattttgaaggacaatg agcaagtcaagattgcccagattgactgcactgaggaaaaggagttgtgtcaaggttacgagattaagggttaccctaccttga aggtgtttcacggtgaagttgaggttccatctgactaccaaggacaaagacagtcccaatccatcgtttcctacatgctgaagca atctcttccacctgtatccgagatcaacgcaactaaggacttggatgacaccattgcagaggctaaagagccagttatcgtcca agtccttccagaagatgcatccaacttggagtccaatacgacctttacggagttgcaggtacattgcgtgagaagttcacctttg tctctaccaagtctactgactacgctaagaagtacacctctgactctactccagcttacctgttggttagacctggagaagagcc atccgtctactctggtgaggaattggatgagactcaccttgttcactggatagacatcgagtccaaacctctgtttggagacattg atggttccaccttcaagtcttatgctgaggctaacattcctcttgcctactatttctacgagaacgaagagcaaagagcagcagc tgctgacatcattaagccatttgctaaggagcaaagaggcaagattaacttcgttggtttggatgcagtcaagttcggcaaacat gccaagaaccttaacatggatgaggagaagttgccactgttcgttatccatgacttggtttccaacaagaaattcggtgttcctc aagaccaagaactgaccaacaaggacgttacagagctgattgagaagttcattgcaggtgaagcagaacccattgtcaagtct gagcctattccagagatacaagaggagaaggtcttcaagttggtaggtaaagctcacgatgaagtcgttttcgacgaatccaa ggacgttttggtcaagtactatgctccttggtgtggtcactgtaagagaatggcaccagcttacgaagagcttgctactttgtacg ccaatgatgaggatgcatcttccaaggtggttattgccaagcttgatcacactcttaacgacgttgataacgtggacatacaggg atacccaaccttgatcctttatcctgctggtgacaagtctaacccacagttatacgacggttccagagaccttgagtctttggctg agtttgtcaaggaaagaggtactcacaaggttgacgctttagctttgagacctgttgaagaggagaaagaagctgaggaagaa gctgagtctgaagctgatgctcacgacgaattgtaagcggccgctcaagaggatgtcagaatgccatttgcctgagagatgcag gcttcatttttgatacttttttatttgtaacctatatagtataggatttttttgtcattttgtttcttctcgtacgagcttgctcctgatc

	agcctatctcgcagcagatgaatatcttgtggtaggggtttgggaaaatcattcgagtttgatgtttttcttggtatttcccactcct cttcagagtacagaagattaagtgagaccttcgtttgtgcggatccttcagtaatgtcttgtttcttttgttgcagtggtgagccatt ttgacttcgtgaaagtttctttagaatagttgtttccagaggccaaacattccacccgtagtaaagtgcaagcgtaggaagacca agactggcataaatcaggtataagtgtcgagcactggcaggtgatcttctgaaagtttctactagcagataagatccagtagtc atgcatatggcaacaatgtaccgtgtggatctaagaacgcgtcctactaaccttcgcattcgttggtccagtttgttgttatcgatc aacgtgacaaggttgtcgattccgcgtaagcatgcatacccaaggacgcctgttgcaattccaagtgagccagttccaacaatc tttgtaatattagagcacttcattgtgttgcgcttgaaagtaaaatgcgaacaaattaagagataatctcgaaaccgcgacttca aacgccaatatgatgtgcggcacacaataagcgttcatatccgctgggtgactttctcgctttaaaaaattatccgaaaaaattt tctagagtgttgttactttatacttccggctcgtataatacgacaaggtgtaaggaggactaaaccatggctaaactcacctctgc tgttccagtcctgactgctcgtgatgttgctggtgctgttgagttctggactgataggctcggtttctcccgtgacttcgtagagga cgactttgccggtgttgtacgtgacgacgttaccctgttcatctccgcagttcaggaccaggttgtgccagacaacactctggcat gggtatgggttcgtggtctggacgaactgtacgctgagtggtctgaggtcgtgtctaccaacttccgtgatgcatctggtccagct atgaccgagatcggtgaacagccctggggtcgtgagtttgcactgcgtgatccagctggtaactgcgtgcatttcgtcgcagaa gagcaggactaacaattgacaccttacgattatttagagagtatttattagttttattgtatgtatacggatgttttattatctattt atgcccttatattctgtaactatccaaaagtcctatcttatcaagccagcaatctatgtccgcgaacgtcaactaaaaataagctt tttatgctcttctctctttttttcccttcggtataattataccttgcatccacagattctcctgccaaattttgcataatcctttacaac atggctatatgggagcacttagcgccctccaaaacccatattgcctacgcatgtataggtgtttttttccacaatattttctctgtgc tctctttttattaaagagaagctctatatcggagaagcttctgtggccgttatattcggccttatcgtgggaccacattgcctgaat tggtttgccccggaagattggggaaacttggatctgattaccttagctgcaggtaccactgagcgtcagaccccgtagaaaaga tcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtt tgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgta gccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgcca gtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggt tcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgct tcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggg gaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcgga gcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttgc

7.2. Vector maps

Sequences of the pictured vectors are listed in Table 29.

7.2.1.1. Genomic Situation of *TKL1*

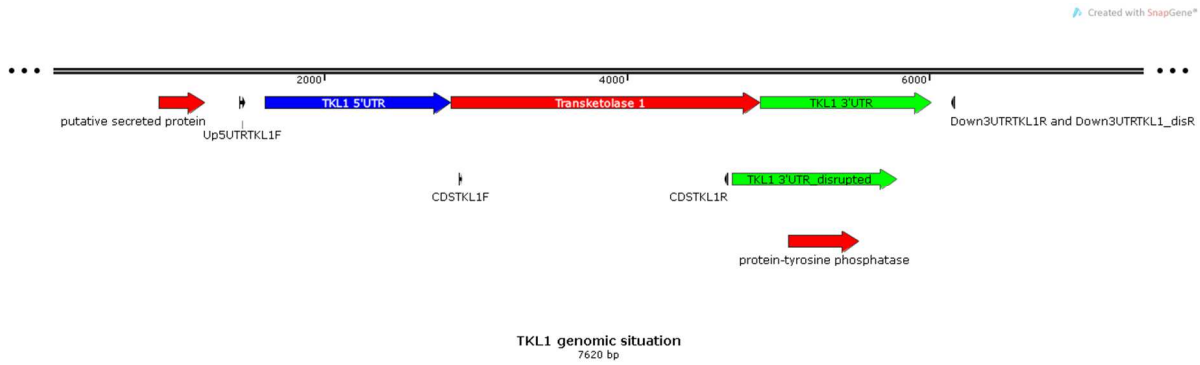


Figure 49 Genomic situation of the transketolase 1 (*TKL1*) gene. *TKL1* is located on chromosome 1 of *P. pastoris* CBS 7435. 5' of the gene a putative secreted protein and 3' a tyrosine phosphatase are coded. The tyrosine phosphatase gene lies within the 3'UTR HRS.

7.2.2. Knock-out vectors

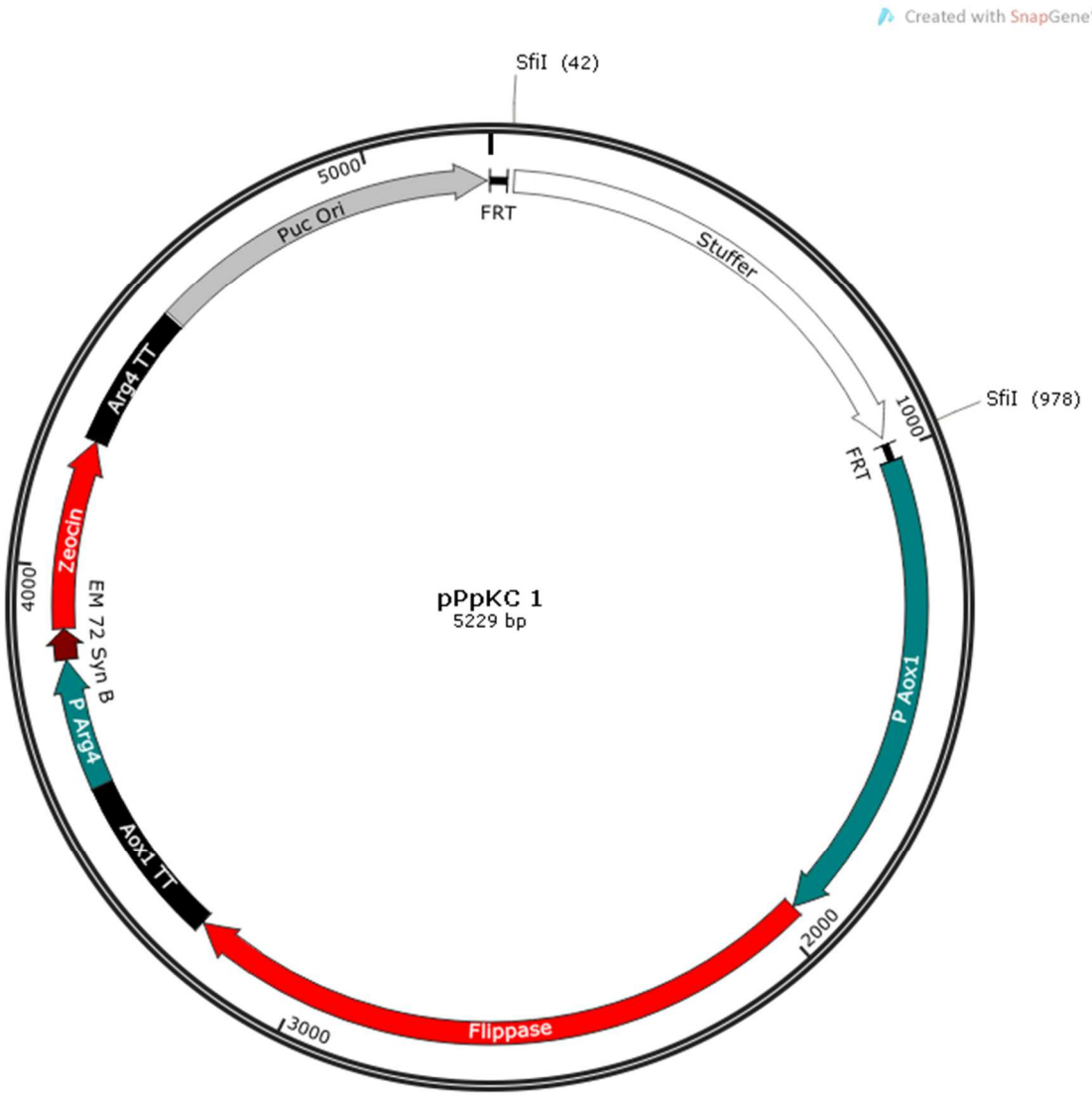


Figure 50 Plasmid map of pPpKC1
pPpKC1 vector harbours a stuffer fragment flanked by two *SfiI* sites for cloning homologous recombination sites (HRS) for integration in the desired region of the *P. pastoris* genome. Further 2 FRT regions are besides the *SfiI* cutting sites. The FRT sites are recognized by the flippase from *S. cerevisae* under the control of *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT respectively) for excision of the marker after successful deleting of the GOI. As selection marker Zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used for expression in *P. pastoris*. The synthetic promoter EM72SynB was used for expression of Zeocin-resistance-protein expression. The plasmid also harbored a pUCori for replication in *E.coli*.

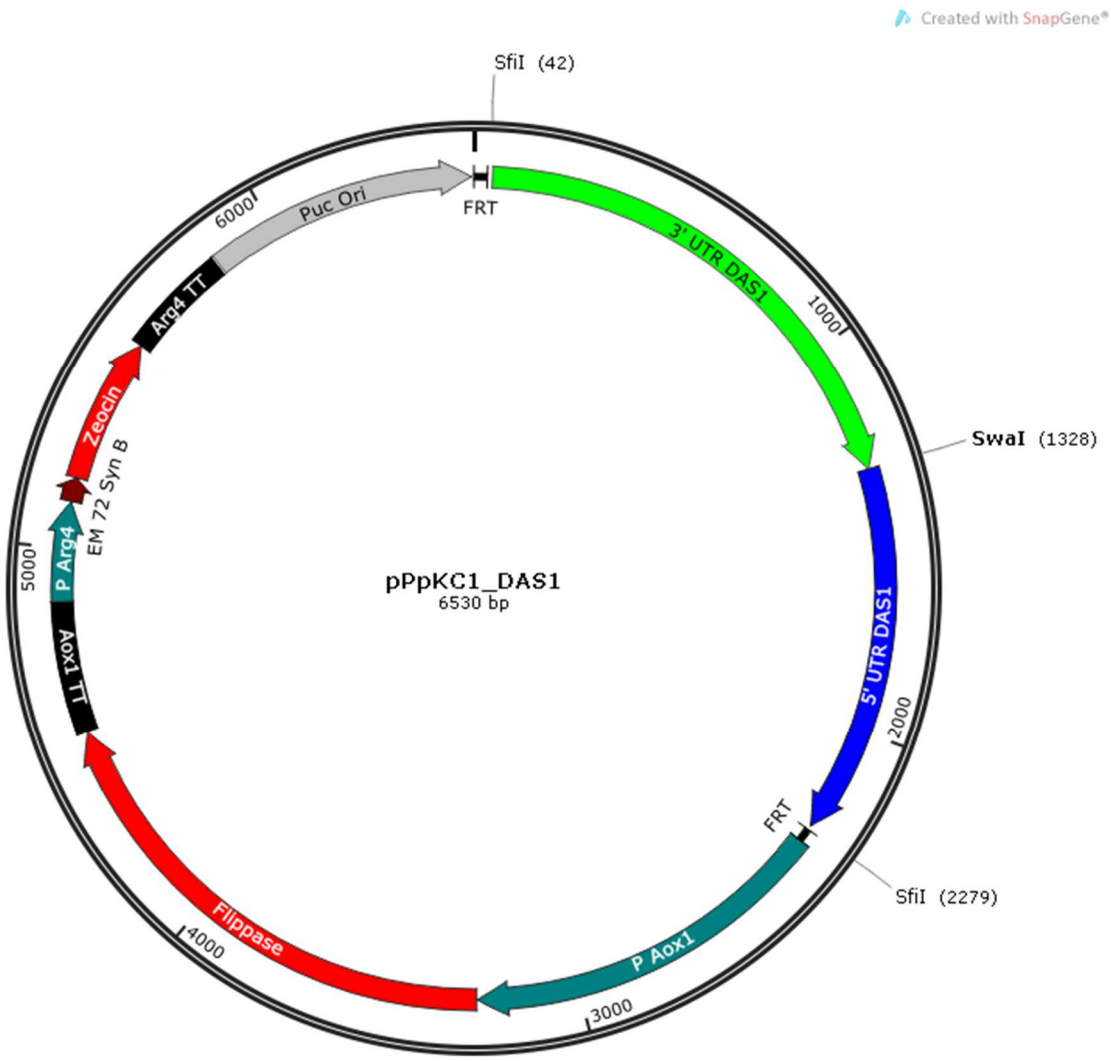


Figure 51 Plasmid map of pPpKC1_DAS1
pPpKC1_DAS2 vector harbours 2 homologous recombination sites located at the 5' UTR and 3'UTR of *DAS1* in the *P. pastoris* genome. In between these 2 regions a *SwaI*/*SmiI* cutting site has been introduced via constructing, which required for later linearization of the vector. The 2 *SfiI* sites for cloning flank the 2 HRS as well as 2 FRT sites. The FRT sites are recognized by the flippase from *S. cerevisae* under the control of *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT respectively) for excision of the marker after successful deleting of the GOI. As selection marker zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used for expression in *P. pastoris*. The synthetic promoter EM72SynB was used for expression of Zeocin-resistance-protein expression. The plasmid also harbored a pUCori for replication in *E.coli*.

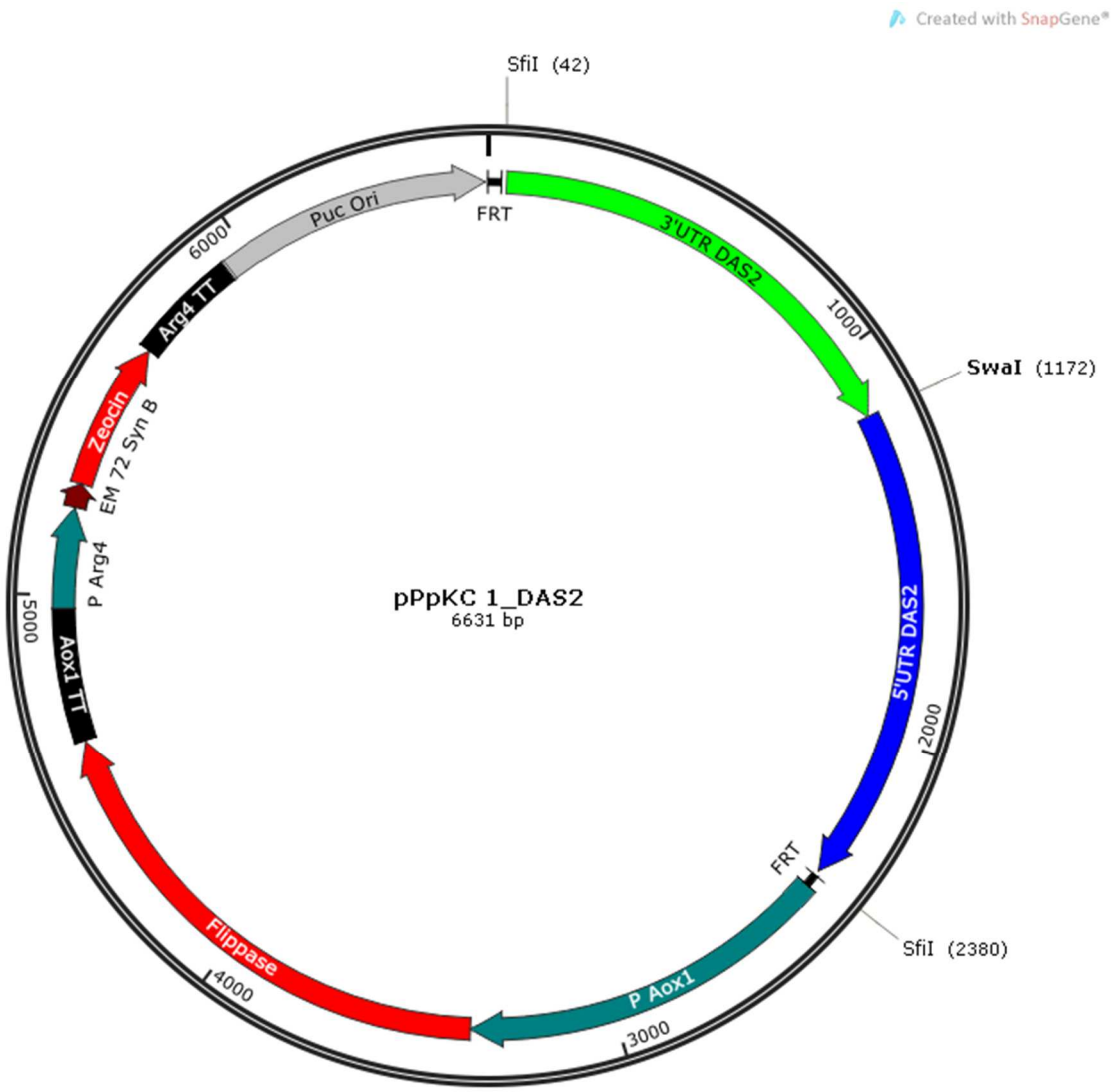


Figure 52 Plasmid map of pPpKC1_DAS2
pPpKC1_DAS1 vector harbours 2 homologous recombination sites located at the 5' UTR and 3'UTR of *DAS2* in the *P. pastoris* genome. In between these 2 regions a *SwaI*/*SmiI* cutting site has been introduced via constructing, which required for later linearization of the vector. The 2 *SfiI* sites for cloning flank the 2 HRS as well as 2 FRT sites. The FRT sites are recognized by the flippase from *S. cerevisae* under the control of *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT respectively) for excision of the marker after successful deleting of the GOI. As selection marker zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used for expression in *P. pastoris*. The synthetic promoter EM72SynB was used for expression of Zeocin-resistance-protein expression. The plasmid also harbored a pUCori for replication in *E.coli*.

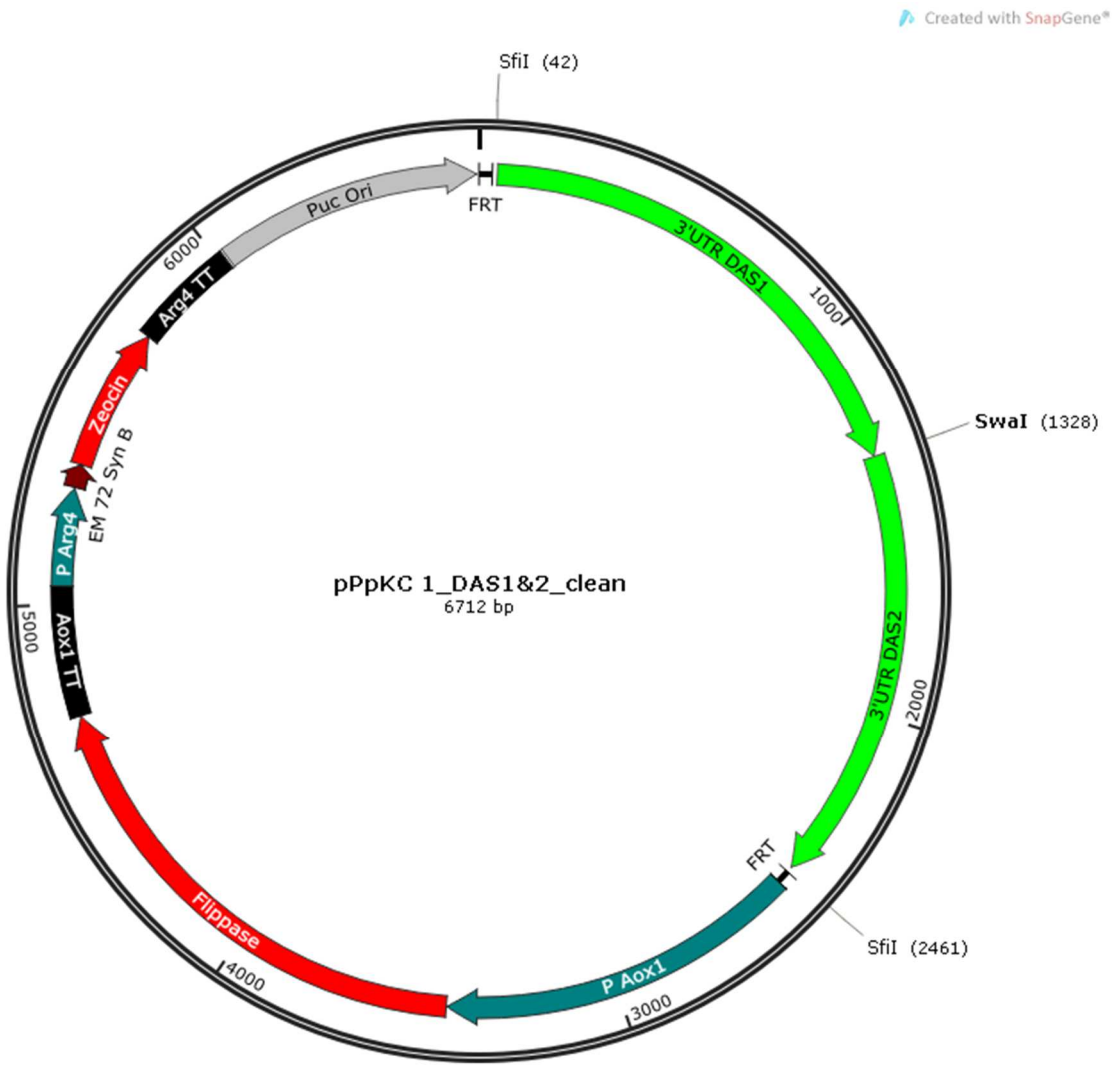


Figure 53 Plasmid map of pPpKC1_DAS1&2_clean
pPpKC1_DAS1&2_clean vector harbors 2 homologous recombination sites located at the 3' UTR and 3'UTR of *DAS1* and *DAS2* respectively in the *P. pastoris* genome. The 3'UTR *DAS2* in this vector corresponds to the 5'UTR in the other vectors i.e. pPpKC1_DAS1, because *DAS2* gene is coded on the complement strand in the genome pictured in Figure 8. In between these 2 regions a *SwaI*/*SmiI* cutting site has been introduced via constructing, which required for later linearization of the vector. The 2 *SfiI* sites for cloning flank the 2 HRS as well as 2 FRT sites. The FRT sites are recognized by the flippase from *S. cerevisae* under the control of *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT respectively) for excision of the marker after successful deleting of the GOI. As selection marker zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used for expression in *P. pastoris*. The synthetic promoter EM72SynB was used for expression of Zeocin-resistance-protein expression. The plasmid also harbored a pUCori for replication in *E.coli*.

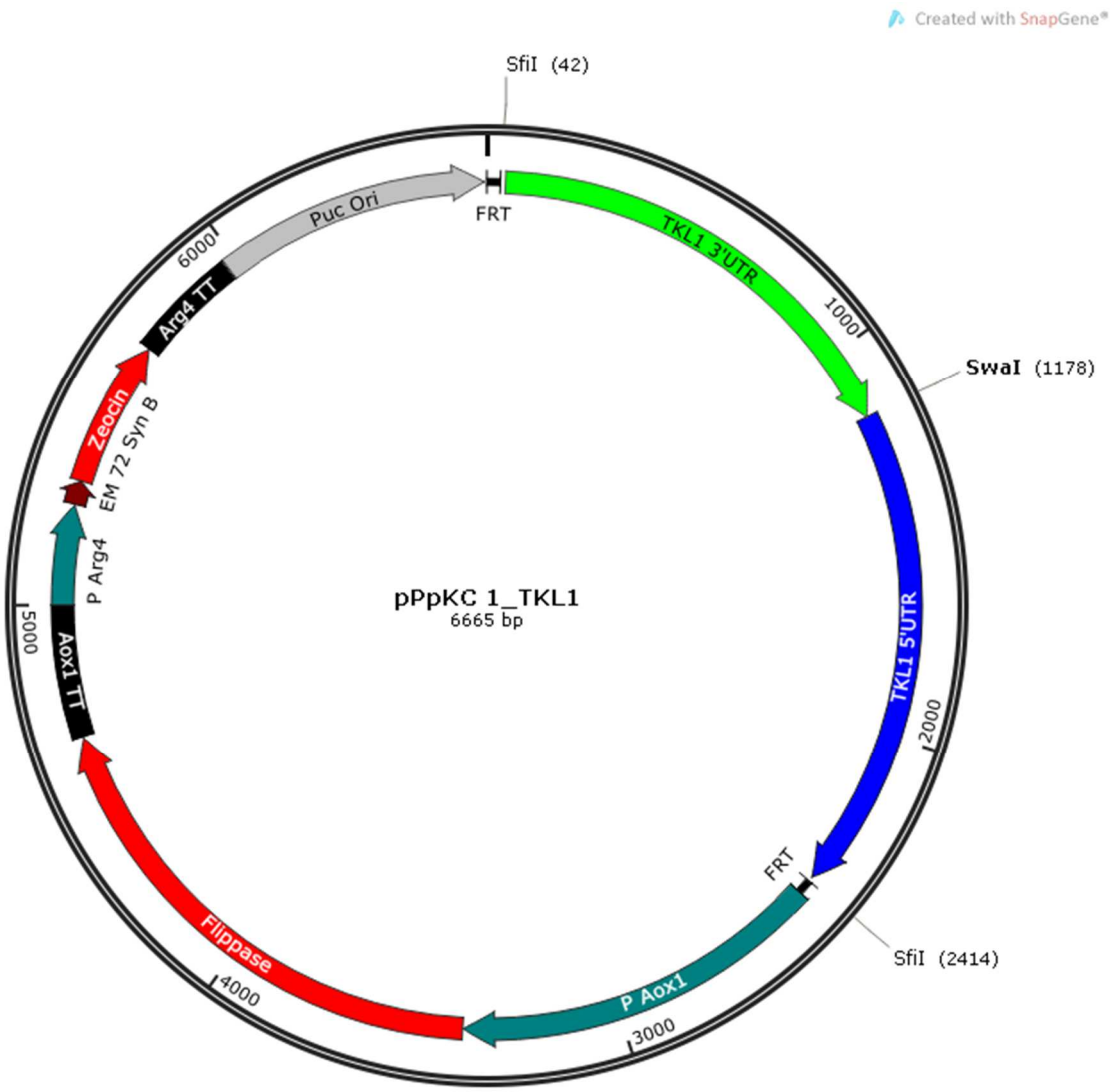


Figure 54 Plasmid map of pPpKC1_TKL1
pPpKC1_TKL1 vector harbours 2 homologous recombination sites located at the 5' UTR and 3'UTR of *TKL1* in the *P. pastoris* genome. In between these 2 regions a *SwaI*/*SmiI* cutting site has been introduced via constructing, which required for later linearization of the vector. The 2 *SfiI* sites for cloning flank the 2 HRS as well as 2 FRT sites. The FRT sites are recognized by the flippase from *S. cerevisae* under the control of *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT respectively) for excision of the marker after successful deleting of the GOI. As selection marker zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used for expression in *P. pastoris*. The synthetic promoter EM72SynB was used for expression of Zeocin-resistance-protein expression. The plasmid also harbored a pUCori for replication in *E.coli*.

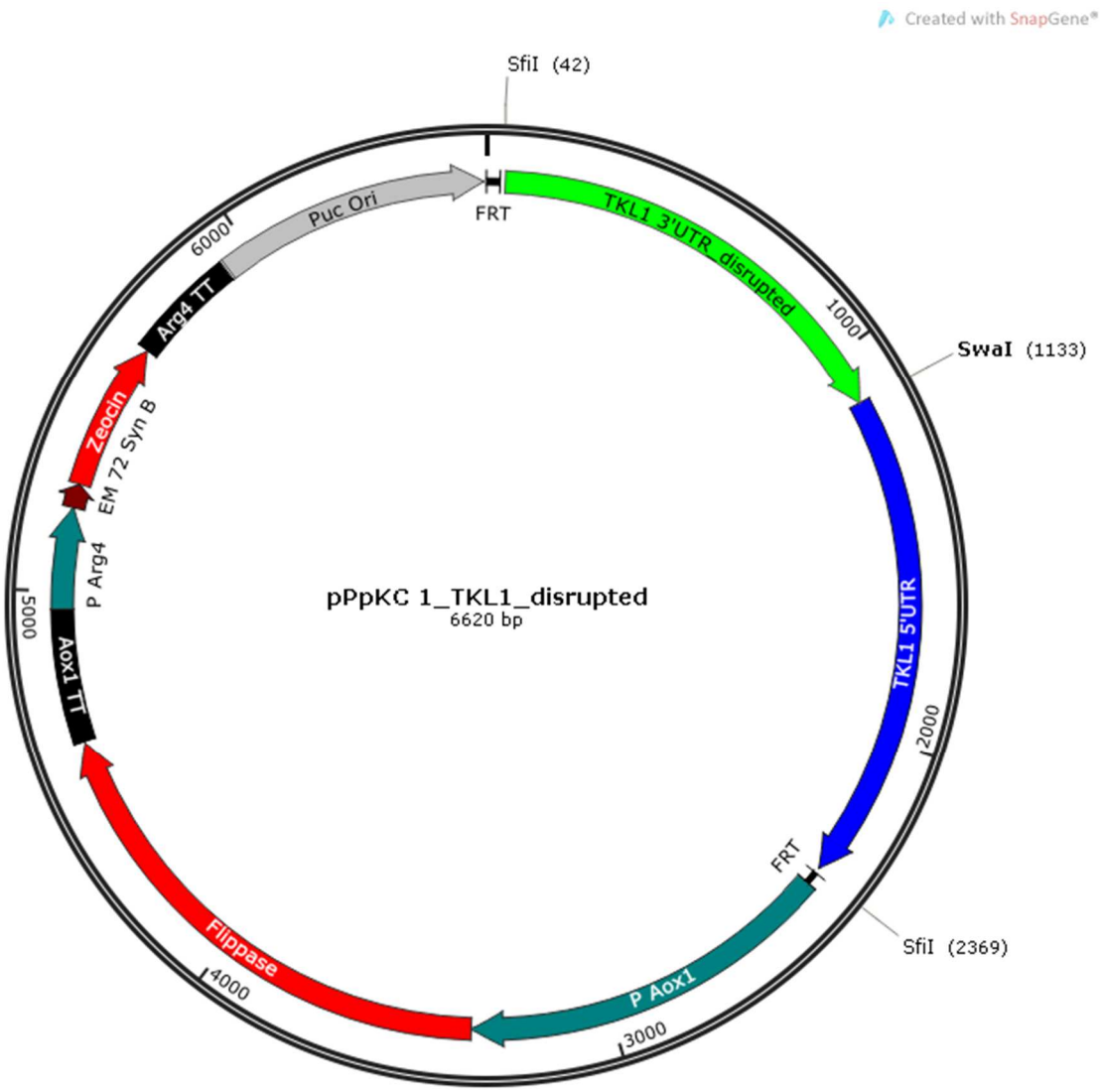


Figure 55 Plasmid map of pPpKC1_TKL1_disrupted (pPpKC1_TKL1_dis)
pPpKC1_TKL1_dis vector harbours 2 homologous recombination sites located at the 5' UTR and 3'UTR (and a small part of the *TKL1* gene as well) of *TKL1* in the *P. pastoris* genome. In between these 2 regions a *SwaI*/*SmiI* cutting site has been introduced via constructing, which required for later linearization of the vector. The 2 *SfiI* sites for cloning flank the 2 HRS as well as 2 FRT sites. The FRT sites are recognized by the flippase from *S. cerevisae* under the control of *AOX1* promoter and terminator (P_{AOX1} and AOX1 TT respectively) for excision of the marker after successful deleting of the GOI. As selection marker zeocin-resistance protein (JQ519690.1) with *ARG4* promoter and terminator (P_{ARG4} and ARG4 TT) was used for expression in *P. pastoris*. The synthetic promoter EM72SynB was used for expression of Zeocin-resistance-protein expression. The plasmid also harbored a pUCori for replication in *E.coli*.

7.2.3. Vectors for model protein expression

7.2.3.1. eGFP vectors

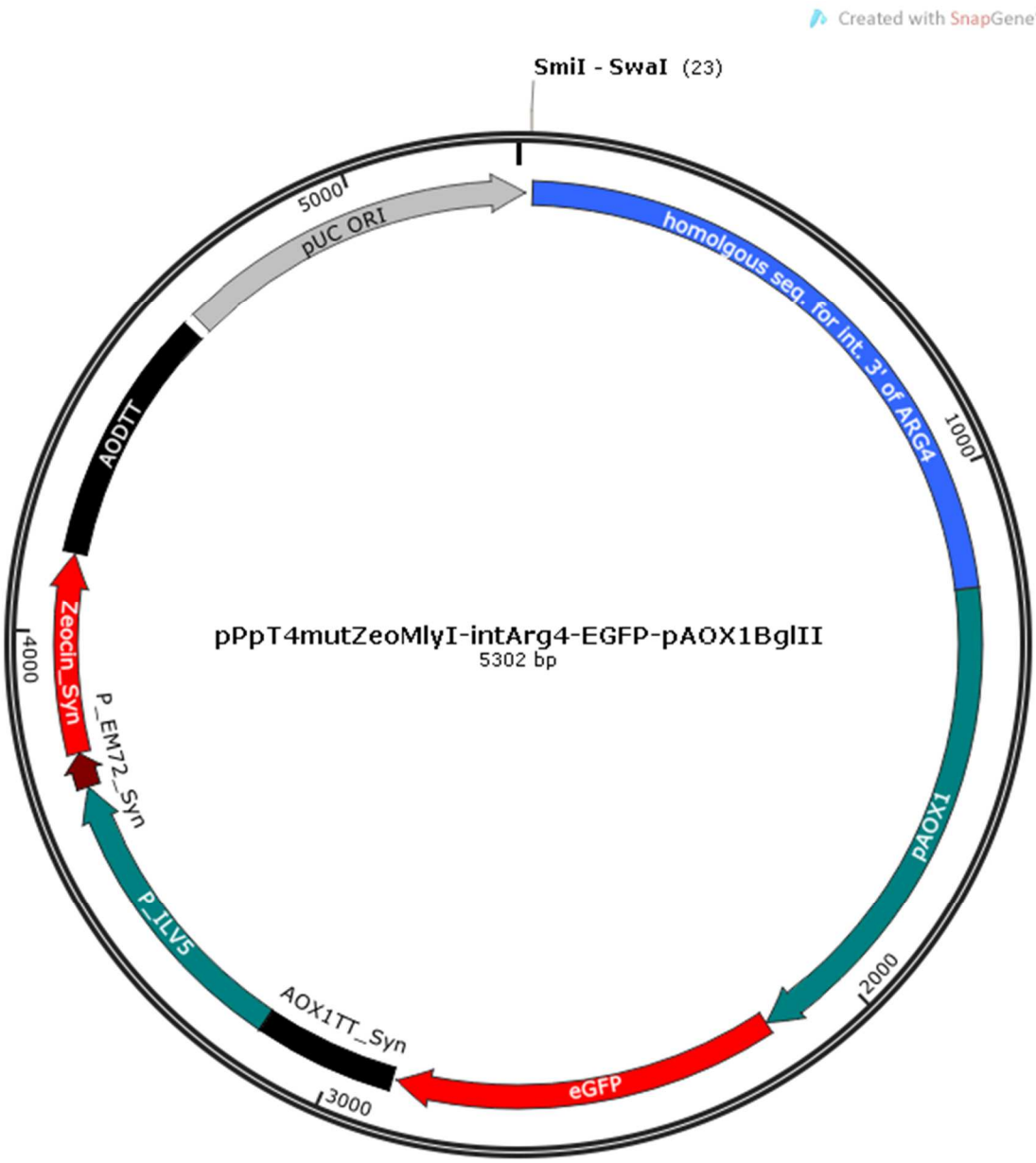


Figure 56 Vector map of the eGFP expression vector pPpT4mutZeoMlyI-intArg4-EGFP-pAOX1BglII.
The vector is based on a pPpT4 vector with a homologous site for integration at 3' of *ARG4* in *P. pastoris* and the pUCori for replication in *E. coli*. Zeocin-resistance-protein under the control of the P_{ILV5} and AODTT for expression in *P. pastoris* and pEM72 for expression in *E. coli.* The expression of the model protein eGFP (enhanced green fluorescent protein) is under the control of P_{AOX1} (*AOX1* promoter from *P. pastoris*) and the terminator of *AOX1* (AOX1TT).

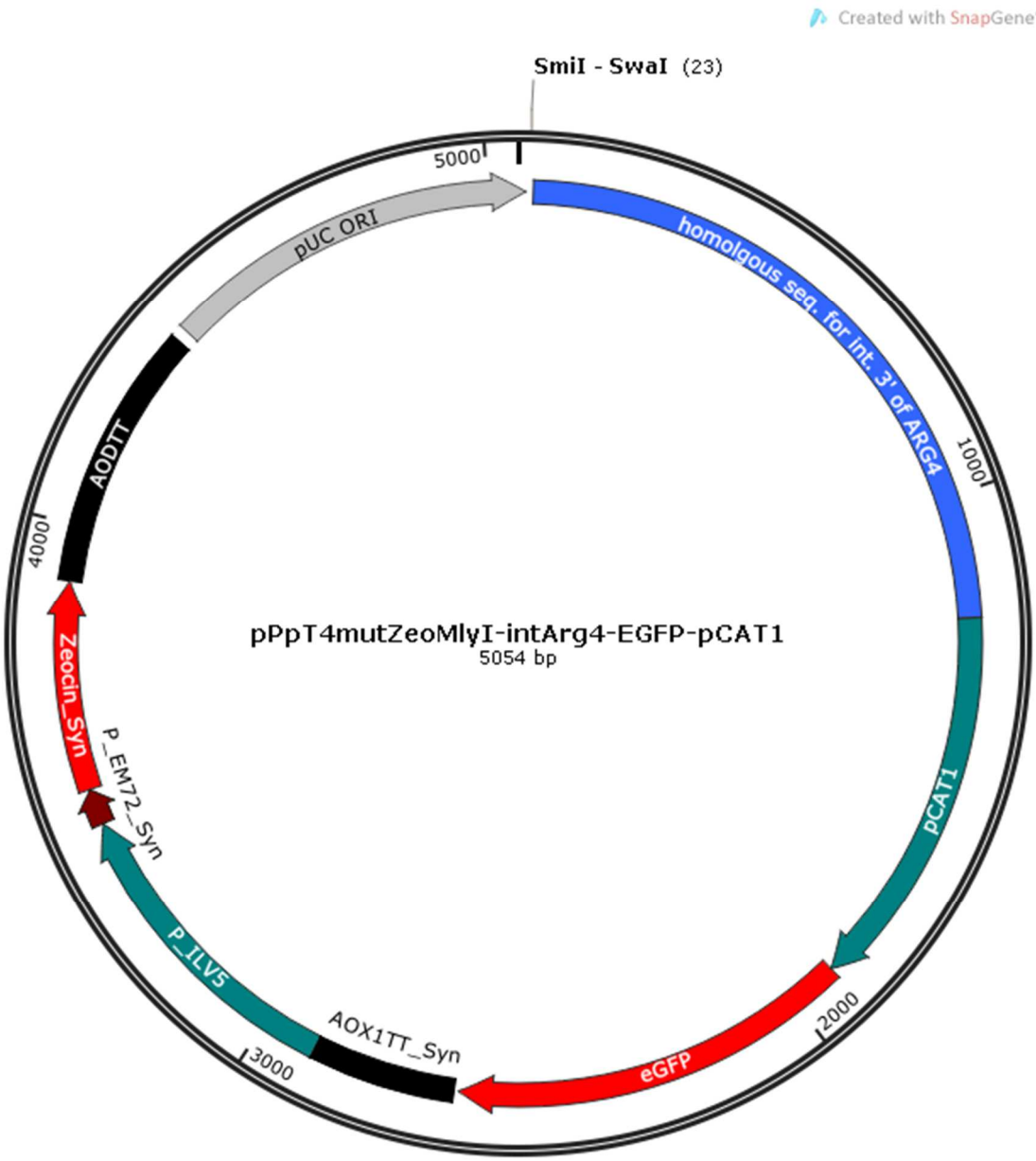


Figure 57 Vector map of the eGFP expression vector pPpT4mutZeoMlyI-intArg4-EGFP-pCAT1.
The vector is based on a pPpT4 vector with a homologous site for integration at 3' of *ARG4* in *P. pastoris* and the pUCori for replication in *E. coli*. Zeocin-resistance-protein under the control of the P_{ILV5} and AODTT for expression in *P. pastoris* and pEM72 for expression in *E. coli.* The expression of the model protein eGFP (enhanced green fluorescent protein) is under the control of P_{CAT1} (catalase promoter from *P. pastoris*) and the terminator of *AOX1* (AOX1TT).

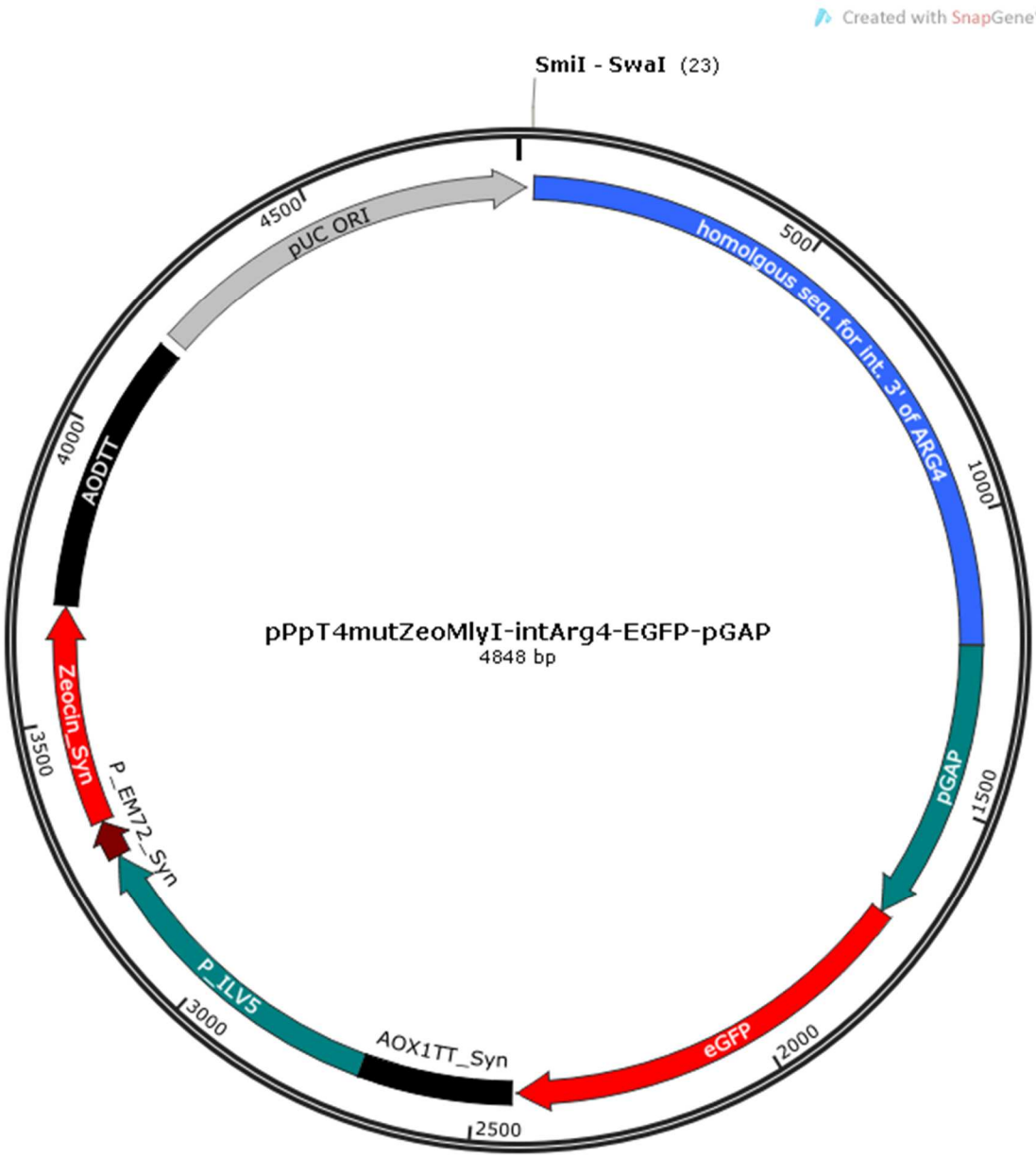


Figure 58 Vector map of the eGFP expression vector pPpT4mutZeoMlyI-intArg4-EGFP-pGAP.
The vector is based on a pPpT4 vector with a homologous site for integration at 3' of *ARG4* in *P. pastoris* and the pUCori for replication in *E. coli*. Zeocin-resistance-protein under the control of the P_{ILV5} and AODTT for expression in *P. pastoris* and pEM72 for expression in *E. coli.* The expression of the model protein eGFP (enhanced green fluorescent protein) is under the control of P_{GAP} (*GAP* promoter from *P. pastoris*) and the terminator of *AOX1* (AOX1TT).

7.2.3.2. BDH1 Vectors

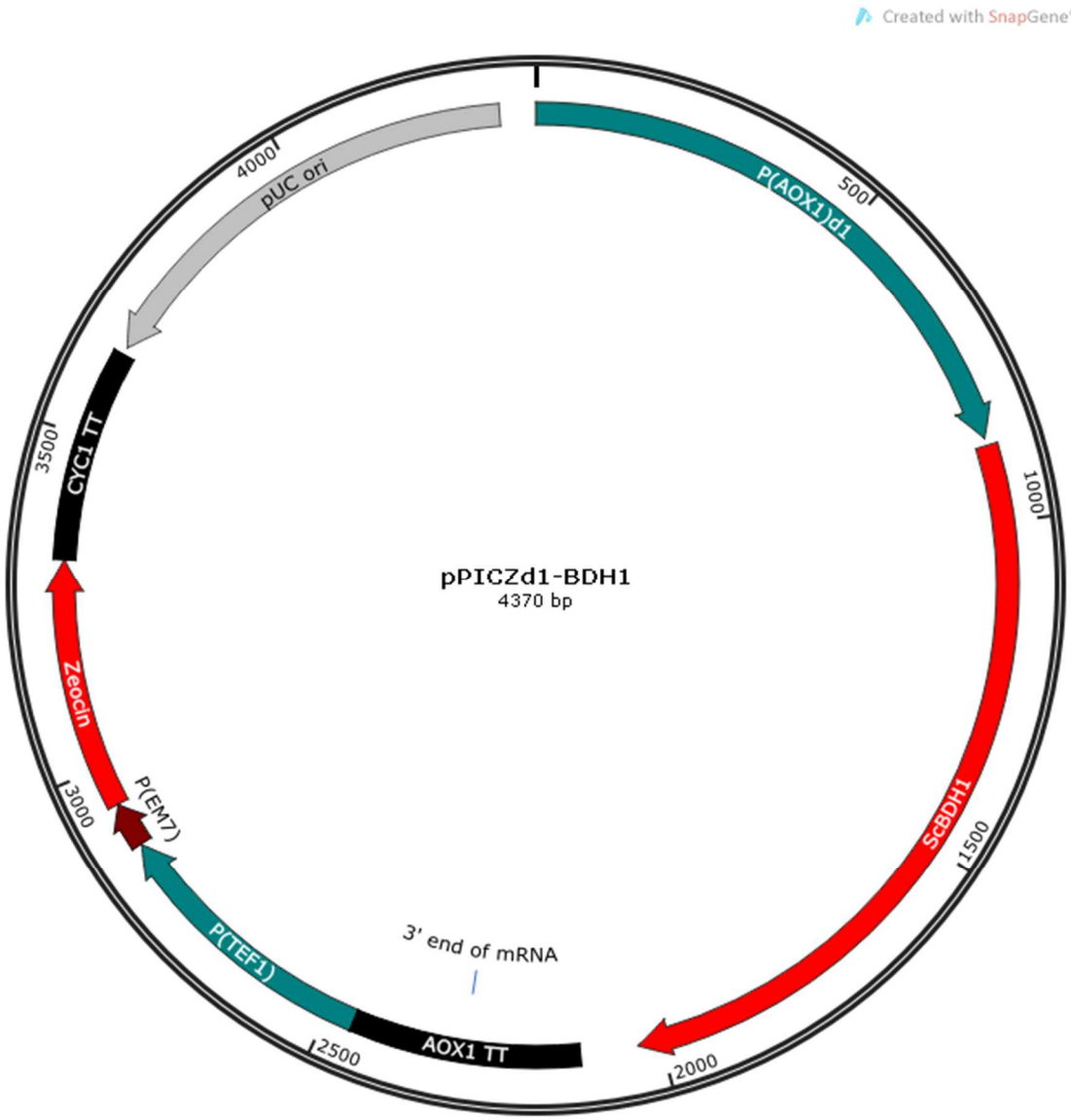


Figure 59 Vector map of pPICZd1-BDH1 (which was used as template for *BDH1* gene from *S. cerevisiae*).
The pPICZd1 vector has a homologous site for integration at P_{AOX1} in *P. pastoris* and the pUCori for replication in *E. coli*. Zeocin-resistance-protein under the control of the P_{TEF1} and CYC1TT for expression in *P. pastoris* and pEM7 for expression in *E. coli.* The expression of *BDH1* from *S. cerevisiae* (butanediol dehydrogenase) is under the control of the constitutive P_{AOX1} (from *P. pastoris*) and the terminator of *AOX1* (AOX1TT)..

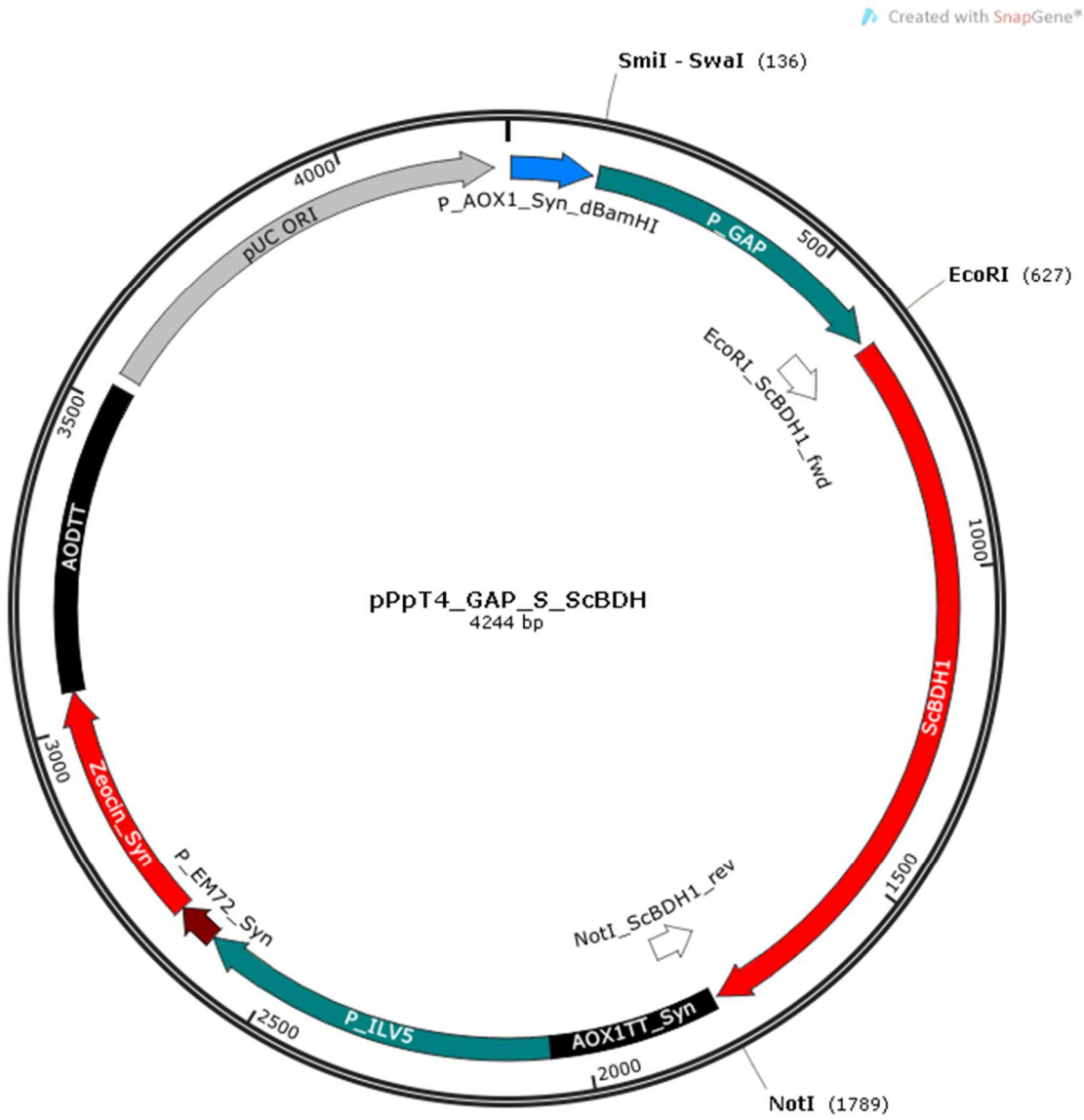


Figure 60 Vector map of pPpT4_GAP_S_ScBDH1.
The pPpT4 vector has a homologous site for integration at P_{AOX1} in *P. pastoris* and the pUCori for replication in *E. coli*. Zeocin-resistance-protein under the control of the P_{ILV5} and AODTT for expression in *P. pastoris* and pEM72 for expression in *E. coli.* The expression of *BDH1* from *S. cerevisiae* (butanediol dehydrogenase) is under the control of the constitutive P_{GAP} (from *P. pastoris*) and the terminator of *AOX1* (AOX1TT). NotI_ScBDH1_rev and EcoRI_ScBDH1_fwd indicate the primers used to amplify *BDH1* gene from pPICZBd1-BDH1.

7.2.3.3. CalB vectors

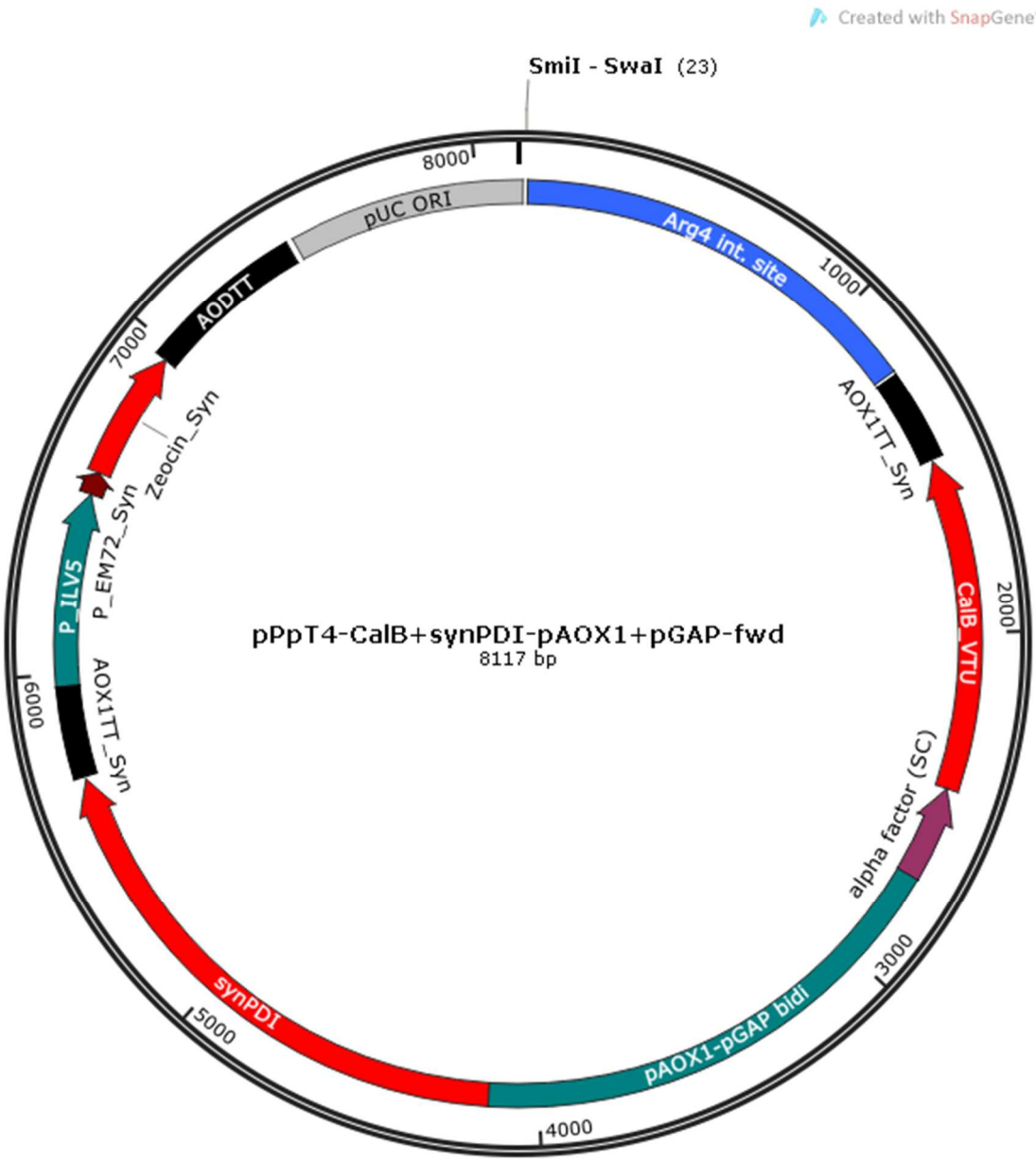


Figure 61 Vector Map pPpT4-CalB+synPDI-pAOX1+pGAP-fwd
The vector is based on the pPpT4 with a pUCori for replication in *E. coli*, the zeocin-resistance-protein under the control of P_{ILV5} and AODTT for expression in *P. pastoris* and under the control of the pEM72 for expression in *E. coli* respectively. For integration in *P. pastoris* a homologous region of *ARG4* gene is available and a *SwaI*/*SmiI* site for linearizing the vector. Two further proteins namely CalB_VTU with the α-factor from *S. cerevisae* for secretion (*C. Antarctica* lipase B; LC026014.1) and syn_PDI (protein disulphide isomerase from *P. pastoris*; CAC33587.1) under the control of a bidirectional promoter P_{AOX1}-P_{GAP} (P_{AOX1} for CalB and P_{GAP} for synPDI) and both terminated by an AOX1TT.

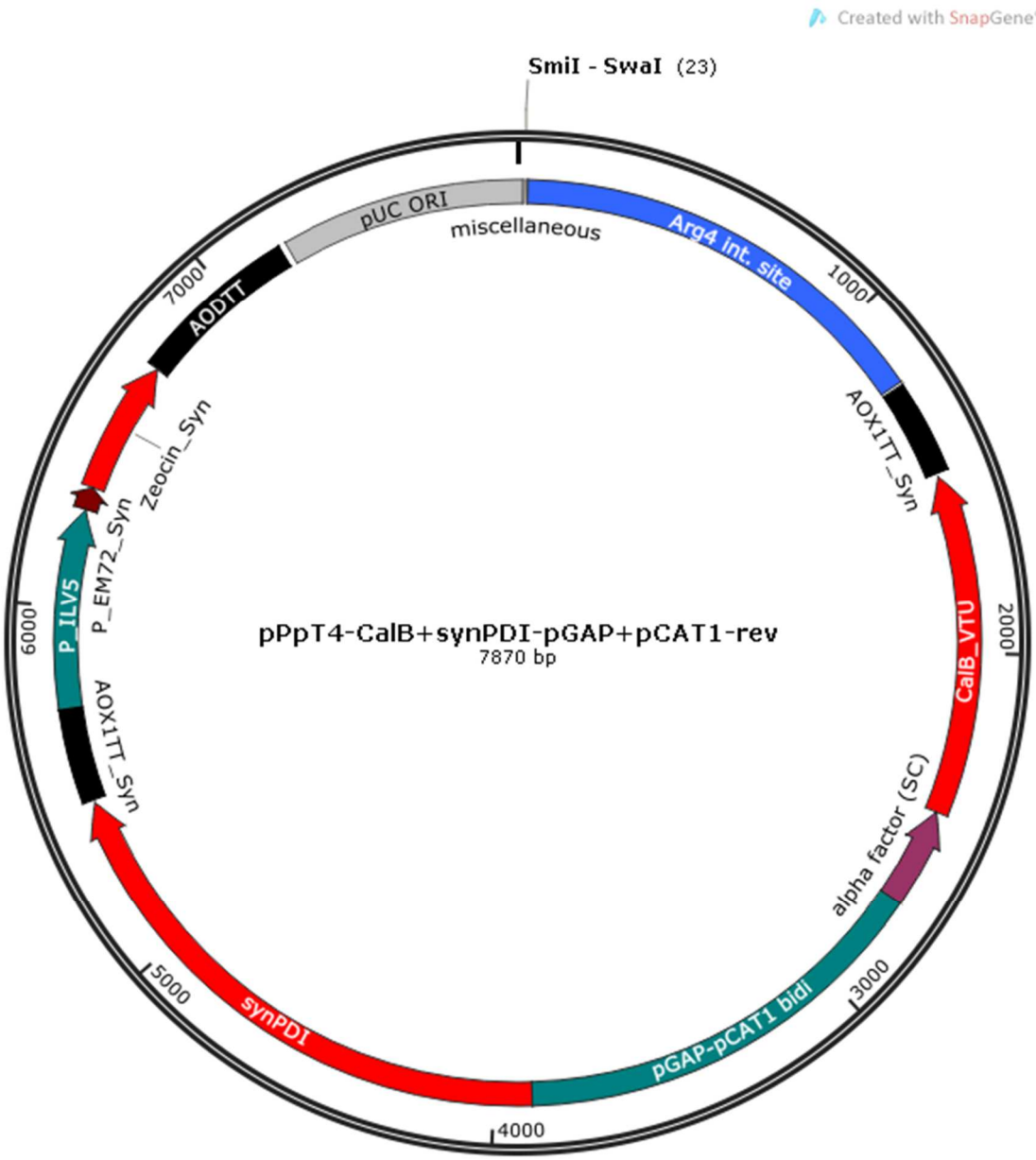


Figure 62 Vector map of pPpT4-CalB+synPDI-pGAP+pCAT1-rev
The vector is based on the pPpT4 with a pUCori for replication in *E. coli*, the zeocin-resistance-protein under the control of P_{ILV5} and AODTT for expression in *P. pastoris* and under the control of the pEM72 for expression in *E. coli* respectively. For integration in *P. pastoris* a homologous region of *ARG4* gene is available and a *SwaI*/*SmiI* site for linearizing the vector. Two further proteins namely CalB_VTU with the α-factor from *S. cerevisae* for secretion (*C. Antarctica* lipase B; LC026014.1) and syn_PDI (protein disulphide isomerase from *P. pastoris*; CAC33587.1) under the control of a bidirectional promoter P_{GAP}-P_{CAT1} (P_{CAT1} for CalB and P_{GAP} for synPDI) and both terminated by an AOX1TT.

7.3.Generated Strains

Table 30 Strains stored at the strain collection of University of Technology, Institute of Molecular Biotechnology.

Hosts train	Host strain: designation	Plasmid	Collection No.
E. coli	K12 Top10F’	pPpKC1-DAS1-verkürzte 5’UTR	7120
E. coli	K12 Top10F’	pPpKC1-DAS2	7121
E. coli	K12 Top10F’	pPpKC1-DAS1/2-clean	7122
E. coli	K12 Top10F’	pPpKC1-TKL1	7123
E. coli	K12 Top10F’	pPpKC1-TKL1-dis	7124
E. coli	K12 Top10F’	pPpT4-GAP-S-ScBDH1	7125
E. coli	K12 Top10F’	pPpT4-GAP-S-CpXYL1opt	7126
E. coli	K12 Top10F’	pPpT4-S-pCAT1-MAO-DS-DNA-2.0	7127
E. coli	K12 Top10F’	pPpKC1-DAS2	7128
P. pastoris	CBS 7435	pPpKC1-DAS1-verkürzte 5’UTR int.	7060
P. pastoris	CBS 7435	pPpKC1-DAS2 int.	7061
P. pastoris	CBS 7435	pPpKC1-DAS1/2-clean int.	7072
P. pastoris	CBS 7435 Δdas1		7073
P. pastoris	CBS 7435 Δdas2		7074
P. pastoris	CBS 7435 Δdas1Δdas2		7072
P. pastoris	CBS 7435	pPpT4_GAP_S_SCBDH1, C7	7181
P. pastoris	CBS 7435 Δdas1Δdas2	pPpT4_GAP_S_SCBDH1, F3	7182