universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Expression of a Putative Pyranose Dehydrogenase from *Coprinopsis cinerea* in *Pichia pastoris*"

Verfasser

Kristoffer Kurt Wollinger

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie UniStG
Betreuerin / Betreuer:	Dr. Angela Witte

1 CURRICULUM VITAE

Europass Curriculum Vitae

Personal information

First name(s) / Surname	**Kristoffer Kurt WOLLINGER**
Address	85/360/C773 Gymnasiumstraße, 1190, Vienna, Austria
Telephone	Mobile: +43 6506678974
E-mail	k.kurt.wollinger@gmail.com
Nationality	Austrian
Date of birth	22. 10. 1982
Gender	male

Education and training

Dates	March 2006 onwards
Title of qualification awarded	Bakkalaureus der technischen Wissenschaften (Food- and Biotechnology)
Principal subjects/occupational skills covered	In particular: Chemistry, Biology and Process Technology
Name and type of organisation providing education and training	Universität für Bodenkultur, 33, Gregor Mendel-Straße, 1180, Vienna, Austria
Level in national or international classification	ISCED 5
Dates	October 2002 onwards
Title of qualification awarded	Magister rerum naturalium (Molecularbiology)
Principal subjects/occupational skills covered	Emphasis: Biochemistry, Microbiology and Bioorganic Chemistry
Name and type of organisation providing education and training	Universität Wien, 1, Dr. Karl Lueger Ring, 1010, Vienna, Austria
Level in national or international classification	ISCED 5
Dates	June 2001
Title of qualification awarded	Matura
Principal subjects/occupational skills covered	- German, English, Mathematics, Biology, Physics and History Emphasis: Biology, Chemistry and Physics
Name and type of organisation providing education and training	Bundesrealgymnasium Linz, 4, Fadingerstraße, 4020, Linz, Austria
Level in national or international classification	ISCED 3

Work experience (Excerpt; for more information see 12.15)

Dates	October 2010 onwards February – June 2010
Occupation or position held	Tutor
Main activities and responsibilities	- Teaching and direction of students in a practical course in enzyme technology
Name and address of employer	Universität für Bodenkultur, 33, Gregor Mendel-Straße, 1180, Vienna, Austria
Type of business or sector	Education
Dates	October 2008 – February 2009 October 2007 – July 2008
Occupation or position held	Tutor
Main activities and responsibilities	- Teaching and direction of students in an organic chemical course
Name and address of employer	Universität Wien, 1, Dr. Karl Lueger Ring, 1010, Vienna, Austria
Type of business or sector	Education

Personal skills and competences

Mother tongue: **German**

Other language

Self-assessment	Understanding				Speaking				Writing	
European level (*)	Listening		Reading		Spoken interaction		Spoken production			
English	C2	Proficient user	C2	Proficient user	C1	Proficient user	C1	Proficient user	C2	Proficient user

(*) Common European Framework of Reference for Languages

Social skills and competences
- TEAM WORK: During my work as a tutor I taught and guided a group of students in German and English. I also worked in a team of volunteer firemen under stressful conditions.
- INTERCULTURAL SKILLS: During my work as a tutor and during my diploma thesis I worked with people from various countries and communicated with them in English.

Organisational skills and competences: As a tutor I was responsible for updating the manual, prepartions and directing efforts to complete tasks in time.

Technical skills and competences
- As a volunteer fireman I can operate common fire brigade equipment, generators and hydraulic tools.
- In my spare time I repaired maintained my car and motorcycle.

Computer skills and competences: Competent with most Microsoft Office programs and other minor software.

Other skills and competences
- Forklift truck driver licence
- Crane operator licence

Driving licence: I am a holder of an Austrian driver licence. Categories A, B and CE.

Annexes
- Complete work experience list (see 12.15)

2 SUMMARY

In this diploma thesis a pyranose dehydrogenase gene from *Coprinopsis cinerea* was expressed in *Pichia pastoris* and its protein analyzed.

Pyranose dehydrogenase is a sugar oxidoreductase with a flavin-domain. It is structurally and catalytically related to fungal pyranose oxidase and cellobiose dehydrogenase. While it is able to oxidize sugars it cannot transfer the electrons onto O_2 and has to use substituted benzoquinones and (organo) metallic ions instead.

Pyranose dehydrogenase is able to monooxidize various sugars at the positions C1, C2 or C3 or dioxidize them at the position C2,3 or C3,4. It is not easy to modify sugars in the way the Pyranose dehydrogenase does. The modified sugars could be used as intermediates for the synthesis of rare sugars, novel drugs or fine chemicals. Therefore the discovery and characterization of new Pyranose dehydrogenases from different organisms is a promising area of research.

The basidomycete *Coprinopsis cinerea* is expected to possess such a Pyranose dehydrogenase. A putative Pyranose dehydrogenase gene (NCBI Reference Sequence: XP_001833871.1 and XP_001833871.2; hypothetical protein CC1G_01548 [*Coprinopsis cinerea* okayama7#130]) was investigated during this diploma thesis:

After modifying the microorganism *Pichia pastoris* to produce and export four different versions of this putative Pyranose dehydrogenase, the proteins were checked for existence and the correct DNA sequence they originated from with positive results.

The Pyranose dehydrogenase activity was determined by two different methods and none of the proteins showed Pyranose dehydrogenase activity. Two of the proteins were also tested for aryl alcohol oxidase activity with negative results.

3 ZUSAMMENFASSUNG

In dieser Diplomarbeit wurde ein Pyranose Dehydrogenase Gen von *Coprinopsis cinerea* in *Pichia pastoris* exprimiert und dessen Protein analysiert.

Pyranose Dehydrogenase ist eine Zucker Oxidoreduktase mit einer Flavin-Domäne und strukturell und katalytisch mit der Pyranose Oxidase und Cellobiose Dehydrogenase aus Pilzen verwandt. Sie oxidiert Zucker, überträgt dabei die Elektronen jedoch nicht auf Sauerstoff, sondern auf substituierte Benzoquinone oder (organo)-metallische Ionen.

Pyranose Dehydrogenasen sind in der Lage verschiedene Zucker entweder einzeln an den Positionen C1, C2 oder C3 oder doppelt an den Positionsgruppe C2,3 und C3,4 zu oxidieren was mit anderen Methoden schwer zu realisieren ist. Die so modifizierten Zucker könnten als Zwischenstufe für die Synthese von seltenen Zuckern, neuartigen Medikamenten oder Feinchemikalien dienen. Aus diesem Grund ist die Entdeckung und Charakterisierung von neuen Pyranose Dehydrogenasen aus unterschiedlichen Organismen ein viel versprechendes Forschungsgebiet.

Es wird vermutet, dass der Basidienpilz *Coprinopsis cinerea* eine Pyranose Dehydrogenase besitzt. Ein dafür verantwortlich gemachtes Gen (NCBI Referenz Sequenz: XP_001833871.1 und XP_001833871.2; hypothetisches Protein CC1G_01548 [*Coprinopsis cinerea* okayama7#130]) wurde in dieser Diplomarbeit untersucht:

Nachdem das Gen in vier verschiedenen Versionen in die Hefe *Pichia pastoris* eingebracht wurde, wurden die von den Hefen produzierten Proteine auf ihr Vorhandensein geprüft. Außerdem wurden die DNA-Sequenzen, die den Proteinen zu Grunde lagen, auf korrekte Basenabfolge überprüft. Alle Prüfungen lieferten positive Ergebnisse.

Die Pyranose Dehydrogenase Aktivität wurde durch zwei verschiedene Methoden bestimmt. Überdies wurden zwei der Proteine auf Aryl Alkohol Oxidase Aktivität getestet. Alle Proteine zeigten keinerlei messbare Aktivität.

4 ACKNOWLEGEMENTS

First, I would like to thank Univ. Prof. Dipl. Ing. Dr. Dietmar Haltrich, the head of the Division of Food Biotechnology in the Department of Food Sciences and Technology at the University of University of Natural Resources and Life Sciences in Vienna, for giving me a very warm welcome and the opportunity to work in this lab.
Second, my thank is due to Ao. Univ.-Prof. Dipl.-Biol. Dr. Angela Witte who made it possible for me to conduct this thesis at the University of University of Natural Resources and Life Sciences.
Special thank is due to my practical supervisors Dipl. Ing. Dr. Magdalena Kujawa, who supervised me in a detailed way for two months, introduced me into the basics of my thesis and into the lab. It also is due to Priv. Doz. Dr. Clemens Karl Peterbauer who supervised me for the rest of the time, showed me the path, but let me develop the thesis into its final stage.
Many thanks are due to all my colleagues from the lab for introducing me into lab customs, providing me with procedural knowledge and their help.

I also want to thank Werner for escorting me through the study by providing me with friendship, support and – no hard feelings – a lot of poker money.
Moreover I want to thank Oliver for accompanying me during the diploma thesis and his famous burgers.
I am also deeply thankful for all my friends who guided me through the past years.

A big thank is due to my mother for her ongoing financial and general support over the long years of study.
Thanks also are due to my family for their continuous support throughout the whole time.

5 CONTENTS

6 INTRODUCTION

6.1 Pyranose Dehydrogenase (PDH)

6.1.1 Sugar Oxidoreductases

Fungal oxidoreductases of sugars are investigated for their technological potential. Their biological function however is unknown. Glucose 1-oxidase (EC 1.1.3.4) oxidizes D-glucose at C1 and can be found in Ascomycetes and Fungi Imperfecti. Pyranose 2-oxidase (EC 1.1.3.10) consists of four subunits, each containing a FAD domain. It oxidizes aldopyranoses at the position C2. It is mainly found in white-rot Basidiomycetes. Cellobiose dehydrogenase (EC 1.1.99.18) oxidizes -1,4-linked di- or oligosaccharides such as cellobiose at C1. It can be found in Basidiomycetes and Ascomycetes. Pyranose dehydrogenases are also a member of this group. (1)

6.1.2 Pyranose Dehydrogenase (PDH)

The fungal pyranose dehydrogenase carries a flavin domain and is catalytically related to pyranose oxidase and cellobiose dehydrogenase. It is a secretory monomeric glycoprotein. It can be found in a small group of litter-decomposing basidiomycetes, but its biological function is unknown. The amino acid sequences at the N-termini are thought to be signal peptides for extracellular secretion. In contrast to pyranose 2-oxidase it does not use oxygen as electron acceptor, but substituted benzoquinones and (organo)-metallic ions. On the other hand its substrate specificity is broader. Pyranose dehydrogenases are able to mono- or dioxidize a sugar as a mono-, di-, oligosaccharide or as a glycoside at C1, C2, C3, C2,3 or C3,4 depending on the sugar-structure and the origin of the enzyme. These enzymes make it possible to produce di- and tri-carbonyl sugar derivates as precursors for synthesis of rare sugars, novel drugs and fine chemicals. (2)

6.1.3 Biological Functions of CDH, P2O and PDH

For CDH from *Trametes versicolor* it could be shown that hyphae from a CDH disruptant strain could not penetrate wood anymore. The biological function of P2O and PDH remains unknown. However P2O is thought to provide H_2O_2 to peroxidases. Another function for CDH, P2O and PDH is thought to be the reduction of quinone or radical intermediates to prevent them from repolymerization or the

production of Fenton's reagent, which then may degrade cellulose, xylan and lignin. (1)
The reduction of toxic quinones that are produced by plants could also protect PDH, CDH and P2O producing organisms. (2)

6.2 Aryl-Alcohol Oxidase

Aryl-alcohol oxidases are fungal oxidoreductases that contain FAD. They provide H_2O_2 to peroxidases for lignin degradation. For the *Pleurotus* species the peroxide is produced from the extracellular metabolite *p*-anisalcohol. However the enzyme can also oxidize other polyunsaturated primary alcohols. (3)

6.3 *Coprinopsis cinerea*

6.3.1 Basidomycota

The phylum Basidiomycota consists of fungi, molds, mutualists, rusts and smuts. A Basidium is a cell in the fungal life cycle in which there is a transient diploid stage. They are also called club fungus, because the Basidium is formed like a club. They play an important role in decomposing plant material such as wood. (4)

6.3.2 *Coprinopsis cinerea* (former *Coprinus cinereus*)

Coprinus cinereus is a typical mushroom with small edible value. Along with *Schizophyllum commune* it is a model organism for homobasidomycetous fungi, because it has a life cycle of two weeks, grows and forms fruiting bodies under lab conditions. Naturally it grows on horse dung, but it also grows and fruits on artificial media. *C. cinerus* has been isolated under a lot of different names, which often leads to confusion and makes analyzes difficult. The genus *Coprinus* has been used to describe saprophytic mushrooms whose fills and often the entire cap auto-digest in the mature state. The so forming inky black fluid gave them the name ink caps. Phylogenetic studies have indicated that the genus *Coprinus* is polyphyletic. (5)
Therefore, since 2001 *Coprinus cinereus* is referred to as *Coprinopsis cinerea*. (6)

6.4 *Pichia pastoris*

Today the production of functional proteins in huge amounts in a cheap way is of commercial and scientific interest. The methylotrophic yeast *Pichia pastoris* is a

suitable organism to express milligram-to-gram quantities. It is also possible to scale up the fermentation to meet higher demands. *P. pastoris* is easy to manipulate genetically and grows on simple and cheap media with high cell densities. It is able to produce high levels of intra- or extracellular protein with higher eukaryotic protein modification including glycosylation, disulphide bond formation and proteolytic processing. The DNA-sequence for heterologous protein expression is integrated into the genome making it genetically very stable, which is important for long or continuous fermentation processes. (7)

6.4.1 Background

In the 1970s *P. pastoris* was considered to be a source of protein, because it could utilize methanol made from natural gas as a carbon and energy source. Unfortunately the gas price increased while the price for soybeans as an alternative protein source decreased. The organism was then evaluated as a system for heterologous protein expression with a positive outcome. The very strong and selective alcohol oxidase promoter was isolated and used for hetereologous protein expression. Today *P. pastoris* also is a model organism in cell biology. As an expression system *P. pastoris* is available from Invitrogen (Carlsbad, CA, USA). (7)

6.4.2 Methanol Metabolism in *P. pastoris*

All methylotrophic yeasts share one methanol utilization pathway with several enzymes involved. First, in the peroxisome the methanol is processed into formaldehyde by alcohol oxidase. Formaldehyd is then oxidized in the cytoplasm to CO_2 providing the cell with NADH or it is used in the peroxisome as a carbon source by the enzyme dihydroxyacetone synthase. (7)

6.4.3 The *P. pastoris* Expression System

To express a foreign gene in *P. pastoris* the gene is inserted into an expression vector. Then the expression vector is incorporated into *P. pastoris*. After a screening the potential strains are tested whether they contain and express this gene.

Usually for protein expression the AOX promoter is used, because of its tight regulation through a repression-derepression mechanism. With this very strong promoter it is possible to express a high level of even toxic proteins by the addition of methanol which activates the promoter. In the absence of methanol the promoter can

be considered silent. The only disadvantages are the methanol itself and the use of two different carbon sources for growth and expression, which makes a transitional period necessary. Methanol is flammable, its concentration difficult to determine and for food products unsuited, because it is mainly produced by the petrochemical industry. (7)

6.5 Histidine-Tag

Immobilized metal ion affinity chromatography (IMAC) is a very easy and simple method to purify recombinant proteins. Six histidines at the N- or C-terminus allow a protein to bind to a nickel ion. This nickel ion is immobilized on a resin and therefore the protein is bound to this resin. After washing this resin the proteins can be obtained again by increasing the amount of imidazole as a competitor to histidine, stripping of the nickel by a strong chelator like EDTA or protonating the histidines with acid. (8)

7 PROJECT DEFINITION

7.1 Initial Situation

The DNA-sequence for the putative PDH of *Coprinopsis cinerea* (NCBI Reference Sequence: XP_001833871.1; hypothetical protein CC1G_01548 [*Coprinopsis cinerea* okayama7#130]) was ordered at the company “Mr. Gene” that sent the plasmid pKKW1 (see 12.1) containing this sequence (will be referred to as CcPDH).
An alternative amino acid sequence for CcPDH was proposed by the Broad institute (Cambridge, MA, USA). (see XP_001833871.2; Pyranose dehydrogenase CC1G_01548 [*Coprinopsis cinerea* okayama7#130]). The sequence coding for this protein will be referred to as CcPDH-mod.

7.2 Task Definitions

(1) Modifying the restriction sites in Mr. Gene’s vector and inserting the CcPDH into a suitable expression vector (pPICZ B).
(2) Preparation of 2 expression vectors: The first carrying for the protein identical to the NCBI amino acid sequence (CcPDH) and the second coding for the same amino acid sequence with an additional C-terminal histidine-tag (CcPDH-HIS).
(3) Modification of the 2 existing expression vectors: The new sequences should code for the protein sequence proposed by Broad institute without (CcPDH-mod) or with histidine-tag (CcPDH-mod-HIS).
(4) Insertion of the four expression vectors into the genome of *P. pastoris* (strain X-33) creating modified *P. pastoris* able to express CcPDH, CcPDH-HIS the Broad institute protein (CcPDH-mod) and the C-terminal histidine-tagged Broad institute protein (CcPDH-mod-HIS).
(5) Validation of the constructed DNA-sequences.
(6) Expression of all four proteins originating from the expression vectors.
(7) Validation of the amino-acid-sequence of the expressed CcPDH-mod.
(8) Screening for PDH-activity or alternative enzymatic activities.

7.3 Task Motivation

If expression of the original protein was successful in *P. pastoris* it would be easy to produce large amounts of protein. This protein could be analyzed for putative PDH

activity or other functions. After further purification the enzymatic properties could be determined.

For the untagged protein a purification method would have to be established first. Purification protocols for the native Pyranose dehydrogenases from *A. bisporus* (9) and *A. meleagris* (10) could be used as template.

The C-terminal histidine-tagged protein could simplify purification.

8 MATERIALS AND METHODS

8.1 Chemicals

Laboratory chemical and ingredients for culture media originated from the companies Sigma-Aldrich (St. Louis, MO USA), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany).

8.2 Buffers, Media and Solutions

8.2.1 TAE-Buffer

Component	Final Concentration	Remark
Tris	0.04M	
EDTA	0.05M	pH8
Acetic acid	5.71%v/v	
RO water		solvent

Table 1 **Recipe** TAE-buffer

8.2.2 High Molecular Weight Running Buffer

Component	Amount [g/l]	Final Concentration	Remark
MOPS	10.45	50mM	
Tris	6.05	50mM	
SDS	1	0.1%w/v	
EDTA-Na_2 x H_2O	1.86	5mM	
RO water			solvent

Table 2 **Recipe** Protein gel High molecular weight running buffer

8.2.3 Staining Solution

Component	Amount	Final Concentration	Remark
Perchloric acid	175ml/l	3.5%	20%
Coomassie Brilliant Blue R250	400mg/l	0.04%w/v	
in methanol	20ml/l		
RO water			solvent

Table 3 **Recipe** Protein gel Staining Solution

8.2.4 Destaining Solution

Component	Amount	Final Concentration	Remark
Acetic acid	50ml/l	5%v/v	
RO water			solvent

Table 4 **Recipe** Protein gel Destaining Solution

8.2.5 LB Media or Agar-Plates

Component	Concentration [g/l]	Remark
Peptone from Casein	10	
Yeast Extract	5	
NaCl	5	
(Agar)	15 to 20	only for agar plates

Table 5 **Recipe** LB

After sterilization (see 8.15.1) of the components and cooling down to 60°C antibiotics were added if needed to the stated final concentrations:

Antibiotic	Concentration [mg/l]
Kan	30
Zeo	12.5 to 25

Table 6 **Recipe** antibiotics for LB

After completion of the medium, it was stored at 4°C and, if it contained Zeo, protected from light. Respectively, the agar plates were poured and stored the same way.

8.2.6 YPD Media or Agar-Plates

2 different YPD-recipes were used. Both led to appropriate growth of the *P. pastoris* cells.

8.2.6.1 Low Glucose Concentration Recipe

Component	Concentration [g/l]	Remark
Peptone from Casein	20	
Yeast Extract	10	
Glucose	4	
(Agar)	15 to 20	only for agar plates

Table 7 **Recipe** low glucose concentration YPD

After sterilization (see 8.15.1) of the components and cooling down to 60°C, if needed, Zeo was added to a final concentration of 100mg/l.
After completion of the medium, it was stored at 4°C and, if it contained Zeo, protected from light. Respectively, the agar plates were poured and stored the same way.

8.2.6.2 High Glucose Concentration Recipe

Component	Amount	Remark
Peptone from Casein	20g	
Yeast Extract	10g	
RO water	800ml	
(Agar)	15 to 20g	only for agar plates

Table 8 **Recipe** high glucose concentration YPD

After sterilization (see 8.15.1) of the components and cooling down to 60°C, 200ml of sterile 60°C warm glucose solution (20g/l) were added. If needed, Zeo was added to a final concentration of 100mg/l to complete the medium.
After completion of the medium, it was stored at 4°C and, if it contained Zeo, protected from light. Respectively, the agar plates were poured and stored the same way.

8.2.7 10x YNB Medium

Component	Final Concentration	Remark
YNB without ammonium-sulphate and amino acids	34g/l	
Ammonium sulphate	100g/l	
RO water		solvent

Table 9 **Recipe** 10x YNB medium

After dissolving, the mixture was filter sterilized (see 8.15.2).

8.2.8 Pre-BMMY Medium

Component	Final Concentration	Remark
Peptone from casein	25g/l	
Yeast extract	12.5g/l	
RO water		solvent

Table 10 **Recipe** Pre-BMMY medium

After dissolving, the mixture was sterilized (see 8.15.1).

8.2.9 BMMY Medium without Methanol

Component	Volume for about 1l [ml]	Remark
Pre-BMMY	800	see 8.2.8
10x YNB	100	see 8.2.7
Potassium phosphate buffer	100	1M; pH6; sterilized (see 8.15.1)
Biotin	2	200mg/l; filter sterilized (see 8.15.2); stored at 4°C

Table 11 **Recipe** BMMY medium

8.2.10 BBG Medium

Component	Amount	Remark
H_3PO_4	26.7ml/l	85%
$CaSO_4$	930mg/l	
K_2SO_4	18.2g/l	
$MgSO_4 \cdot 7\ H_2O$	14.9g/l	
KOH	4.13g/l	
Glycerol	40g/l	
Antifoam-204	500µl/l	
H_2O		solvent

Table 12 **Recipe** BBG medium

After dissolving, the mixture was sterilized (see 8.15.1).

8.2.11 MN solution

Component	Amount	Remark
$CuSO_4 \cdot 5\ H_2O$	6mg/ml	
KI	100µg/ml	
$MNSO_4 \cdot H_2O$	3mg/ml	
$Na_2MoO_4 \cdot 2\ H_2O$	200µg/ml	
$B(OH)_3$	20µg/ml	
$CoCl_2 \cdot 7\ H_2O$	980µg/ml	
$FeSO_4 \cdot 7\ H_2O$	65mg/ml	
Biotin	200µg/ml	
H_2SO_4	5µl/ml	
$ZnCl_2$	20mg/ml	
H_2O		solvent

Table 13 **Recipe** MN solution

After dissolving, the mixture was filter sterilized (see 8.15.2).

8.2.12 GF medium

A 50%w/v glycerol solution was sterilized (see 8.15.1) and 12ml/l MNS (see 8.2.11) was added.

8.3 Kits

The kits used during the diploma thesis are listed in the following table.

Purpose	Manufacturer	Kit Name	Catalogue Number	Remarks
E. coli transformation	NEB (Ipswich, MA, USA)	NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987H	
E. coli Plasmid Mini Preparation	Sigma-Aldrich (St. Louis, MO, USA)	GeneElute™ Plasmid Miniprep Kit	PLN350	4ml of overnight-culture in LB-medium with the appropriate antibiotic (see 8.2.5) were used per preparation.
	Promega (Madison, WI, USA)	PureYield™ Plasmid Miniprep System	A1222	3ml of overnight-culture in LB-medium with the appropriate antibiotic (see 8.2.5) were used per preparation.
Agarose Gel and PCR Purification System	Promega (Madison, WI, USA)	Wizard® SV Gel and PCR Clean-Up System	A9282	
Protein Expression in *P. pastoris*	Invitrogen (Carlsbad, CA, USA)	EasySelect™ *Pichia* Expression Kit		For the creation of protein expression competent cells the vector pPICZ B (see 12.6) out of this kit was used.

Table 14 **List** of the used kits

8.4 Organisms

8.4.1 *Escherichia coli*

The used bacterium was *E. coli* „NEB 5-alpha Competent *E. coli* (High Efficiency)“ originated from NEB (Ipswich, MA, USA).

8.4.2 *Pichia pastoris*

The used yeast was *P. pastoris* strain X-33 from Invitrogen (Carlsbad, CA, USA).

8.5 Transformation of *P. pastoris*

To integrate the expression vector into the *P. pastoris* genome, the organism was electroporated. When *P. pastoris* was electroporated by being in a strong electric field for a short period of time, it took up foreign DNA. *P. pastoris* was then able to integrate the expression vector or its linear form even better into its genome.
The device "MicroPulser™" and the cuvettes "Gene Pulser®/MicroPulser™ Cuvettes 0.2cm gap" used originated from Biorad (Hercules, CA USA).
For the electroporation, electrocompetent cells on ice (prepared as described in the manual, but stored at -70°C after freezing in liquid nitrogen) were mixed with DNA. Then the mixture was collected at the bottom of an icecold electroporation-cuvette and the electroporation was performed. Immediately after electroporation, 500µl of icecold sterile 1M sorbitol solution and 500µl of icecold sterile YPD-medium (see 8.2.6) were added.
After a recuperation phase in which the mixture was shaken at 30°C for up to 4 hours with up to 100rpm the cells were spread onto YPD-agar-plates and cultivated at 30°C for at least 48h.

8.6 PCR

PCRs were used to amplify sections of DNA for analytical reasons or to change the DNA-sequence in small sections.
"Phusion® High-Fidelity DNA Polymerase" (in the Lab-journal referred to as Pfu-Polymerase or Phu-Polymerase) with the catalogue number "F-530" originated from Finnzymes (Espoo, Finland).
dNTPs originated from Fermentas (Burlington, Canada).
Phusion®-Mastermix for 150 PCRs was made from the Phusion® Polymerase kit and the dNTPs:

Component	Volume [µl]
Buffer HF 5x	750
Phusion® Polymerase	37.5
dNTPs (10mM each)	75
RO water	1012.5

Table 15 **Recipe** Phusion® mastermix

Primers for the PCRs originated from VBC-Biotech (Wien, Austria). The sequences of the Primers are listed in 12.9.

8.6.1 *P. Pastoris* Colony PCR

Colony PCR was used to amplify sections of genomic DNA from *P. pastoris*.
First, the organism was cultivated on an YPD-plate with the appropriate antibiotic (see 8.2.6) for about 3 days. Then a rather big lump of cells was then taken with a toothpick and suspended in 30µl of RO-water. The cells were boiled for 10 minutes and centrifuged for 2 minutes with about 13000g. The supernatant contained the genomic DNA that could be amplified by PCR.

8.7 DNA-Gel-Electrophoresis

8.7.1 General

“6x MassRuler™ Loading Dye”, “MassRuler™ DNA Ladder Mix” and “GeneRuler™ DNA Ladder Mix” originated from Fermentas (Burlington, Canada).

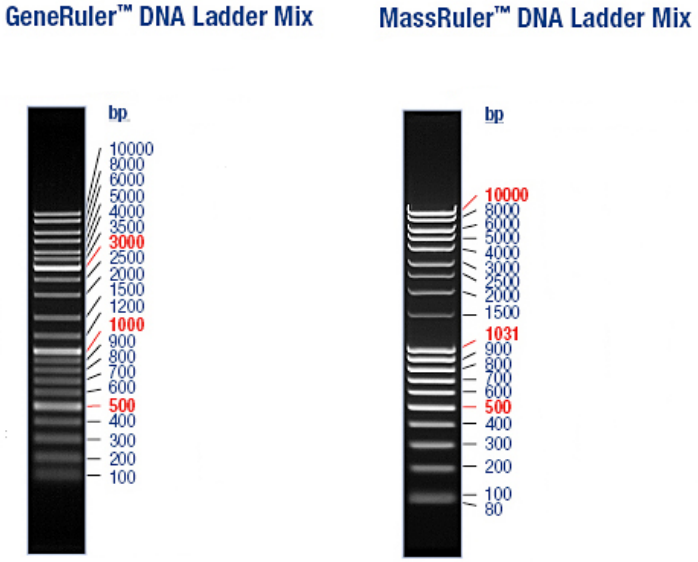

Figure 1 **Ladders** GeneRuler™ and MassRuler™

“StarPure AGAROSE Low EEO Standard” originated from Starlabs (Ahrensburg, Germany).
“Ethidiumbromidlösung 0.025% in der Tropfflasche 15ml“ originated from Roth (Karlsruhe, Germany).

The gel-electrophoresis-equipment was manufactured by Biorad (Hercules, USA). It consisted of “PowerPac 300“, “Mini-Sub® Cell GT”, “Wide Mini-Sub® Cell GT“ Gel Doc™ 2000” and “Quantity One 4.3.0”.

8.7.2 Procedure

0.8%m/v to 1%m/v agarose was dissolved in boiling 1x TAE-buffer (see 8.2.1). After cooling down to about 60°C, one drop of 0.025% ethidiumbromid-solution per 30ml of gel-solution was added. The gel was cast from this solution.
After mixing the DNA-samples with loading dye (final concentration 1x), the samples and usually 5µl of the marker were put into the gel slots and ran in 1x TAE-buffer at about 75V to 100V for about 30 minutes to 180 minutes.
DNA was made visible with UV-light and, if needed, photographed and cut out.

8.7.3 Quantification of DNA

The amount of DNA was quantified with the help of the Software “Quantity One 4.3.0”. Known amounts of MassRuler™ DNA Ladder Mix and sample were separated on the gel and photographed with an exposure time that prevented saturated pixels. The brightness of the marker bands, with similar in length to the sample band, was used to create a calibration curve. The amount of DNA in the sample band was derived from the brightness of the sample band.

8.8 Protein-Gel-Electrophoresis

To analyze the size of proteins SDS-protein-gel-electrophoresis combined with a Coomassie staining was used.
To make a gel the system “PerfectBlue Doppelgelsystem Twin S” from PeqLab (Erlangen, Germany) was used.

The ladder „Precision Plus Protein™ Standards Dual Color" originated from Biorad (Hercules, CA USA).

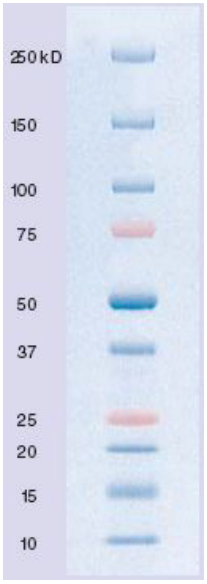

Figure 2 **Ladder** Precision Plus Protein™ Standards Dual Color

8.8.1 Procedure

First the separating gel was cast with the system and the following recipe for 2 gels:

Component	Amount	Final Concentration	Remark
BisTris	3.43ml	0.417M	1.25M, pH6.5-6.8
Acrylamid:Bisacrylamid	5ml	12.5%w/v	ratio 37.5:1, 30%w/v
RO water	3.45ml		
TEMED	11µl		
Ammonium persulfate	114µl		10%w/v

Table 16 **Recipe** Protein gel Separating gel

After casting, the gel was covered with 2-propanol.

When the separating gel was hardened the stacking gel was cast on top of it with a comb in it. 2 stacking gels had the following recipe:

Component	Amount	Final Concentration	Remark
BisTris	1.43ml	0.417M	1.25M, pH6.5-6.8
Acrylamid:Bisacrylamid	860µl	5.16%w/v	ratio 37.5:1, 30%w/v
RO water	2.65ml		
TEMED	7µl		
Ammonium persulfate	54µl		10%w/v

Table 17 **Recipe** Protein gel Stacking gel

When the stacking gel was hardened, the comb was removed and the gel was ready for use.

After filling the system with high molecular weight running buffer (see 8.2.2) and cooling down the system to 10°C, the samples were prepared the following way:

10µl sample were mixed with 10µl 2x Laemmli buffer. The mixture was incubated at 99°C for 3 minutes and cooled down to 0°C. After centrifugation with 9300g at 4°C for 2 minutes, the supernatant was ready to be loaded on the gel.

After loading the gel with 5µl of protein ladder and 15µl of each sample the electrophoresis was started with 100V and the voltage was increased to 200V when the proteins reached the separating gel.

After the dye front reached the bottom of the gel the electrophoresis was stopped and the gel was stained the following way:

The gel was washed in RO water 3 times for 1 minute each. After that the gel was stained with staining solution (see 8.2.3) for about 1 hour and destained with destaining solution (see 8.2.4) 2 times for 15 minutes each. After keeping the gel in RO water overnight, it could be scanned.

8.9 Protein-Sequencing

To get sequence information of a certain protein from a solution, this solution analyzed by Dr. Johannes Stadlmann (Altmann-Glykobiologie Gruppe, University of Natural Resources and Life Sciences, Wien, Austria). First all proteins were deglycosylated and then separated by protein-gel-electrophoresis. The protein from the band was isolated and digested by trypsin. The digested fragments were

analyzed by a mass spectrometer. This was done by Dr. Johannes Stadlmann (Altmann-Glykobiologie Gruppe, University of Natural Resources and Life Sciences, Wien, Austria). The result was a file describing the fragments that were detected.
This file was analyzed with the Mascot search engine at www.matrixscience.com (11). Therefore a Mascot MS/MS Ion search with the following options:

Name	Option 1	Option 2
Your name	Kristoffer Kurt Wollinger	
Email	k.kurt.wollinger@gmail.com	
Search title		
Database(s)	NCBInr	
Enzyme	Trypsin	
Allow up to _ missed cleavages	1	
Quantification	None	
Taxonomy	All entries	
Fixed modifications	Carbamidomethyl (C)	
Variable modifications	Deamidated (NQ)	
	Oxidation (M)	
Peptide tol. ±	200	ppm
# ^{13}C	0	
MS/MS tol. ±	0.3	Da
Peptide charge	2+	
Monoisotopic	“checked”	
Average	“unchecked”	
Data file	<name of file from Stadlmann>	
Data format	Micromass (.PKL)	
Precursor		
Instrument	ESI-QUAD-TOF	
Error tolerant	“unchecked”	
Decoy	“unchecked”	
Report top _ hits	AUTO	

Table 18 **Options** for Mascot search

The proteins consisting of the fragments were listed and sorted by likelihood in the Mascot search results.

8.10 Restriction Enzymes

Restriction enzymes and their corresponding buffers originated from Fermentas (Burlington, Canada) and were used as recommended.

8.11 Optical Density 600 (OD_{600})

To determine the concentration of cells present in a solution, the absorbance of the solution or its dilution at 600nm was measured. The result shows scattering of light caused by the size and amount of cells.

8.12 Bradford Assay for Protein Concentration

If Coomassie Brilliant Blue G-250 in acidic solution binds to protein the absorbance maximum shifts from 465 nm to 595 nm. (12)

Bradford reagent had the article number “500-0006” and was manufactured by Biorad (Hercules, CA USA).

Bovine serum albumin was used as protein standard. It had the article number “P0914” and was provided by Sigma-Aldrich (St. Louis, MO, USA).

Bradford reagent was prepared in the lab and the calibration curve stored in the photometer by lab personnel. 1000µl of Bradford reagent were mixed with 20µl of sample or its dilution. After 15 minutes at room temperature the absorption at 595nm was measured and the corresponding protein concentration noted.

8.13 PDH Activity Assays

The PDH activity was measured with two photometric methods in an UV/VIS photometer.

8.13.1 Ferricenium-Hexafluorophosphate-Assay

PDH is able to oxidize one hydroxyl-group of glucose by reducing 2 ferricenium-ions. The reduction of the ferricenium-ion to ferrocene leads to a decrease in the absorption at 300nm. (13)

33.1mg ferricenium hexafluorophosphate were dissolved in 100ml 5mM HCl.

For the assay 770µl of 100mM potassium phosphate buffer (pH7.5) were mixed with 200µl of ferricenium solution and incubated at 30°C for 10 minutes. After that, 20µl of sample or its dilution were added and finally the reaction was started with 10µl of 2.5M glucose solution. The molar absorption coefficient for the ferricenium-ion is $4.3 mM^{-1} cm^{-1}$.

8.13.2 Benzoquinone-Assay

Pyranose-dehydrogenase oxidises D-glucose to 2- and/or 3-Keto-derivate, at the same time it reduces 1,4-Benzoquinone to Hydroquinone. (9)

For the assay 940µl of 75mM sodium citrate buffer (pH4.5) were mixed with 20µl of 2.5M glucose solution and 20µl of 50mM benzoquinone in sodium citrate buffer solution. The mixture was incubated at 30°C for 10 minutes. The reaction was started with 20µl of sample or its dilution. The molar absorption coefficient at 290nm for hydroquinone is $2.24 mM^{-1} cm^{-1}$.

8.14 Aryl-Alcohol Oxidase Assays

The aryl-alcohol oxidase activity was measured with a designed assay. Aryl-alcohol oxidase oxidizes anisyl alcohol better than the usually used veratryl alcohol (14). If one molecule anisyl alcohol is oxidized to the corresponding aldehyde, one molecule H_2O_2 will be formed out of O_2. After that, horseradish peroxidase reduces one molecule H_2O_2 to 2 molecules OH^- and oxidizes 2 molecules ABTS to $ABTS^+$. $ABTS^+$ ($\varepsilon_{420}=43200 M^{-1} cm^{-1}$) is then detected at 420nm (15).

For the assay peroxidase solution was made. The activity of the peroxidase was 1428U/ml. The enzyme was dissolved in 1M $(NH_4)_2SO_4$ made with 50mM potassium phosphate buffer (pH6.5). A reaction mixture was made consisting of the following components:

Component	Amount	Final Concentration	Remark
Peroxidase	100µl	5.7U/ml	1428U/ml
ABTS	14.7mg	1mM	Diamonium salt
Anisyl alcohol	3.1µl	1mM	molten
Potassium phosphate buffer	25ml	~100mM	Solvent; 100mM; pH6

Table 19 **Recipe** for aryl alcohol oxidase assay reaction mixture

The addition of 10µl sample to 990µl reaction mixture (30°C) started the reaction that was monitored at 420nm for 3 minutes.

8.15 Sterilization

8.15.1 Thermal Sterilization

Sterilization was performed by autoclaving for 20 minutes at 120°C.

8.15.2 Filter Sterilization

The two types of filters used were „Steritop™" with the article number "SCGVT05RE" and "Rotilabo®" with the article number "P666.1". They were manufactured by Millipore (Billerica, MA USA) and Roth (Karlsruhe, Germany).respectively.
The solution was pressed through the filter by a syringe or sucked through by vacuum, which led to sterilization of the solution.

8.16 Culture Collection

Organisms were stored for the further future in the culture collection. The organisms were cultivated at listed temperature in listed medium with listed antibiotic (see 8.2.5 and 8.2.6) until visible turbidity. After that, one part of culture was mixed with one part of sterilized 30% glycerol in RO water solution. Then, after freezing in liquid nitrogen the cultures were stored at -70°C to -80°C. If the organism harbored a plasmid, the plasmid was isolated and stored additionally at -70°C to -80°C. For a list of all organisms that were added to the culture collection see 12.12.

8.17 DNA Insert Ligation into Vector DNA

The "T4 DNA Ligase" and its "T4 DNA Ligase Buffer" originated from Fermentas (Burlington, Canada).

8.18 DNA-Sequencing

Sequencing of DNA was done by the company "AGOWA" (later "LGC Genomics"; Berlin, Germany).
For plasmid-DNA-sequence-analysis the plasmid-DNA was sent to the company without added primer. The company sent back a text-file with the determined DNA-sequence.

For DNA that originated from PCR the DNA was sent to the company with the recommended DNA concentration either with or without added primer. The company sent back a text-file with the determined DNA-sequence.

8.19 DNA-Sequence-Comparison

For comparisons of DNA-sequences the internet tool bl2seq BLAST (16) from the NCBI-homepage was used.

8.20 Protein-Sequence-Comparison

For searching the NCBI protein database using a translated nucleotide query the internet tool blastx BLAST (16) from the NCBI-homepage was used.

8.21 Protein Expression with the Pump-Tube-System

To express proteins, modified *P. pastoris* was cultivated in small amounts and then transferred into an expression medium. A pump added methanol to the expression medium which let *P. pastoris* express the target protein.

The pump used was called “MINIPULS Evolution®” and manufactured by Gilson (Middleton, WI USA).

First the modified *P. pastoris* was cultivated on YPD or YPD-Zeo agar plates (see 8.2.6) at 30°C for about 3 days.

After that the fresh grown cells were transferred into 25ml of YPD medium (see 8.2.6) with a toothpick and cultivated for 1 day at 30°C and 100rpm in a baffled flask.

All 25ml of cell suspension were then transferred into 200ml BMMY without methanol (see 8.2.9). The hose of the pump were filled with methanol delivering a small amount of methanol into the flask. After 33.5h of breeding at 30°C and 120rpm in a baffled flask, the methanol addition through the pump was started. Every 3 hours the pump added a degassed 50%v/v methanol solution for 15min with the pump speed of .79rpm. By that, about 2%v/v methanol were added per day to the cells. After 10 days the protein expression was ended and the cells separated from the supernatant by centrifugation with 4000g for 5 minutes.

Samples were taken out daily from the beginning to the end of the protein expression. The OD_{600} (see 8.11) of the sample was measured and parallel to that, the sample was separated into cell pellet and supernatant by centrifugation with 16000g at 4°C for 3 minutes.

The protein concentration of the supernatant was determined using the Bradford assay (see 8.12).

8.22 Protein Expression with the Multifors-System

To express proteins, a fermenter was run with a modified *P. pastoris* culture, because in a fermenter a lot of parameters can be monitored and adjusted to optimize the protein expression process.

The fermenter system used for this purpose was called “Multifors” and manufactured by Infors HT (Bottmingen, Switzerland).

The chosen culture was cultivated, transferred into the fermenter cultivated with a batch and then with a fed batch process. Both were glycerol based. After that the protein expression was started by methanol addition.

First the modified modified *P. pastoris* was cultivated on YPD or YPD-Zeo agar plates (see 8.2.6) at 30°C for about 3 days.

After that, fresh grown cells were transferred into 25ml of YPD or YPD-Zeo medium (see 8.2.6) with a toothpick and cultivated overnight at 30°C and about 100rpm in a baffled flask.

All components were considered sterile, either because of sterilization or toxicity (NH_3, methanol). The fermenter was filled with 175ml BBGM (see 8.2.10), closed and sterilized (see 8.15.1). The temperature was set to 30°C, the O_2-level to 30% (0% being saturated with N_2 and 100% being saturated with air) and the pH to the desired value. Samples were taken to monitor the fermentation process. The foam-formation was prevented by the Antifoam-204 already present in the BBGM and by further Antifoam-204 additions of 100µl with a syringe if necessary. The following solutions were connected to the fermenter to automatically control the pH and manually the water level throughout the entire fermentation process:

Connection	Solution	Controlled Parameter
“Acid”	6M H_3PO_4	pH
“Base”	NH_3 (25%)	pH
“AF”	H_2O	water level

Table 20 **Connection overview** at the beginning

The fermenter was inoculated with 12ml of preculture solution through a syringe and the batch process started. The O_2-level rising again to about 40% indicated that the batch process was finished.

20ml of GFM (see 8.2.12) were connected to the “Feed” connection and the fed batch process was started with a feed rate of 1ml/h. After the GFM was consumed, the O_2-level rose back to about 40% indicating that the fed batch process was finished.

After emptying the feeding tube by reverse pumping, 150ml methanol mixed with 1.8ml MNS (see 8.2.11) were connected to the “Feed” connection and protein expression was started with a rate of 0.2ml/h. The feed rate was increased step by step to a final of 0.8ml. Higher methanol levels brought the O_2-level down to slightly above 0% indicating that methanol started to accumulate. After the methanol was consumed, the O_2-level rose back to about 40% indicating that the fed batch process was finished.

The fermentation broth was centrifuged for 10 minutes at 4°C with 2704g to separate the cells from the rest of the liquid.

9 CONDUCT

9.1 Transformation of *E. coli* with pKKW1

All 5µg of the original vector pKKW1 from "Mr. Gene" (see 12.1) were dissolved in 100µl of sterile RO-water and stored at -20°C.

1µl of pKKW1-solution (equals 50ng of plasmid DNA) was transformed into *E. coli* (see 8.3). The cells were then spread on LB-Kan-plates (see 8.2.5) and cultivated overnight at 37°C.

One colony of pKKW1 containing *E. coli* was named KKW1.2 and transferred onto a LB-Kan-agar-plate (see 8.2.5) and cultivated overnight at 37°C.

Plasmid DNA from KKW1.2 (referred to as pKKW1.2) was isolated by a Plasmid mini preparation (see 8.3).

After a restriction digestion (see 8.10) of the DNA with EcoRI and NotI for 2 hours at 37°C which led to linearization of the plasmid DNA, a DNA-gel-electrophoresis (see 8.7 and Figure 3) of the digested DNA was performed.

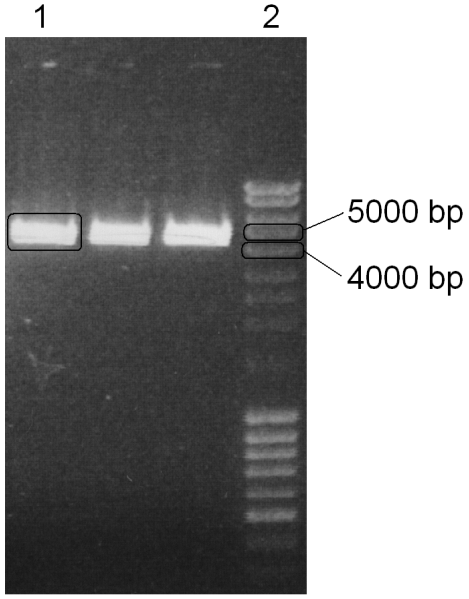

Figure 3 **Restriction digestion agarose gel** for pKKW1.2 with EcoRI and NotI MassRuler™ (2) as standard and pKKW1.2 (1)

After another restriction digestion (see 8.10) of the DNA with EcoRI and NcoI for 2 hours at 37°C which led to 2 DNA-fragments (1069bp and 2963bp long), a DNA-gel-electrophoresis (see 8.7 and Figure 4) of the digested DNA was performed.

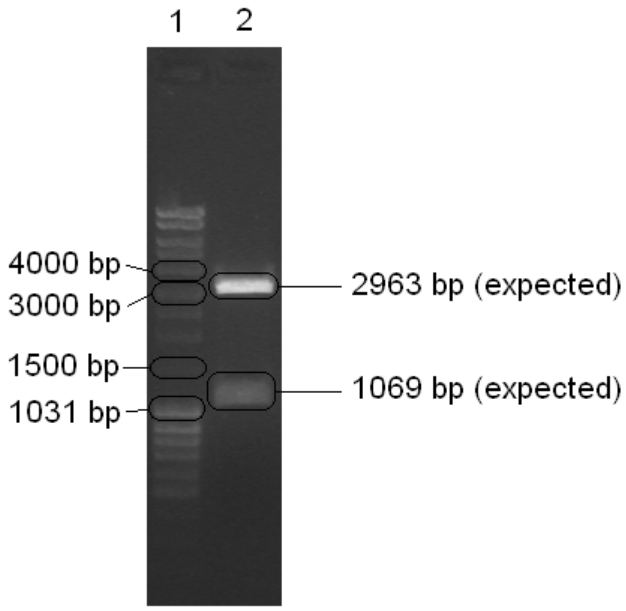

Figure 4 **Restriction digestion agarose gel** for pKKW1.2 with EcoRI and NcoI MassRuler™ (1) as standard and pKKW1.2 (2)

The data from both digestions confirmed that KKW1.2 harbored the 4032bp long plasmid KKW1.
KKW1.2 was added to the culture collection (see 8.16 and 12.12.1).

9.2 Mutagenesis of pKKW1.2 into pKKW4 and pKKW5

pKKW1 was carrying CcPDH. CcPDH should be expressed through the expression vector pPICZ B - one time without (CcPDH) and another time with the coexpressed histidine-tag (CcPDH-HIS) from the pPICZ B vector. Therefore two constructs were necessary.

9.2.1 pKKW4

To get CcPDH into the expression vector pPICZ B without the histidine-tag it had to be cut out just the way it was and inserted into pPICZ B. At the 5'-end of the insert an EcoRI-restriction-site was already present. At the 3'-end an XhoI-restriction-site was going to be inserted by PCR-mutagenesis.

For this purpose the primers KKWp3s and KKWp3a were used on pKKW1.2 as template. This is shown in the following table:

Name	Sequence
KKWp3a	<gccctatagtaattccggctgaccagaatc
pKKW1.2 (bp2080-bp2126)	-gccgatatcattaaggccgactggtcttaggaccattaattaactg- -cggctatagtaattccggctgaccagaatcctggtaattaattgac-
KKWp3s	cgactggtcttagCTCGAGGGaattaactg>

Table 21 **PCR-mutagenesis** for pKKW4
The **new bases** are colored in green and the **ones to replace** are colored in red.

9.2.2 pKKW5

To get CcPDH-HIS into the expression vector pPICZ B it had to be cut out and inserted into pPICZ B in a way so that the histidine tag would be in the same ORF. At the 5'-end of the insert an EcoRI-restriction-site was already present. At the 3'-end an XhoI-restriction-site was going to be inserted by PCR-mutagenesis. Moreover the stop-codon TAG had to be replaced by the base G.
For this purpose the primers KKWp4s and KKWp4a were used on pKKW1.2 as template shown in Table 22:

Name	Sequence
KKWp4a	<cgacggctatagtaattccggctgaccaga
pKKW1.2 (bp2077-bp2125)	-gctgccgatatcattaaggccgactggtcttagggaccattaattaact- -cgacggctatagtaattccggctgaccagaatccctggtaattaattga-
KKWp4s	gccgactggtctGCTCGAGGCttaattaact>

Table 22 **PCR-mutagenesis** for pKKW5
The **new bases** are colored in green and the **ones to replace** are colored in red.

9.2.3 PCR-Mutagenesis

Both PCRs were going to deliver mostly linear DNA fragments. They should be different from the original circular plasmid only in the lack of DNA-methylation-sites and the new XhoI-restriction-site (see green marked sequence in Table 21; referred to as pKKW4-I) or the new XhoI-restriction-site and the by G replaced stop-codon (see green marked sequence in Table 22; referred to as pKKW5-I).

The PCRs (see 8.6 and Table 23) were carried out and the DNAs from the PCR-samples were purified by DNA-gel-electrophoresis (see 8.7).

Component Type	Construct Name	
	pKKW4-I	pKKW5-I
Template DNA	pKKW1.2	
Forward primer	KKWp3s	KKWp4s
Reverse primer	KKWp3a	KKWp4a

Table 23 **PCR template-DNA and primer-DNA** for pKKW4-I and pKKW5-I

The gel slices containing pKKW4-I and pKKW5-I were cut out and its DNA eluted (see 8.3).

To get rid of the original vector, the elutions were digested (see 8.10) with DpnI for 2 hours at 37°C. This led to removal of the template DNA pKKW1.2 which was methylated.

After the digestions, the restriction enzyme was inactivated for 20 minutes at 65°C and the mixtures were purified by DNA-gel-electrophoresis (see 8.7).

The gel slices containing pKKW4-I and pKKW5-I were cut out and its DNA eluted (see 8.3) in 30µl RO water.

9.2.4 Transformation

In each case 5µl of pKKW4-I and pKKW5-I (see 9.2.3) were transformed into *E. coli* (see 8.3). The cells were then spread on LB-Kan-plates (see 8.2.5) and cultivated overnight at 37°C.

Two colonies of *E. coli* transformed with pKKW4-I and pKKW5-I were named KKW4.1 and KKW5.1, respectively. Then they were transferred onto a separate LB-Kan-agar-plate (see 8.2.5) and cultivated overnight at 37°C. Plasmid DNAs from KKW4.1 (referred to as pKKW4.1) and from KKW5.1 (referred to as pKKW5.1) were isolated by a plasmid mini preparation (see 8.3).

9.3 Insert-Transfer out of pKKW4.1 and pKKW5.1 into pPICZ B

The plasmid pKKW4.1 (see 9.2) carried CcPDH, while pKKW5.1 (see 9.2) carried CcPDH-HIS with a G instead of the stop-codon. Both inserts were flanked by EcoRI-restriction-sites at the 5'-ends and XhoI-restriction-sites at the 3'-ends. The plan was

to cut out the inserts with EcoRI and XhoI and integrate them into opened – by EcoRI and XhoI - blank pPICZ B-vectors.

9.3.1 Preparation of the Insert

Therefore pKKW4.1 and pKKW5.1 were digested (see 8.10) with EcoRI and XhoI for 2.5 hours at 37°C which led to 2 fragments each (1734bp and 2298bp long for pKKW4.1 and 1732bp and 2300bp long for pKKW5.1), the shorter always being the insert.

The mixtures were purified by DNA-gel-electrophoresis (see 8.7).

The gel slices containing pKKW4.1 and pKKW5.1 were cut out and their DNAs (referred to as pKKW4.1-f1 and pKKW5.1-f1) eluted (see 8.3) in 50µl RO-water each.

The lengths of the fragments concurred with the expected lengths and therefore the DNAs pKK4.1-f1 and pKKW5.1-f1 were considered to be the inserts of interest.

9.3.2 Preparation of the Vector

The *E. coli* carrying the pPICZ B vector (see 12.6) was taken out of the culture collection (see 12.12.1) and transferred onto a LB-Zeo-plate and into LB-Zeo-medium (see 8.2.5).

Plasmid DNA (referred to as pPICZ B) was isolated by a plasmid mini preparation (see 8.3). The DNA was then digested (see 8.10) with EcoRI and XhoI which led to 2 fragments (38bp and 3290bp long), the longer being the open vector.

The mixture was purified by DNA-gel-electrophoresis (see 8.7).

The gel slice containing the open vector was cut out and its DNA (referred to as pPICZ B-f1) eluted (see 8.3) in 50µl RO water.

9.3.3 Ligation of Inserts with Vectors, Transformations and Preparations

Before the ligations, the insert pKKW4.1-f1 or pKKW5.1-f1 was mixed with pPICZ B-f1 and RO water and then incubated at 65°C for 20 minutes. After that, the mixtures were ligated for 1 hour (see 8.17 and Table 24).

Component	Volume [µl]
Vector pPICZ B-f1	2
Insert	6
RO water	9.8
T4 DNA-Ligase	0.2
T4-DNA-Ligase-Buffer	2

Table 24 **Ligation formula** for pKKW6 and pKKW7

3µl of each ligated DNA-construct were transformed into *E. coli* (see 8.3). The cells were then spread on LB-Zeo-plates (see 8.2.5) and cultivated overnight at 37°C.
One colony containing the vector with the insert from pKKW4.1 was named KKW6.3. Another colony containing the vector with the insert from pKKW5.1 was named KKW7.4. Both were transferred onto LB-Zeo-agar-plates (see 8.2.5) and cultivated overnight at 37°C. Plasmid DNAs from KKW6.3 and KKW7.4 (referred to as pKKW6.3 and pKKW7.4) were isolated by plasmid mini preparations (see 8.3).
KKW6.3 and KKW7.4 were added to the culture collection (see 8.16 and 12.12.1).

9.3.4 Verification of the Vectors pKKW6.3 and pKKW7.4

To verify the validity of the vectors pKKW6.3 and pKKW7.4, 2 restriction-digestions (see 8.10) with EcoRI and XhoI were conducted which each led to 2 fragments (1734bp and 3290bp long for pKKW6.3 and 1732bp and 3290bp long for pKKW7.4).

A DNA-gel-electrophoresis (see 8.7 and Figure 5) of the digested DNAs was performed.

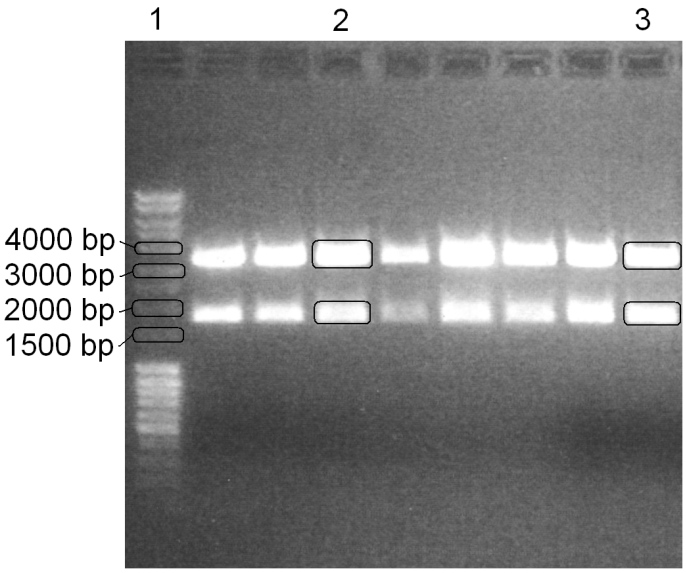

Figure 5 **Restriction digestion agarose gel** for pKKW6.3 and pKKW7.4 with EcoRI and XhoI MassRuler™ (1) as standard, pKKW6.3 (2) and pKKW7.4 (3)

The data from the digestions confirmed that KKW6.3 and KKW7.4 harbored the construct consisting of pPICZ B and the inserts of interest.

Additionally for each of pKKW6.3 and pKKW7.4, 2 plasmid-DNA-sequence-analyses (see 8.18) were ordered. Sequencing order information and formulas are in Table 25. For the sequencing results see 12.10.

Number	Primer	DNA	Volumes [µl]		
			DNA	RO Water	Primer
s1	5AOX	pKKW6.3	3	9	-
s2	3AOX	pKKW6.3	3	9	-
s3	5AOX	pKKW7.4	3	9	-
s4	3AOX	pKKW7.4	3	9	-

Table 25 **Sequencing order information and formulas** for pKKW6.3 and pKKW7.4

The sequence comparisons (see 8.19) of the data with the expectation can be seen in 12.10.1 and Table 63 for pKKW6.3 and Table 64 and Table 65 for pKKW7.4.

All four analyses confirmed the expected sequences for pKKW6.3 and pKKW7.4 in the regions that had been modified through PCR-mutagenesis (see 9.2), restriction-digestions (see 9.3.1 and 9.3.2) and ligation (see 9.3.3). All four mismatches on the

edges of the received sequences could be neglected, because in these areas the method used for sequencing had lower fidelity and the affected bases were out of the ORF for the gene-coding sequences of interest.

9.4 Mutagenesis of the NCBI-Sequence into the Broad Institute-Sequence

After the construction of expression vectors (pKKW6.3 and pKKW7.4) which encoded for the NCBI sequence XP_001833871.1 (CcPDH and CcPDH-HIS), the aim of the mutagenesis was to get expression vectors for the Broad Institute sequence XP_001833871.2 (CcPDH-mod and CcPDH-mod-HIS).

In Table 26 the proteins CcPDH and CcPDH-mod are compared. The same amino acids are different for histidine tagged proteins CcPDH-HIS and CcPDH-mod-HIS.

```
1:     1 MGVFSRIALSGLYINLALGAVYNSVAELPTDVEFDFIVAGGGTAGPVIASRLAENPDFQV   60
2:     1 MGVFSRIALSGLYINLALGAVYNSVAELPTDVEFDFIVAGGGTAGPVIASRLAENPDFQV   60

1:    61 LLVEAGGD-----NEGDINFVVPGFQRRLSSSYNWGFQTIGQTGLNGRTLNYARGKVLGG  115
2:    61 LLVEAGGERYPLSNEGDINFVVPGFQRRLSSSYNWGFQTIGQTGLNGRTLNYARGKVLGG  120

1:   121 SSSTNGMVYNRGSAQDYNRWANVTGDDGWKWENLLPSIKRGEKWVLPADGRSVDGAYNPD  175
2:   116 SSSTNGMVYNRGSAQDYNRWANVTGDDGWKWENLLPSIKRGEKWVLPADGRSVDGAYNPD  180

1:   181 AHGYDGELLITNFNTPPTDFDRRVQDNFNEEFPFCLDVNDGNNIGACPTQYTIGYGERSS  235
2:   176 AHGYDGELLITNFNTPPTDFDRRVQDNFNEEFPFCLDVNDGNNIGACPTQYTIGYGERSS  240

1:   241 AATAFVSTEHRNRPNFHVLLNTYVTRVLGTGDNALDFRTIEVAADSASPRQTIVASKEVV  295
2:   236 AATAFVSTEHRNRPNFHVLLNTYVTRVLGTGDNALDFRTIEVAADSASPRQTIVASKEVV  300

1:   301 LSAGAFGSPQILLNSGIGPREELEEVGVESVLDIPDVGKNLQDHPASFAMWLANGQPSPA  355
2:   296 LSAGAFGSPQILLNSGIGPREELEEVGVESVLDIPDVGKNLQDHPASFAMWLANGQPSPA  360

1:   361 VDEAEAFAQWQQNRSGPLTDPGSHYIVWSRIPANASIFQEYPDDQTAPGAPHIELAISGS  415
2:   356 VDEAEAFAQWQQNRSGPLTDPGSHYIVWSRIPANASIFQEYPDDQTAPGAPHIELAISGS  420

1:   421 GPTVAASVLLLNPASRGSVKIRSNNPFDPPVIDLGFLTHRYDILAFVEGIRSAWRYFAGD  475
2:   416 GPTVAASVLLLNPASRGSVKIRSNNPFDPPVIDLGFLTHRYDILAFVEGIRSAWRYFAGD  480

1:   481 GFKDHVVAPITANPDTTPLEEIEQQLRNGVGTTLHVSGSVAMSARGASNGVLDPDLKVKG  535
2:   476 GFKDHVVAPITANPDTTPLEEIEQQLRNGVGTTLHVSGSVAMSARGASNGVLDPDLKVKG  540

1:   541 ATGLRVADASIMPYITTGHTVGAVYVIGERAADIIKADWS                      575
2:   536 ATGLRVADASIMPYITTGHTVGAVYVIGERAADIIKADWS                      580
```

Table 26 **Protein sequence comparison of XP_001833871.1 and XP_001833871.2**
The sequence XP_001833871.1 (CcPDH) (1) and the new sequence XP_001833871.2 (CcPDH-mod) (2). The **new amino acids** are colored in green.

To change the protein sequence the DNA sequence had to be changed. The old and the new DNA sequence can be seen in Table 27.

```
1: 1141 GCCGGTGGAGAC---------------AACGAGGGAGACATCAACTTCGTCGTCCCTGGT 1185
2:      GCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAGACATCAACTTCGTCGTCCCTGGT
```

Table 27 **DNA sequence comparison of CcPDH and CcPDH-mod**
The sequence (pKKW6.3 and pKKW7.4; CcPDH and CcPDH-HIS) (1) and the new sequence (CcPDH-mod and CcPDH-mod-HIS) (2). The **new bases** are colored in green, the **old bases** in red.

To accomplish the mutagenesis a modified insert was constructed from the original vector by several PCRs. The modified insert was then inserted back into the corresponding empty original vector. This was done twice - one time for pKKW6.3 and the other time for pKKW7.4.

9.4.1 Insert Construction

For this purpose 2 PCR-constructs were formed and fused together by PCR again twice - one time for pKKW6.3 and the other time for pKKW7.4. After that the constructs were prepared for insertion by cutting with restriction enzymes.

9.4.1.1 PCR Short Fragment

The primers 5AOX and KKWp6a (see 12.9) were used on pKKW6.3 (see 9.3) or on pKKW7.4 (see 9.3) as template. This is shown in the following table:

Name	Sequence
5AOX	gactggttccaattgacaagc>
Template (bp855-bp1149)	-gactggttccaattgacaagc...ttccaggtcttgttggttgaggccggtgga- -ctgaccaaggttaactgttcg...aaggtccagaacaaccaactccggccacct-
KKWp6a	<aaggtccagaacaaccaactccggccacct

Table 28 **PCR** for short construct from pKKW6.3 or pKKW7.4

The PCRs were going to deliver short unmodified fragments of the template DNAs which will be referred to as pKKW6.3-sc or pKKW7.4-sc.
The PCRs (see 8.6) were carried out and the DNA from the PCR-samples was purified by DNA-gel-electrophoresis (see 8.7).
The gel slices containing pKKW6.3-sc and pKKW7.4-sc were cut out and their DNA eluted (see 8.3).

9.4.1.2 PCR Long Fragment

The primers KKWp6s and 3AOX (see 12.9) were used on pKKW6.3 (see 9.3) or on pKKW7.4 (see 9.3) as template. This is shown in the following table:

Name	Sequence
KKWp6s	tgttggttgaggccggtggagaAAGATACCCATTGTCTaacgagggag>
Template (bp1130 -bp2874 [pKKW6.3]) -bp2872 [pKKW7.4])	-tgttggttgaggccggtggagac----------aacgagggag...ggatgtcagaatgccatttgc- -acaaccaactccggccacctctg----------ttgctccctc...cctacagtcttacggtaaacg-
3AOX	<cctacagtcttacggtaaacg

Table 29 **PCR** for long construct from pKKW6.3 or pKKW7.4
The **new bases** are colored in green and the **ones to replace** are colored in red.

The PCRs were going to deliver long modified fragments of the template DNA which will be referred to as pKKW6.3-lc and pKKW7.4-lc.
The PCRs (see 8.6) were carried out and the DNA from the PCR-samples was purified by DNA-gel-electrophoresis (see 8.7).
The gel slices containing pKKW6.3-lc and pKKW7.4-lc were cut out and its DNA eluted (see 8.3).

9.4.1.3 PCR Fusion

To fuse the short (see 9.4.1.1) and long construct (see 9.4.1.2) together a PCR was used twice – one time for pKKW6.3 and the other time for pKKW7.4.
Therefore the primers 5AOX and 3AOX were used on pKKW6.3-sc and pKKW6.3-lc or pKKW7.4-sc and pKKW7.4-lc simultaneously as templates. This is shown in the following table:

Name	Sequence
5AOX	gactggttccaattgacaagc>
1st template (sc)	gactggttccaattgacaagc...tgttggttgaggccggtgga>
2nd template (lc)	<acaaccaactccggccacctctTTCTATGGGTAACAGAttgctccctc...cctacagtcttacggtaaacg
3AOX	<cctacagtcttacggtaaacg

Table 30 **PCR** for fusion from pKKW6.3
The **new bases** are colored in green.

The PCRs were going to deliver fusions of the two fragments of the template DNA which will be referred to as pKKW6.3-fu or pKKW7.4-fu.
The PCRs (see 8.6) were carried out and the DNA from the PCR-samples was purified by DNA-gel-electrophoresis (see 8.7).
The gel slices containing pKKW6.3-fu and pKKW7.4-fu were cut out and its DNA eluted (see 8.3).

9.4.1.4 Preparation of the PCR Fusion Constructs

Therefore pKKW6.3-fu (see 9.4.1.3) and pKKW7.4-fu (see 9.4.1.3) were digested (see 8.10) with EcoRI and XhoI for 2.5 hours at 37°C which led to 3 fragments each (90bp, 197bp and 1749bp for pKKW6.3-fu or 1747bp for pKKW7.4-fu), the longest always being the prepared insert.
The mixtures were purified by DNA-gel-electrophoresis (see 8.7).
The gel slices containing pKKW6.3-fu and pKKW7.4-fu were cut out and their DNA (referred to as pKKW6.3-fur or pKKW7.4-fur) eluted (see 8.3).

9.4.1.5 Quantification of pKKW6.3-fur and pKKW7.4-fur

To determine the concentration of DNA in the elution of pKKW6.3-fur and pKKW7.4-fur a quantification DNA gel electrophoresis (see 8.7.3) was conducted.
The so determined DNA concentrations are for pKKW6.3-fur and pKKW7.4-fur are listed in Table 31:

Name	DNA Concentration [ng/µl]	DNA Concentration [ng*µl^{-1}*kbp^{-1}]
pKKW6.3-fur	4.26	2.44
pKKW7.4-fur	1.02	0.58

Table 31 **DNA concentrations of** pKKW6.3-fur and pKKW7.4-fur

9.4.2 Vector Construction

For this purpose the original vectors (pKKW6.3 and pKKW7.4) were cut by the same restriction enzymes as the prepared inserts (see 9.4.1.4) and separated from their former inserts.

9.4.2.1 Preparation of the Vectors

Therefore pKKW6.3 (see 9.3) and pKKW7.4 (see 9.3) were digested (see 8.10) with EcoRI and XhoI for 2.5 hours at 37°C which led to 2 fragments each (3290bp and 1734bp for pKKW6.3 or 1732bp for pKKW7.4), the longer always being the prepared vector.

The mixtures were purified by DNA-gel-electrophoresis (see 8.7).

The gel slices containing pKKW6.3 and pKKW7.4 were cut out and their DNA (referred to as pKKW6.3-vr or pKKW7.4-vr) eluted (see 8.3).

9.4.2.2 Quantification of pKKW6.3-vr and pKKW7.4-vr

To determine the concentration of DNA in the elution of pKKW6.3-vr and pKKW7.4-vr a quantification DNA gel electrophoresis (see 8.7.3) was conducted.

The so determined DNA concentrations are for pKKW6.3-vr and pKKW7.4-vr are listed in Table 32:

Name	DNA Concentration [ng/µl]	DNA Concentration [ng*µl^{-1}*kbp^{-1}]
pKKW6.3-vr	6.05	1.84
pKKW7.4-vr	2.31	0.70

Table 32 **DNA concentrations of** pKKW6.3-vr and pKKW7.4-vr

9.4.3 Ligation of Inserts with Vectors, Transformations and Preparations

The insert pKKW6.3-fur or pKKW7.4-fur was mixed with the corresponding vector pKKW6.3-vr or pKKW7.4-vr and ligated for 1 hour (see 8.17, Table 33 and Table 34).

DNA type	Ligated Construct	
	pKKW10	pKKW11
Vector	pKKW6.3-vr	pKKW7.4-vr
Insert	pKKW6.3-fur	pKKW7.4-fur

Table 33 **Ligation components** for pKKW10 and pKKW11

Component	Volume [µl]		Remarks
	pKKW10	pKKW11	
Vector	5.5 (=33.28ng)	3.9 (=9.01ng)	
Insert	12.3	13.9	1:3 molar ratio over vector
T4 DNA-Ligase	0.2		
T4-DNA-Ligase-Buffer	2		

Table 34 **Ligation formula** for pKKW10 and pKKW11

5µl of each ligated DNA-construct were transformed into *E. coli* (see 8.3). The cells were then spread on LB-Zeo-plates (see 8.2.5) and cultivated overnight at 37°C.
One colony containing the vector with the insert from pKKW6.3-fur was named KKW10.1. Another colony containing the vector with the insert from pKKW7.4-fur was named KKW11.1. Both were transferred onto LB-Zeo-agar-plates (see 8.2.5) and cultivated overnight at 37°C. Plasmid DNAs from KKW10.1 and KKW11.1 (referred to as pKKW10.1 and pKKW11.1) were isolated by plasmid mini preparations (see 8.3). KKW10.1 and KKW11.1 were added to the culture collection (see 8.16 and 12.12.1).

9.4.4 Verification of the Vectors pKKW10.1 and pKKW11.1

To verify the validity of the vectors pKKW10.1 (see 9.4.3) and pKKW11.1 (see 9.4.3), 2 restriction-digestions (see 8.10) with EcoRI and XhoI were conducted which each led to 2 fragments (1749bp and 3290bp long for pKKW10.1 and 1747bp and 3290bp long for pKKW11.1).

A DNA-gel-electrophoresis (see 8.7 and Figure 6) of the digested DNAs was performed.

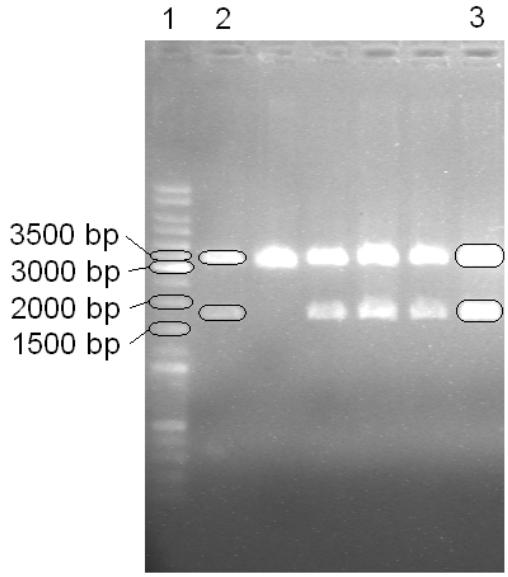

Figure 6 **Restriction digestion agarose gel** for pKKW10.1 and pKKW11.1 with EcoRI and XhoI
GeneRuler™ (1) as standard, pKKW10.1 (2) and pKKW11.1 (3)

The data from the digestions confirmed that KKW10.1 and KKW11.1 harbored the construct consisting of pPICZ B and the inserts of interest.
Additionally for each of pKKW10.1 and pKKW11.1, 2 plasmid-DNA-sequence-analyses (see 8.18) were ordered. Sequencing order information and formulas are in Table 35. For the sequencing results see 12.10.

Number	Primer	DNA	Volumes [µl]		
			DNA	RO Water	Primer
s5	5AOX	pKKW10.1	6	6	-
s6	5AOX	pKKW11.1	6	6	-
s7	3AOX	pKKW10.1	6	6	-
s8	3AOX	pKKW11.1	6	6	-

Table 35 **Sequencing order information and formulas** for pKKW10.1 and pKKW11.1

The sequence comparisons (see 8.19) of the data with the expectation can be seen in Table 66 and Table 67 for pKKW10.1 and in Table 68 and Table 69 for pKKW11.1. All four analyses confirmed the expected sequences for pKKW10.1 and pKKW11.1 in the regions that had been modified through PCR-mutagenesis (see 9.4.1), restriction-

digestions (see 9.4.1.4 and 9.4.2.1) and ligation (see 9.4.3). The mismatches on the edges of the received sequences could be neglected, because in these areas the method used for sequencing had lower fidelity and the affected base was either out of the ORF for the gene-coding sequences of interest or it was identical to an adjacent base.

9.5 Transformations of *P. pastoris* with pKKW6.3, pKKW7.4, pKKW10.1 and pKKW11.1

P. pastoris was going to be transformed with pKKW6.3 (see 9.3), pKKW7.4 (see 9.3), pKKW10.1 (see 9.4) and pKKW11.1 (see 9.4). All Constructs were formed by the pPICZ B vector and an insert (for further detail see Table 36).

Vector	Insert
pKKW6.3	CcPDH
pKKW7.4	CcPDH-HIS
pKKW10.1	CcPDH-mod
pKKW11.1	CcPDH-mod-HIS

Table 36 **Insert overview** of pKKW6.3, pKKW7.4, pKKW10.1 and pKKW11.1

After integration of the vectors into the genome of *P. pastoris*, the organism should be able to express the corresponding protein. To integrate the vector, it had to be linearized by a restriction-digest (see 8.10) first. Next the DNA was taken up by *P. pastoris*, after electroporation (see 8.5).

9.5.1 Linearization

For linearization of pKKW6.3, pKKW7.4, pKKW10.1 and pKKW11.1, they were digested (see 8.10) with SacI for about 18 hours at 37°C, which led to 1 fragment for each vector (see Table 37).

Vector	Fragment Length [bp]
pKKW6.3	5024
pKKW7.4	5022
pKKW10.1	5039
pKKW11.1	5037

Table 37 **Fragment length** of linearized pKKW6.3, pKKW7.4, pKKW10.1 and pKKW11.1

The mixture was purified by DNA-gel-electrophoresis (see 8.7).

The gel slices containing the linearized DNA were cut out and its DNAs (referred to as pKKW6.3-l, pKKW7.4-l, pKKW10.1-l and pKKW11.1-l) eluted (see 8.3) in RO water (see Table 38).

Fragment	Elution Volume [µl]
pKKW6.3-l	30
pKKW7.4-l	30
pKKW10.1-l	50
pKKW11.1-l	50

Table 38 **Elution volume** of pKKW6.3-l, pKKW7.4-l, pKKW10.1-l and pKKW11.1-l

9.5.2 Transformation

The linearized DNA-vectors pKKW6.3-l, pKKW7.4-l, pKKW10.1-l and pKKW11.1-l were transformed into *P. pastoris* by electroporation (see 8.5). Therefore the transformation-mixtures were prepared (see Table 39) and electroporated under specified conditions (see Table 40).

Component	Volume [µl]			
	KKW6.3.1	KKW7.4.1	KKW10.1.1	KKW11.1.1
DNA	2		8	
Electrocompetent cells	50		100	

Table 39 **Electroporation formula** for KKW6.3.1, KKW7.4.1, KKW10.1.1 and KKW11.1.1

Voltage [kv]	Resistance [ohm]	Pulse Length [ms]
1.5	125	3

Table 40 **Electroporation conditions** for KKW6.3.1, KKW7.4.1, KKW10.1.1 and KKW11.1.1

The cells were then spread on YPD-Zeo-agar-plates (see 8.2.6) and cultivated at 30°C for a certain amount of time (see Table 41).

Component	Cultivating Time [d]
KKW6.3.1	3
KKW7.4.1	3
KKW10.1.1	5
KKW11.1.1	5

Table 41 **Breeding time** for KKW6.3.1, KKW7.4.1, KKW10.1.1 and KKW11.1.1

One colony of each *P. pastoris* transformed with pKKW6.3-I, pKKW7.4-I, pKKW10.1-I and pKKW11.1-I were transferred onto YPD-Zeo-agar-plates (see 8.2.6) and cultivated for 3 days at 30°C.

These organisms were called KKW6.3.1, KKW7.4.1, KKW10.1.1 and KKW11.1.1. They should be able to express the protein of interest with and without a C-terminal histidine-tag.

KKW6.3.1, KKW7.4, KKW10.1.1 and KKW11.1.1 were added to the culture collection (see 8.16 and 12.12.2).

9.5.3 Verification of KKW10.1.1

9.5.3.1 General

After the integration of pKKW10.1 into *P. pastoris* part of its sequence (CcPDH-mod) was confirmed to make sure that the expressed protein would be based on the correct DNA sequence.

Therefore 2 overlapping sections (see Figure 7) of the genomic DNA were amplified by *P. pastoris* colony PCR (see 8.6.1). These sections which covered the whole CcPDH-mod were sequenced (see 8.18) and the sequence data was compared to the expected sequence (see 8.19).

Figure 7 **Sequencing scheme** for KKW10.1.1
Ruler [bp], genomic DNA with the **gene of interest CcPDH-mod** and the 2 **overlapping sections**

9.5.3.2 Preparation of Forward Section

To obtain PCR product from the forward section (referred to as KKW10.1.1-fs), the primers 5AOX and KKWp4a were used on genomic DNA from KKW10.1.1 as template. This is shown in the following table:

Name	Sequence
5AOX	gactggttccaattgacaagc>
KKW10.1.1 (1834bp)	-gactggttccaattgacaagcttttg...agagagctgccgatatcattaaggccgactggtct- -ctgaccaaggttaactgttcgaaaac...tctctcgacggctatagtaattccggctgaccaga
KKWp4a	<cgacggctatagtaattccggctgaccaga

Table 42 **PCR** for KKW10.1.1-fs

The genomic DNA was prepared as described in 8.6.1. To gather enough DNA a big PCR was performed (see 8.6).
The DNA from the PCR-samples was purified by DNA-gel-electrophoresis (see 8.7). The only band visible was cut out and the DNA eluted (see 8.3) in 25µl.
To determine the concentration of DNA in the elution a quantification DNA gel electrophoresis (see 8.7.3) was conducted.
The so determined DNA concentration for KKW10.1.1-fs was 112.14ng/µl.

9.5.3.3 Preparation of Backward Section

To obtain PCR product from the backward section (referred to as KKW10.1.1-bs), the primers KKWp6s and 3AOX were used on genomic DNA from KKW10.1.1 as template. This is shown in the following table:

Name	Sequence
KKWp6s	tgttggttgaggccggtggagaaagatacccattgtctaacgagggag>
KKW10.1.1 (1770bp)	-tgttggttgaggccggtggagaaagatacccattgtctaacgagggagacatc...caagaggatgtcagaatgccatttgc- -acaaccaactccggccacctctttctatgggtaacagattgctccctctgtag...gttctcctacagtcttacggtaaacg-
3AOX	<cctacagtcttacggtaaacg

Table 43 **PCR** for KKW10.1.1-bs

The genomic DNA was prepared as described in 8.6.1. To gather enough DNA a big PCR was performed (see 8.5).
The DNA from the PCR-samples was purified by DNA-gel-electrophoresis (see 8.7). The only band visible was cut out and the DNA eluted (see 8.3) in 25µl.

To determine the concentration of DNA in the elution a quantification DNA gel electrophoresis (see 8.7.3) was conducted.
The so determined DNA concentration for KKW10.1.1-bs was 56.32ng/µl.

9.5.3.4 Sequencing and Sequence Comparison

Sequencing (see 8.18) of the PCR products KKW10.1.1-fs (see 9.5.3.2) and KKW10.1.1-bs (see 9.5.3.3) was ordered. Sequencing order information and formulas are in Table 44. For the sequencing results see 12.10.

Number	Primer	DNA	Volumes [µl]		
			DNA	RO Water	Primer
s9	5AOX	KKW10.1.1-fs	3.57	6.43	-
s10	3AOX	KKW10.1.1-bs	7.1	2.9	-
s11	KKWp4a	KKW10.1.1-fs	3.57	6.43	4
s12	KKWp6s	KKW10.1.1-bs	7.1	2.9	4

Table 44 **Sequencing order information and formulas** for KKW10.1.1-fs and pKKW10.1.1-bs

The sequence comparisons (see 8.19) of the data with the expectation can be seen in 12.10.9.
All four analyses confirmed the expected sequence for KKW10.1.1 in the region that is coding for the protein. The mismatches on the edges of the received sequences could be neglected, because in these areas the method used for sequencing had lower fidelity, the affected base was either out of the ORF for the gene-coding sequences of interest or it was identical to an adjacent base. Moreover for the whole gene sequence every base was confirmed by the received sequences at least once.

9.6 Protein Expression with All Modified Organisms

After the *P. pastoris* organisms were created (see 9.5), they were used for protein expression. For this purpose the pump-tube-system was used (see 8.21) with multiple flasks. The proteins to be produced were expected in the supernatant because they were carrying their original exporting sequence from *C. cinerea*, which should enable *P. pastoris* to export them also.

9.6.1 Used Cultures

Following organisms were prepared as described and used for the protein expression (see 8.21):

Culture Number	Name	Expressed Protein	OD_{600} of Preculture (25ml)		Remark
			Calc.	Diluted (1:20)	
1	AbPDH	AbPDH	11.6	0.578	Positive Control
2	AmPDH	AmPDH	11.3	0.564	Positive Control
3	X33	-	10.9	0.546	Negative Control
4	KKW6.3.1	CcPDH	10.9	0.495	
5	KKW7.4.1	CcPDH-HIS	9.8	0.492	
6	KKW10.1.1	CcPDH-mod	9.4	0.470	
7	KKW11.1.1	CcPDH-mod-HIS	10.6	0.530	

Table 45 **Organsim scheme** for pump-tube-system

9.6.2 Protein Expression Phase

The protein expression was conducted as described in 8.21.

After putting the 25ml culture into the BMMY the tubes were filled with methanol, which already started the protein expression. The starting point of the protein expression is therefore the time of tube-filling. During the protein expression, samples were taken regularly and analyzed. The OD_{600} (see 8.11) of the cell suspension was measured. The protein concentration (see 8.12) and PDH activity (see 8.13.1) of the supernatant were analyzed. For a detailed list with the results see 12.13. The data are shown in Figure 8, Figure 9 and Figure 10.

9.6.3 Cell Density Progression

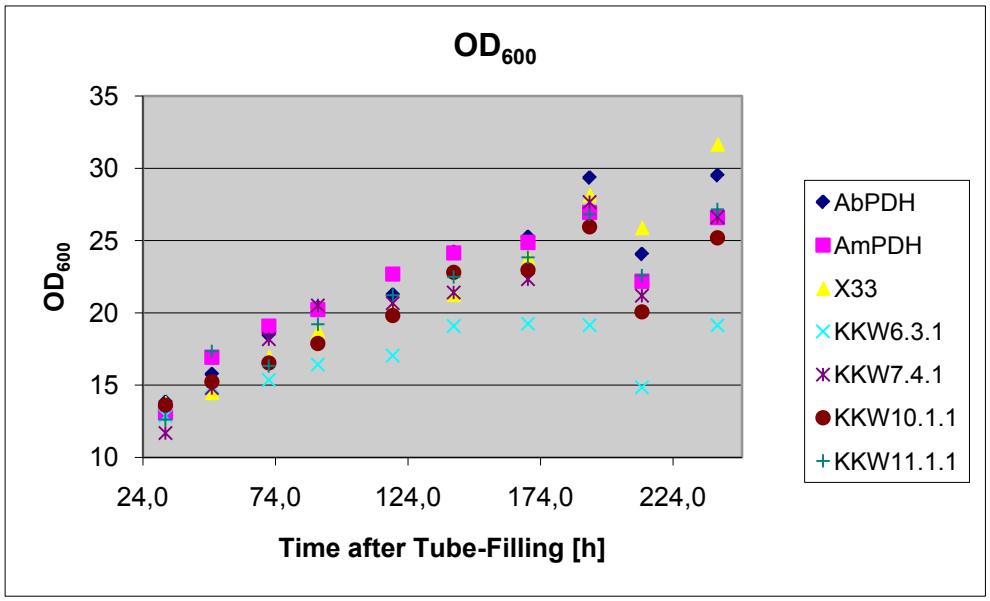

Figure 8 **OD_{600} data** of "Protein Expression with All Modified Organisms"

As can be seen in Figure 8 the cell density increased throughout the whole protein expression. The second last sample is thought to be an outlier. If samples are left alone for too long the cells go down to the bottom and are not able to be brought back into suspension again by vortexing. The result for the cell density is then lower than usual.

9.6.4 Protein Concentration Progression

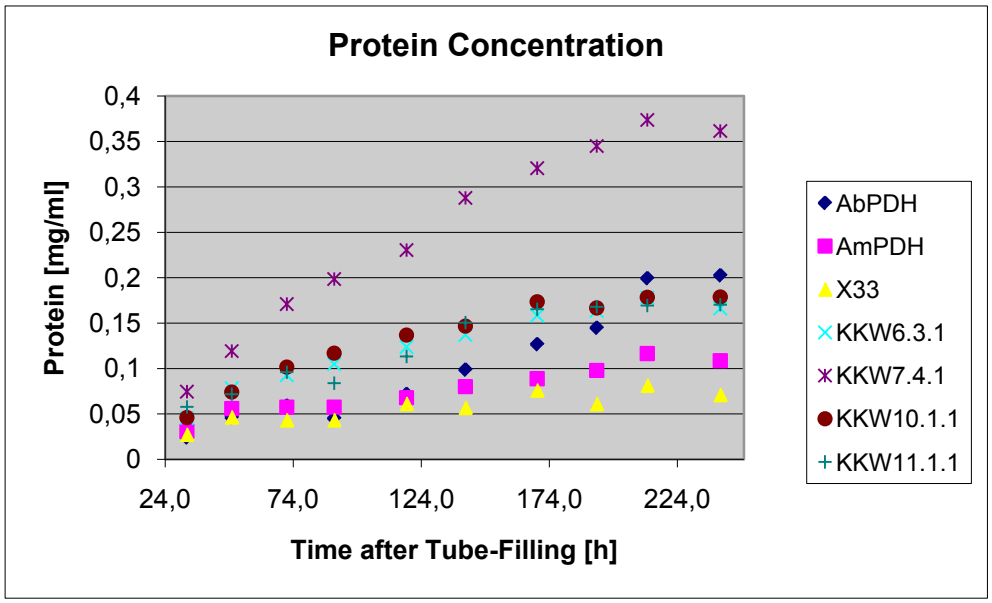

Figure 9 **Protein concentration data** of "Protein Expression with All Modified Organisms"

As you can see in Figure 9 the protein concentration in the supernatant started at a high level and increased throughout the whole protein expression. It should be noted that the protein in the supernatant consisted of mostly yeast extract and peptone from casein at the beginning and expressed protein and lysed cells protein at the end.

9.6.5 PDH Activity Progression

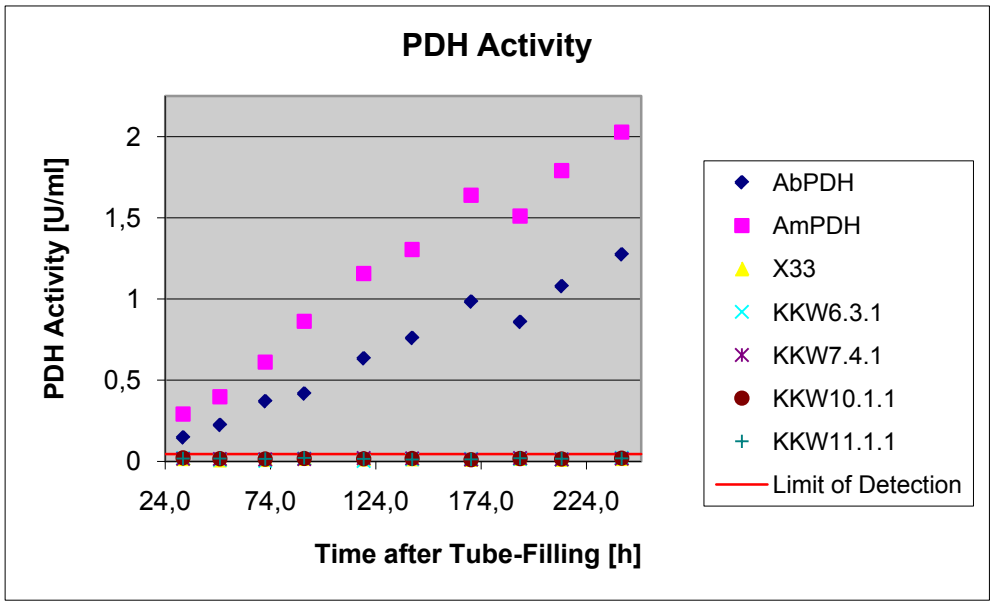

Figure 10 **PDH activity data** of "Protein Expression with All Modified Organisms"

The mean value from all negative control activities (see 12.13) multiplied by 3 represents the limit of detection for PDH activity. The limit of detection is therefore 0.045U/ml.

As can be seen in Figure 10 the PDH activity in the supernatant increased throughout the whole protein expression. As one can see only the positive controls showed activity and moreover only they could exceed the limit of detection for PDH activity (see 12.13). The activity in the samples taken first is due to the small amount methanol that entered the system during the tube-filling.

9.6.6 Protein-Gel-Electrophoresis

To further analyze the expressed protein of the supernatant, a protein-gel-electrophoresis (see 8.8) was conducted and the gel was scanned (see Figure 11).

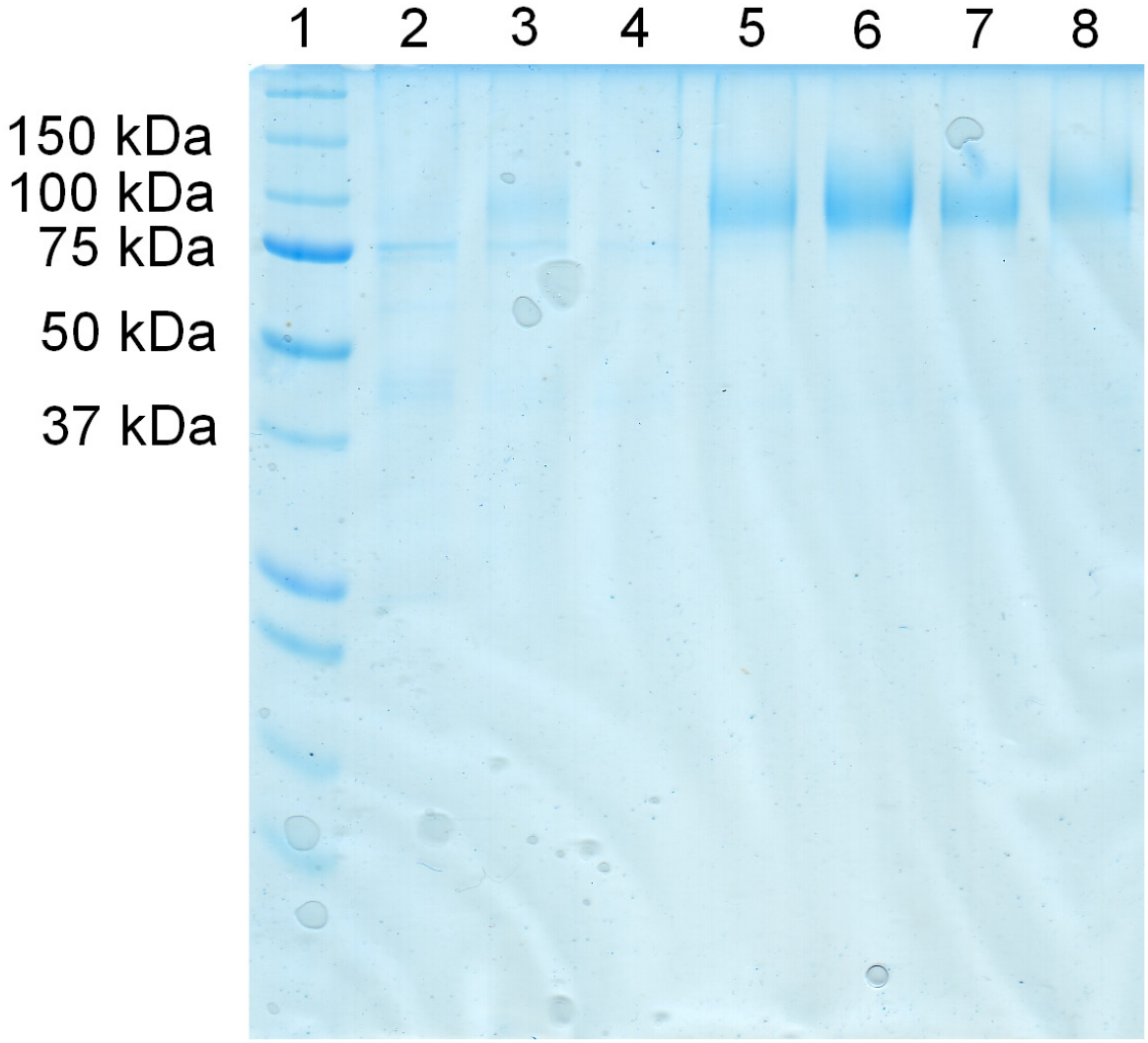

Figure 11 **Protein gel** for AbPDH, AmPDH, X33, KKW6.3.1, KKW7.4.1, KKW10.1.1 and KKW11.1.1
Precision Plus Protein™ Standards Dual Color (1) as standard, AbPDH (2) and AmPDH (3) as positive controls, X33 (4) as negative control and KKW6.3.1 (5), KKW7.4.1 (6), KKW10.1.1 (7) and KKW11.1.1 (8)

The bands at 75kDa in all controls (see Figure 11 lanes 2 to 4) are considered protein from dead cells, because they were present in the negative control.

The bands at around 100kDa in one positive control and all samples (see Figure 11 lane 3 and 5 to 8) are considered PDH. The absence of such a band for AbPDH can be explained by taking a look at the 75kDa bands. These are strongest compared to

all other cultures indicating that most protein originated from dead cells and not expressed PDH.

All samples show a strong broad protein band at the same size as the positive control AmPDH. The proteins of interest are therefore considered to have been expressed and exported out of the cells.

9.6.7 Protein-Sequencing of KKW10.1.1

To ensure that the detected proteins that were considered to be the proteins of interest, protein sequencing was ordered (see 8.9). The protein from the only visible band of KKW10.1.1 (see Figure 11 lane 7) was target of this analyses. The fragment file was analyzed with the Mascot search engine. The results can be seen in the following table:

Rank	Protein	GI-Number	NCBI Reference Sequence	Score
1	CcPDH-mod	299740608	XP_001833871.2	1197
2	various	-	-	139

Table 46 **Protein-sequencing** of KKW10.1.1

The protein CcPDH-mod covered with the received peptides can be seen in the following table:

```
  1 MGVFSRIALS GLYINLALGA VYNSVAELPT DVEFDFIVAG GGTAGPVIAS
 51 RLAENPDFQV LLVEAGGERY PLSNEGDINF VVPGFQRRLS SSYNWGFQTI
101 GQTGLNGRTL NYARGKVLGG SSSTNGMVYN RGSAQDYNRW ANVTGDDGWK
151 WENLLPSIKR GEKWVLPADG RSVDGAYNPD AHGYDGELLI TNFNTPPTDF
201 DRRVQDNFNE EFPFCLDVND GNNIGACPTQ YTIGYGERSS AATAFVSTEH
251 RNRPNFHVLL NTYVTRVLGT GDNALDFRTI EVAADSASPR QTIVASKEVV
301 LSAGAFGSPQ ILLNSGIGPR EELEEVGVES VLDIPDVGKN LQDHPASFAM
351 WLANGQPSPA VDEAEAFAQW QQNRSGPLTD PGSHYIVWSR IPANASIFQE
401 YPDDQTAPGA PHIELAISGS GPTVAASVLL LNPASRGSVK IRSNNPFDPP
451 VIDLGFLTHR YDILAFVEGI RSAWRYFAGD GFKDHVVAPI TANPDTTPLE
501 EIEQQLRNGV GTTLHVSGSV AMSARGASNG VLDPDLKVKG ATGLRVADAS
551 IMPYITTGHT VGAVYVIGER AADIIKADWS
```

Table 47 **Protein sequence coverage** of pKKW10.1.1 with the sequence data
The expected CcPDH-mod covered with the received peptide sequencesa from Mascot search. The **peptides covering the protein** are colored in green.

The only protein that was detected with significant score was CcPDH-mod. This confirmed the previous consideration (see 9.6.6), that the protein was expressed and exported out of the cell successfully.

9.7 Protein Expression with KKW6.3.1 and KKW7.4.1

Before all the *P. pastoris* organisms were used for protein expression (see 9.6), two of them were used for protein expression in a fermenter. For this purpose the Multifors-system was used (see 8.22) with 4 units. The proteins to be produced were expected in the supernatant because of the results from the other protein expression (see 9.6).

9.7.1 Used Cultures

Following organisms were prepared as described and used for the protein expression (see 8.22). All used precultures are listed in Table 48. Cell densities and wet cell mass per ml after centrifugation with 16000g at 4°C for 5 minutes are also listed below:

Culture Number	Name	Expressed Protein	OD_{600} (see 8.11)		Wet Cell Mass [g/l]	Remark
			Calc.	Diluted (1:20)		
1	X33	-	10.2	.511	43.2	neg. control
2	KKW6.3.1	CcPDH	6.64	.332	28.5	
3	KKW7.4.1	CcPDH-HIS	7.64	.382	34.8	
4	AbPDH	AbPDH	9.15	.458	47.4	pos. control

Table 48 **Organsim scheme and data listing** of the precultures for the Multifors-system

9.7.2 Early Fermentation Phase (Cell Production)

The protein expression was conducted as described in 8.22.
The fermenter was set to a pH of 5. The fermenters were inoculated with 12ml of the precultures and the batch process was started.

3 days later the batch process was finished. Cell densities (see 8.11), wet cell masses per ml after centrifugation with 16000g at 4°C for 5 minutes and PDH activities (see 8.13.1) from the supernatant are listed below:

Culture Number	OD_{600}		Wet Cell Mass [g/l]	PDH Activity [U/ml]	Remark
	Calc.	Diluted (1:50)			
1	37.9	.757	76.4	.013	negative control
2	36.9	.737	74.0	.013	
3	41.9	.839	94.3	.017	
4	36.6	.731	89.0	.009	positive control

Table 49 **Data listing** for the finished batch process in the Multifors-system

The glycerol fed batch process was finished after 26 hours. Cell densities (see 8.11), wet cell masses per ml after centrifugation with 16000g at 4°C for 5 minutes and PDH activities (see 8.13.1) from the supernatant are listed below:

Culture Number	OD_{600} (see 8.11)		Wet Cell Mass [g/l]	PDH activity [U/ml]	Remark
	Calc.	Diluted (1:100)			
1	97.3	.973	157	.008	negative Control
2	90.8	.908	180	.005	
3	97.3	.973	185	.010	
4	89.6	.896	168	.050	positive Control

Table 50 **Data listing** for the finished glycerol based fed batch process in the Multifors-system

The PDH activity of the positive control (see culture number 4 in Table 49 and Table 50) showed that protein was hardly expressed as long as there is no methanol present in the medium.

9.7.3 Late Fermentation Phase (Protein Production)

The protein expression was continued as described in 8.22.

The fermenters were fed with methanol and the pH was changed during the fermentation process.

Samples were taken repeatedly: The OD_{600} (see 8.11) of the cell suspension and the PDH activity (see 8.13.1) of the supernatant were measured.

For a detailed list with the results and pH changes see 12.14. The data are illustrated in Figure 12 and Figure 13.

9.7.4 Wet Cell Mass Progression

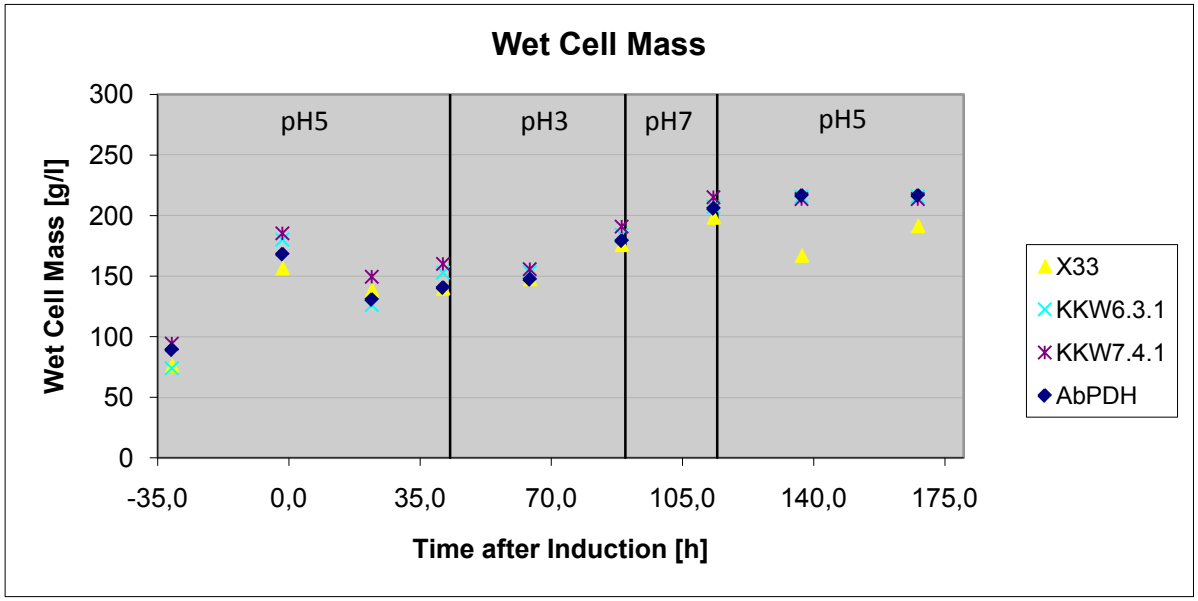

Figure 12 **Wet Cell Mass data** of "Protein Expression with KKW6.3.1 and KKW7.4.1"

As can be seen in Figure 12, the wet cell mass after centrifugation with 16000g at 4°C for 5 minutes increased throughout the whole process to level in the end. The second last sample is thought to be an outlier.

9.7.5 PDH Activity Progression

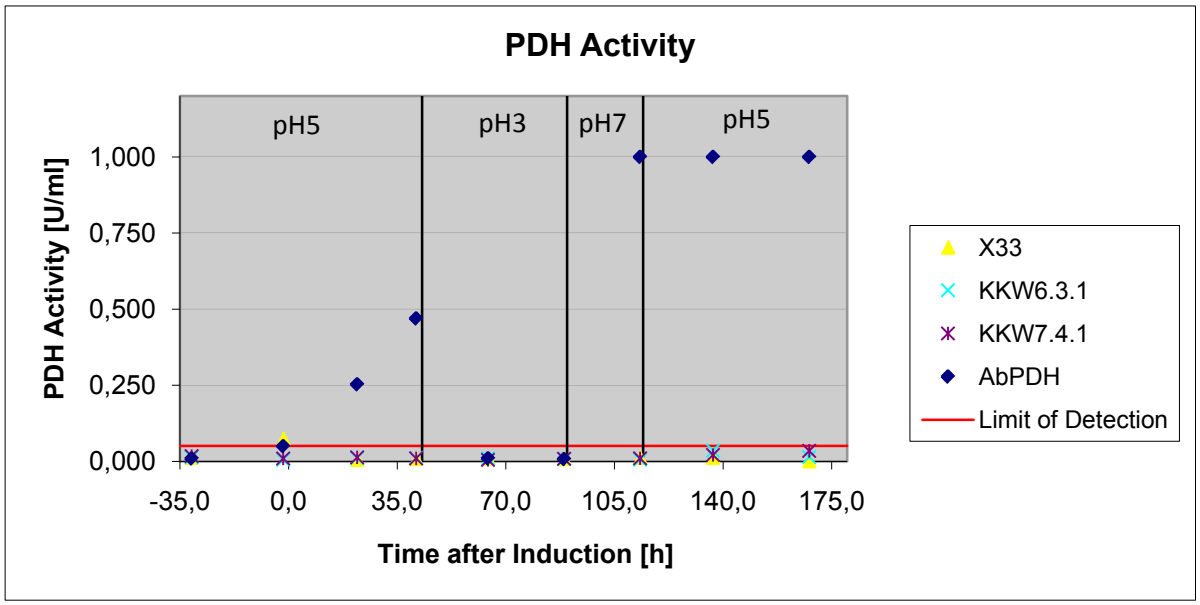

Figure 13 **PDH activity data** of "Protein Expression with KKW6.3.1 and KKW7.4.1"
After a first positive activity assay the activity was not measured with a dilution for a second time. Therefore an activity of 1 actually means an activity greater than 1.

The mean value from all negative control activities (see 12.14) multiplied by 3 represents the limit of detection for PDH activity. The limit of detection is therefore 0.051U/ml.

As can be seen in Figure 13 the PDH activity in the supernatant increased if the pH was 5 or higher. As one can see only the positive control showed activity and moreover only it could exceed the limit of detection for PDH activity (see 12.14). The activitiy in the negative control taken before induction is considered to be an outlier, because the negative control showed more activity than the positive control at this point of time.

9.8 Alternative Enzymatic Activity Measurements

After PDH activity from the expressed proteins could not be detected, a different PDH activity assay was used. Moreover an alternative enzyme activity was taken into consideration. The samples used for those measurements originated from the fermentation (see 9.7).

9.8.1 Identification of Alternative Enzymatic Properties

To identify a possible alternative enzymatic activity of the expressed protein, its sequence (CcPDH) was blasted against the protein database. As can be seen in 12.11, beside pyranose dehydrogenase activity there was aryl-alcohol oxidase activity as an alternative enzymatic activity suggested for CcPDH.

9.8.2 Alternative PDH Activity Assay

To verify the negative PDH activity of CcPDH and CcPDH-HIS the PDH assay using benzoquinone as electron acceptor (see 8.13.2). The samples were taken from the Multifors protein expression 41.1 hours after induction (see 9.7). The results can be seen in the following table:

Culture Number	Protein	PDH activity [U/ml] (see 8.13.2)	Remark
1	-	.017	negative Control
2	CcPDH	.026	
3	CcPDH-HIS	.025	
4	AbPDH	.373	positive Control

Table 51 **Alternative PDH activity assay** for CcPDH and CcPDH-HIS from the Multifors protein expression

If the PDH activity of the negative control is multiplied by 3, the limit of detection will be 0.051. The positive control exceeded the limit of detection showing PDH activity. Neither CcPDH nor CcPDH-HIS exceeded the limit of detection. Therefore the PDH activity of the assayed proteins therefore can be considered as negative.

9.8.3 Aryl-Alcohol Oxidase Activity Assays

The expressed proteins CcPDH and CcPDH-HIS were tested for aryl-alcohol oxidase activity (see 8.14). The samples were taken from the Multifors protein expression 167.8 hours after induction (see 9.7). The results can be seen in the following table:

Culture Number	Protein	Aryl-alcohol oxidase activity (see 8.14)	Remark
1	-	Negative	negative Control
2	CcPDH	Negative	
3	CcPDH-HIS	Negative	
4	AbPDH	Negative	positive Control

Table 52 **Aryl-alcohol oxidase activity assay** for CcPDH and CcPDH-HIS from the Multifors protein expression

The negative control only showed a little absorption change and was considered as a negative reference. The positive control AbPDH did not exceed the threefold absorption change of the negative reference and its aryl-alcohol oxidase activity was therefore considered to be negative. The samples containg CcPDH and CcPDH-HIS also did not exceed the threefold absorption change of the negative reference and their aryl-alcohol oxidase activities were therefore considered to be negative too.

10 RESULTS

At the beginning of the diploma thesis, the vector KKW1 carrying the DNA-sequence CcPDH was received from the company Mr. Gene. CcPDH is coding for a putative PDH from *C. cinerus* (see 9.1).

Originating from this vector 4 expression vectors were created (see 9.2, 9.3 and 9.4). They carry the inserts coding for CcPDH, CcPDH-HIS, CcPDH-mod and CcPDH-mod-HIS. These vectors were tested for correct length and sequence fidelity in sensitive areas with positive results (see 9.3.4 and 9.4.4).

All 4 expression vectors were used to create 4 genetically altered *P. pastoris* organisms (see 9.5). The organism carrying the sequence CcPDH-mod was tested for sequence fidelity covering the whole CcPDH-mod sequence with a positive result (see 9.5.3).

All four organisms were used to produce the proteins of interest (see 9.6 and 9.7):
The cell masses compared to the protein concentration in both protein expressions (see Figure 8 and Figure 9 for 9.6 and Figure 12 for 9.7) showed growing and protein exporting cells. The export was verified by protein-gel-electrophoresis (see 9.6.6) for 9.6. PDH activity could only be detected for the positive controls (see Figure 10). Changes in pH during protein expression did not result in active CcPDH or CcPDH-HIS (see Figure 13).

For the protein CcPDH-mod it was shown that it was produced and exported out of the cells. This was proven by protein-gel-electrophoresis (see 9.6.6) and protein sequencing (see 9.6.7):
The protein was found at around 100kDa. The calculated size for the protein is 62.28kDa. The reason for the high size, it was detected with, is massive glycosylation from *P. pastoris*. The glycosylation pattern is not as distinct as a protein sequence. There are a lot of different ways for the same protein. This explains the wideness of the CcPDH-mod band.
The protein sequencing of this band revealed that it consisted only of the protein of interest, substantiating the results from the protein gel electrophoresis.

The other three proteins are considered to be produced and exported out of the cells. They were also found at the same size and with the same appearance as CcPDH-mod.

The expressed proteins were tested for PDH and aryl-alcohol oxidase activity with negative results (see 9.6.5, 9.7.5 and 9.8). Some reasons for this inactivity of the proteins are discussed in the following chapter.

11 DISCUSSION

Both conducted protein expressions showed that P. pastoris is able to grow and express all four proteins of interest, but only in inactive form. The change of pH during the expression did not lead to the production of active CcPDH or CcPDH either.

It could be proven beyond doubt, that the putative PDH CcPDH-mod was produced and exported.

However, it did show neither PDH activity nor aryl-alcohol oxidase. Reasons for that could be:

- The protein was a PDH and now is relict, because *C. cinerea* does not need it anymore.
- The gene coding for the PDH is not spliced as predicted by *C. cinerea*. Therefore the original mRNA and the in *P. pastoris* transcribed mRNA code for different amino acid sequences. An indication for that gave the change of the amino acid sequence for the gene during the diploma thesis in the NCBI database. That is also the reason why there are CcPDH-mod and CcPDH-mod-HIS. These two sequences represent the actually predicted amino acid sequence.
- It is possible that the protein needs a cofactor to function properly, that was not provided by *P. pastoris*. However it has to be said that *P. pastoris* was chosen especially to counteract such a possibility. The metabolism from *C. cinerea* is not as different from *P. pastoris* as it is from e. g. *E. coli*.
- The high glycosylation and different glycosylation pattern could also be a reason for the inactivity. Glycosylation patterns are different from organism to organism. Also the amount of glycosylation in *P. pastoris* is rather high. This difference could prevent proper folding or impair flexibility of the protein.
- The assistance with folding could be different between *C. cinerea* and *P. pastoris*. A lack of the correct helper protein could make proper and timely folding impossible. However it has to be said that *P. pastoris* was chosen especially to counteract such a possibility. The metabolism from *C. cinerea* is not as different from *P. pastoris* as it is from e. g. *E. coli*.

- The export of the protein in *P. pastoris* could be different from *C. cinerea*. Very often the export sequence is not present after export any more. If it is e. g. truncated differently in the two organisms than this could lead to a change in activity.
- PDH is acting on the key molecule glucose. Therefore it is possible that it is activated posttranslational to prevent its premature activity. This could be realized e. g. by proteolytic cleavage. Therefore it is very likely that *P. pastoris* cannot activate the protein in such a way.
- The instability of the PDH could make detection impossible. This is very unlikely because it is considered to be an extracellular enzyme. Such enzymes are usually very stable.
- Proteases in the extracellular space could have abolished the PDH activity. This is possible, but unlikely, because the positive control would have been subject to proteolytic inactivation as well.
- It is possible, that the PDH does not work at the pH values that were used during activity measurements. This is very unlikely, because pH4.5 with benzoquinone and pH6 with ferricenium were used. Usually enzymes show at least a little activity at pH values that differ from the optimal pH value. It would be reduced, but detectable. Especially extracellular enzymes work in a rather broad range of pH values.
- Both electron acceptors could be unavailable for this PDH. This is unlikely because benzoquinone is a commonly accepted electron acceptor for PDHs.
- Aryl-alcohol oxidase activity was tested, with a designed assay. Because of the lack of aryl-alcohol oxidase, the assay could not be verified by a positive control. Therefore there is the small possibility, that the assay did not detect the existing aryl-alcohol oxidase activity.

P. pastoris was chosen for the production of this PDH, because it is usually able to produce high amounts of functional protein in a rather small volume, at low cost and in an easy way. Now it is obvious, that *P. pastoris* cannot produce this PDH in active form. Therefore, for the time being, it cannot be recommended to do more research in this direction.
First it has to be verified, that a culture of *C. cinerea* is able to show PDH activity.

If so, for further research the PDH will be considered not to be a relict, but to be active. If this is the case, then *C. cinerea* will be able to produce it in its functional form. Therefore it will be reasonable to use *C. cinerea* for production of the protein. It can be expected, that the yield for PDH production in such a way will be low. To counteract this, the used scale will have to be huge. Then the next task will be to establish a method of purification to isolate the protein with PDH activity from the rest. A sequencing of this protein then will reveal its identity.

If in fact CcPDH is responsible for PDH activity, a different organism can be chosen for heterologous protein production. If it is able to produce an active protein in this fashion, it will be possible to further analyze and characterize the PDH.

12 APPENDICES

12.1 Sequence of pKKW1 and pKKW1.2

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGC
CGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGGCCGCTACAGGGCGCTCCCATT
CGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGG
GGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAG
CGCGACGTAATACGACTCACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCGCATAAGCTTGAATTCATGGGAGTC
TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAACTCTGTTGCTGAATTGCCA
ACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTGCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAAC
CCAGATTTCCAGGTCTTGTTGGTTGAGGCCGGTGGAGACAACGAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAG
AGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTGAACGGTAGAACCTTGAACTAC
GCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAAT
AGATGGGCAAATGTCACTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGG
GTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTTACGATGGTGAATTGTTGATC
ACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTG
GACGTCAACGACGGTAACAACATTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCT
ACCGCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACGTCACCAGAGTCTTG
GGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCTAGTCCTAGACAAACAATCGTT
GCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCACCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGA
GAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCT
TCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAATGGCAACAG
AATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTC
CAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTT
GCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCACCT
GTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATAC
TTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACCATTGGAGGAAATC
GAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCA
AACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCCTCCATTATGCCATAC
ATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAGAGAGCTGCCGATATCATTAAGGCCGACTGGTCT
TAGGGACCATTAATTAACTGGGCCTCATGGGCCTTCCTTTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAG
CTGCATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCG
GTCGTTCGGGTAAAGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGA
CAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCG
GATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGT
AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATC
GTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCG
CTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTG
GTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGT
CTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCC
TTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTATTAGAAAAATT
CATCCAGCAGACGATAAAACGCAATACGCTGGCTATCCGGTGCCGCAATGCCATACAGCACCAGAAAACGATCCGCCC
ATTCGCCGCCCAGTTCTTCCGCAATATCACGGGTGGCCAGCGCAATATCCTGATAACGATCCGCCACGCCCAGACGGC
CGCAATCAATAAAGCCGCTAAAACGGCCATTTTCCACCATAATGTTCGGCAGGCACGCATCACCATGGGTCACCACCA
GATCTTCGCCATCCGGCATGCTCGCTTTCAGACGCGCAAACAGCTCTGCCGGTGCCAGGCCCTGATGTTCTTCATCCA
GATCATCCTGATCCACCAGGCCCGCTTCCATACGGGTACGCGCACGTTCAATACGATGTTTCGCCTGATGATCAAACG
GACAGGTCGCCGGGTCCAGGGTATGCAGACGACGCATGGCATCCGCCATAATGCTCACTTTTTCTGCCGGCGCCAGAT
GGCTAGACAGCAGATCCTGACCCGGCACTTCGCCCAGCAGCAGCCAATCACGGCCCGCTTCGGTCACCACATCCAGCA
CCGCCGCACACGGAACACCGGTGGTGGCCAGCCAGCTCAGACGCGCCGCTTCATCCTGCAGCTCGTTCAGCGCACCGC
TCAGATCGGTTTTCACAAACAGCACCGGACGACCCTGCGCGCTCAGACGAAACACCGCCGCATCAGAGCAGCCAATGG
TCTGCTGCGCCCAATCATAGCCAAACAGACGTTCCACCCACGCTGCCGGGCTACCCGCATGCAGGCCATCCTGTTCAA
TCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTA
TTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

Table 53 **Sequence** of pKKW1 and pKKW1.2
CcPDH is colored in cyan.

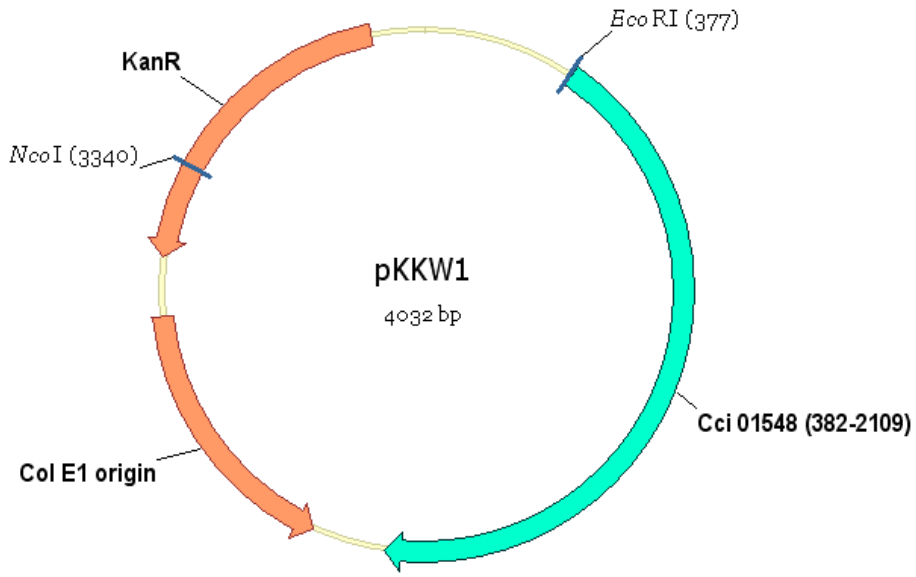

Figure 14 **Vector scheme** for pKKW1 and pKKW1.2 with:
Cci 01548: CcPDH
Col E1 origin: Origin of replication
EcoRI: Restriction site
KanR: Kanamycin resistance gene
XhoI: Restriction site

12.2 Sequence of pKKW4.1

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGC
CGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGGCCGCTACAGGGCGCTCCCATT
CGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGG
GGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAG
CGCGACGTAATACGACTCACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCGCATAAGCTTGAATTCATGGGAGTC
TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAACTCTGTTGCTGAATTGCCA
ACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTGCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAAC
CCAGATTTCCAGGTCTTGTTGGTTGAGGCCGGTGGAGACAACGAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAG
AGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTGAACGGTAGAACCTTGAACTAC
GCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAAT
AGATGGGCAAATGTCACTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGG
GTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTTACGATGGTGAATTGTTGATC
ACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTG
GACGTCAACGACGGTAACAACATTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCT
ACCGCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACGTCACCAGAGTCTTG
GGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCTAGTCCTAGACAAACAATCGTT
GCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCACCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGA
GAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCT
TCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAATGGCAACAG
AATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTC
CAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTT
GCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCACCT
GTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATAC
TTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACCATTGGAGGAAATC
GAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCA
AACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCCTCCATTATGCCATAC
ATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAGAGAGCTGCCGATATCATTAAGGCCGACTGGTCT
TAGCTCGAGGGAATTAACTGGGCCTCATGGGCCTTCCTTTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAG
CTGCATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCG
GTCGTTCGGGTAAAGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGA
CAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCG
GATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGT
AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATC
GTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCG
CTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTG
GTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGT
CTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCC
TTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTATTAGAAAAATT
CATCCAGCAGACGATAAAACGCAATACGCTGGCTATCCGGTGCCGCAATGCCATACAGCACCAGAAAACGATCCGCCC
ATTCGCCGCCCAGTTCTTCCGCAATATCACGGGTGGCCAGCGCAATATCCTGATAACGATCCGCCACGCCCAGACGGC
CGCAATCAATAAAGCCGCTAAAACGGCCATTTTCCACCATAATGTTCGGCAGGCACGCATCACCATGGGTCACCACCA
GATCTTCGCCATCCGGCATGCTCGCTTTCAGACGCGCAAACAGCTCTGCCGGTGCCAGGCCCTGATGTTCTTCATCCA
GATCATCCTGATCCACCAGGCCCGCTTCCATACGGGTACGCGCACGTTCAATACGATGTTTCGCCTGATGATCAAACG
GACAGGTCGCCGGGTCCAGGGTATGCAGACGACGCATGGCATCCGCCATAATGCTCACTTTTTCTGCCGGCGCCAGAT
GGCTAGACAGCAGATCCTGACCCGGCACTTCGCCCAGCAGCAGCCAATCACGGCCCGCTTCGGTCACCACATCCAGCA
CCGCCGCACACGGAACACCGGTGGTGGCCAGCCAGCTCAGACGCGCCGCTTCATCCTGCAGCTCGTTCAGCGCACCGC
TCAGATCGGTTTTCACAAACAGCACCGGACGACCCTGCGCGCTCAGACGAAACACCGCCGCATCAGAGCAGCCAATGG
TCTGCTGCGCCCAATCATAGCCAAACAGACGTTCCACCCACGCTGCCGGGCTACCCGCATGCAGGCCATCCTGTTCAA
TCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTA
TTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

Table 54 **Sequence** of pKKW4.1
CcPDH is colored in **cyan**.

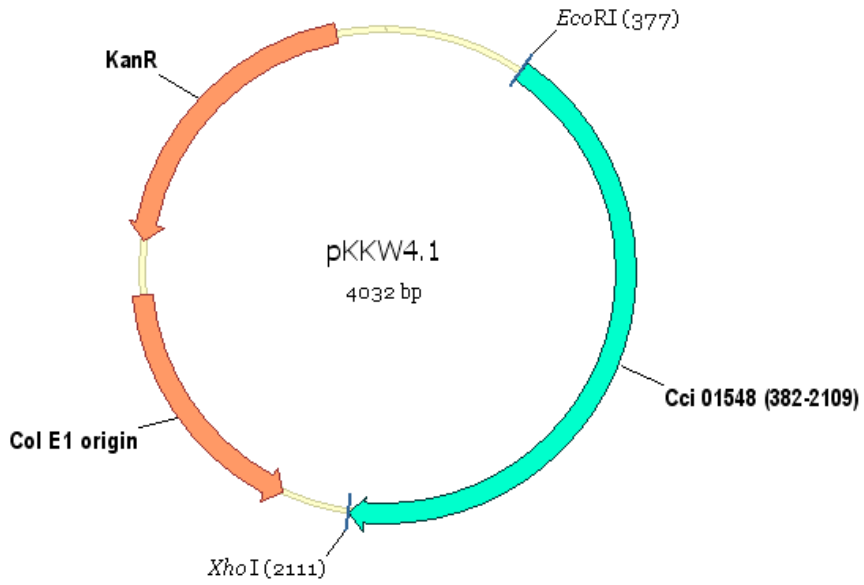

Figure 15 **Vector scheme** for pKKW4.1
with:
Cci 01548: CcPDH
Col E1 origin: Origin of replication
EcoRI: Restriction site
KanR: Kanamycin resistance gene
XhoI: Restriction site

12.3 Sequence of pKKW5.1

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGC
CGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGGCCGCTACAGGGCGCTCCCATT
CGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGG
GGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAG
CGCGACGTAATACGACTCACTATAGGGCGAATTGAAGGAAGGCCGTCAAGGCCGCATAAGCTTGAATTCATGGGAGTC
TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAACTCTGTTGCTGAATTGCCA
ACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTGCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAAC
CCAGATTTCCAGGTCTTGTTGGTTGAGGCCGGTGGAGACAACGAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAG
AGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTGAACGGTAGAACCTTGAACTAC
GCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAAT
AGATGGGCAAATGTCACTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGG
GTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTTACGATGGTGAATTGTTGATC
ACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTG
GACGTCAACGACGGTAACAACATTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCT
ACCGCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACGTCACCAGAGTCTTG
GGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCTAGTCCTAGACAAACAATCGTT
GCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCACCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGA
GAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCT
TCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAATGGCAACAG
AATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTC
CAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTT
GCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCACCT
GTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATAC
TTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACCATTGGAGGAAATC
GAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCA
AACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCCTCCATTATGCCATAC
ATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAGAGAGCTGCCGATATCATTAAGGCCGACTGGTCT
GCTCGAGGCTTAATTAACTGGGCCTCATGGGCCTTCCTTTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAG
CTGCATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCG
GTCGTTCGGGTAAAGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGA
CAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCG
GATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGT
AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATC
GTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCG
CTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTG
GTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGT
CTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCC
TTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTATTAGAAAAATT
CATCCAGCAGACGATAAAACGCAATACGCTGGCTATCCGGTGCCGCAATGCCATACAGCACCAGAAAACGATCCGCCC
ATTCGCCGCCCAGTTCTTCCGCAATATCACGGGTGGCCAGCGCAATATCCTGATAACGATCCGCCACGCCCAGACGGC
CGCAATCAATAAAGCCGCTAAAACGGCCATTTTCCACCATAATGTTCGGCAGGCACGCATCACCATGGGTCACCACCA
GATCTTCGCCATCCGGCATGCTCGCTTTCAGACGCGCAAACAGCTCTGCCGGTGCCAGGCCCTGATGTTCTTCATCCA
GATCATCCTGATCCACCAGGCCCGCTTCCATACGGGTACGCGCACGTTCAATACGATGTTTCGCCTGATGATCAAACG
GACAGGTCGCCGGGTCCAGGGTATGCAGACGACGCATGGCATCCGCCATAATGCTCACTTTTTCTGCCGGCGCCAGAT
GGCTAGACAGCAGATCCTGACCCGGCACTTCGCCCAGCAGCAGCCAATCACGGCCCGCTTCGGTCACCACATCCAGCA
CCGCCGCACACGGAACACCGGTGGTGGCCAGCCAGCTCAGACGCGCCGCTTCATCCTGCAGCTCGTTCAGCGCACCGC
TCAGATCGGTTTTCACAAACAGCACCGGACGACCCTGCGCGCTCAGACGAAACACCGCCGCATCAGAGCAGCCAATGG
TCTGCTGCGCCCAATCATAGCCAAACAGACGTTCCACCCACGCTGCCGGGCTACCCGCATGCAGGCCATCCTGTTCAA
TCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTA
TTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

Table 55 **Sequence** of pKKW5.1
CcPDH with a G instead of the stop-codon is colored in **cyan**

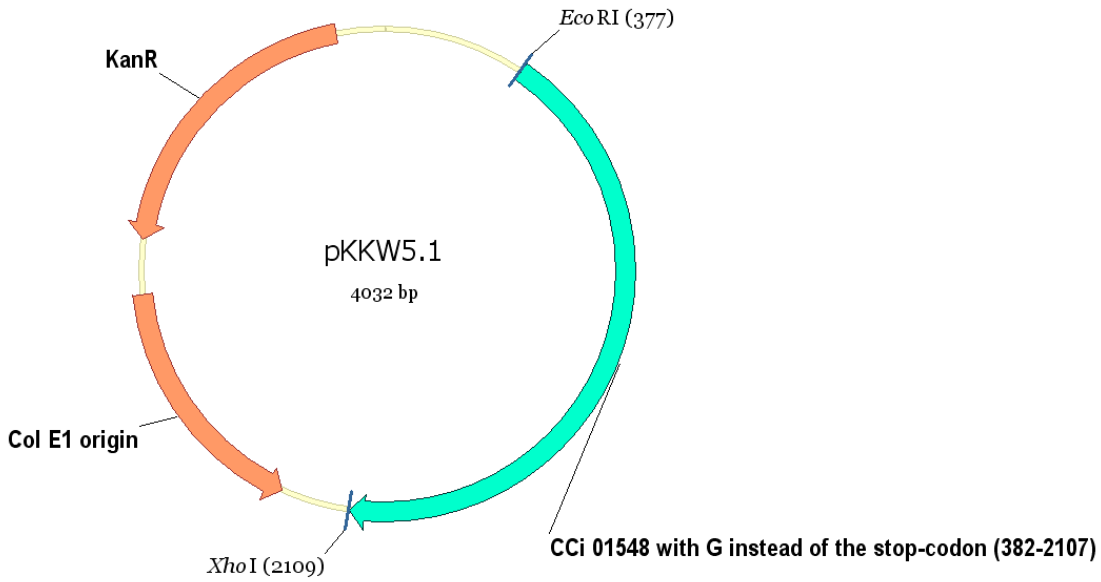

Figure 16 **Vector scheme** for pKKW5.1
with:
Cci 01548 with a G instead of the stop-codon: Modified CcPDH
Col E1 origin: Origin of replication
EcoRI: Restriction site
KanR: Kanamycin resistance gene
XhoI: Restriction site

12.4 Sequence of pKKW6.3

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCATTCTCACACATAA
GTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAAC
ACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCA
ACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCC
AAATGGCCCAAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACT
AAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTT
TGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTG
CACCTGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAA
GATTCTGGTGGGAATACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAAT
ATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTCATAATTGCGACT
GGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGA
AACGAGGAATTCATGGGAGTCTTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTAC
AACTCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTGCTGGTCCTGTCATT
GCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTGAGGCCGGTGGAGACAACGAGGGAGACATCAAC
TTCGTCGTCCCTGGTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTG
AACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTCTACAATAGA
GGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCA
ATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGT
TACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTCAAT
GAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCAACTCAATACACTATTGGATAC
GGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAAC
ACCTACGTCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCT
AGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCACCACAAATCTTGTTG
AATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAA
AACTTGCAGGATCACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAG
GCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGTAGAATC
CCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATC
TCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCC
AACAACCCATTCGATCCACCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT
ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATTACTGCCAACCCAGAC
ACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCT
ATGAGTGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCC
GATGCCTCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAGAGAGCTGCCGAT
ATCATTAAGGCCGACTGGTCTTAGCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGG
ATCTGAATAGCGCCGTCGACCATCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAG
TTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCA
GGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATTTTGTTTCTTCTCGTACG
AGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGAT
GTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTGCGGATCCCCCACAC
ACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTC
AAAACACCCAAGCACAGCATACTAAATTTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTT
GGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTT
CTTGAAATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTTAATAAACGGTCTTCAATTT
CTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCATTAGAAAGAAAGCATAGCAAT
CTAATCTAAGGGGCGGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGGAACT
AAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCG
ACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCA
GCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCG
AGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGG
GGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACTGACACGTCCGAC
GGCGGCCCACGGGTCCCAGGCCTCGGAGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAATTAGTTATGTCACGC
TTACATTCACGCCCTCCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATT
TATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTTTTTTTCTGTACAGACGCGTG
TACGCATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTGGAG
ACCAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTC
CGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
GCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTC
CCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTG
GGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTA
AGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG
TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACC
TTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAG
CAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAA
AACTCACGTTAAGGGATTTTGGTCATGAGATC

Table 56 **Sequence** of pKKW6.3
CcPDH is colored in cyan.

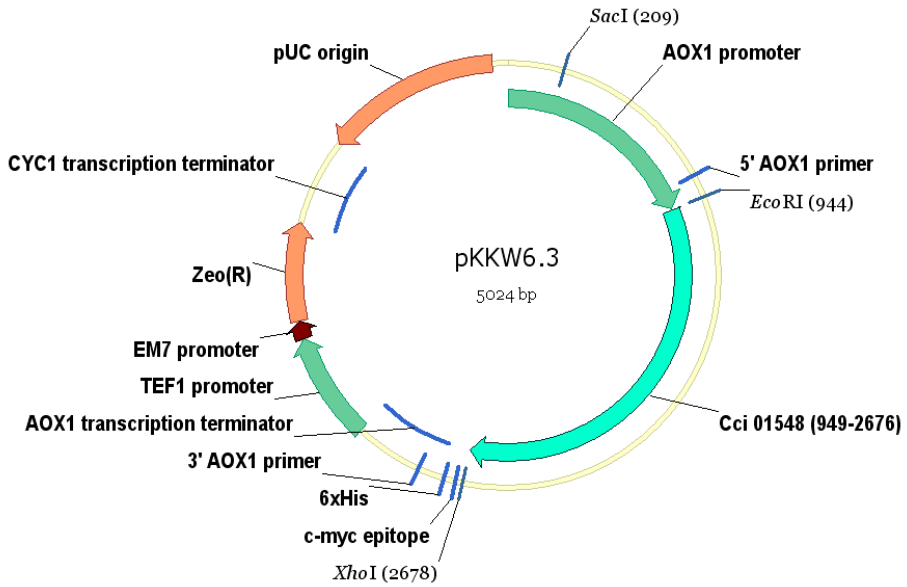

Figure 17 **Vector scheme** for pKKW6.3

with:

Cci 01548: CcPDH (Gene of interest)
3' AOX1 primer: 3AOX (see 12.6)
5' AOX1 primer: 5AOX (see 12.6)
6xHis: Histidine-tag
AOX1 promoter: *P. pastoris* promoter
AOX1 transcription terminator: *P. pastoris* terminator
c-myc epitope: Myc-tag
EcoRI: Restriction site
EM7 promotor: *E. coli* promoter
pUC origin: Origin of replication
SacI: Restriction site
TEF1 promotor: *P. pastoris* promotor
XhoI: Restriction site
Zeo(R): Zeocin™ resistance gene

12.5 Sequence of pKKW7.4

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCATTCTCACACATAA
GTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAAC
ACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCA
ACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCC
AAATGGCCCAAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACT
AAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTT
TGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTG
CACCTGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAA
GATTCTGGTGGGAATACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAAT
ATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTCATAATTGCGACT
GGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGA
AACGAGGAATTCATGGGAGTCTTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTAC
AACTCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTGCTGGTCCTGTCATT
GCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTGAGGCCGGTGGAGACAACGAGGGAGACATCAAC
TTCGTCGTCCCTGGTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTG
AACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTCTACAATAGA
GGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCA
ATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGT
TACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTCAAT
GAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCAACTCAATACACTATTGGATAC
GGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAAC
ACCTACGTCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCT
AGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCACCACAAATCTTGTTG
AATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAA
AACTTGCAGGATCACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAG
GCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGTAGAATC
CCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATC
TCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCC
AACAACCCATTCGATCCACCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT
ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATTACTGCCAACCCAGAC
ACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCT
ATGAGTGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCC
GATGCCTCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAGAGAGCTGCCGAT
ATCATTAAGGCCGACTGGTCTGCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGAT
CTGAATAGCGCCGTCGACCATCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTT
GGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGG
CTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATTTTGTTTCTTCTCGTACGAG
CTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGT
TTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTGCGGATCCCCCACACAC
CATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAA
AACACCCAAGCACAGCATACTAAATTTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGG
AAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCT
TGAAATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTTAATAAACGGTCTTCAATTTCT
CAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCATTAGAAAGAAAGCATAGCAATCT
AATCTAAGGGGCGGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGGAACTAA
ACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGAC
CGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGC
GCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAG
TGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGG
CGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACTGACACGTCCGACGG
CGGCCCACGGGTCCCAGGCCTCGGAGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAATTAGTTATGTCACGCTT
ACATTCACGCCCTCCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATTTA
TTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTTTTTTTCTGTACAGACGCGTGTA
CGCATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTGGAGAC
CAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCG
CCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGC
GTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCC
TTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGG
CTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAG
ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTT
CTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTT
CGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCA
GATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA
CTCACGTTAAGGGATTTTGGTCATGAGATC

Table 57 **Sequence** of pKKW7.4
CcPDH-HIS is colored in **cyan**.

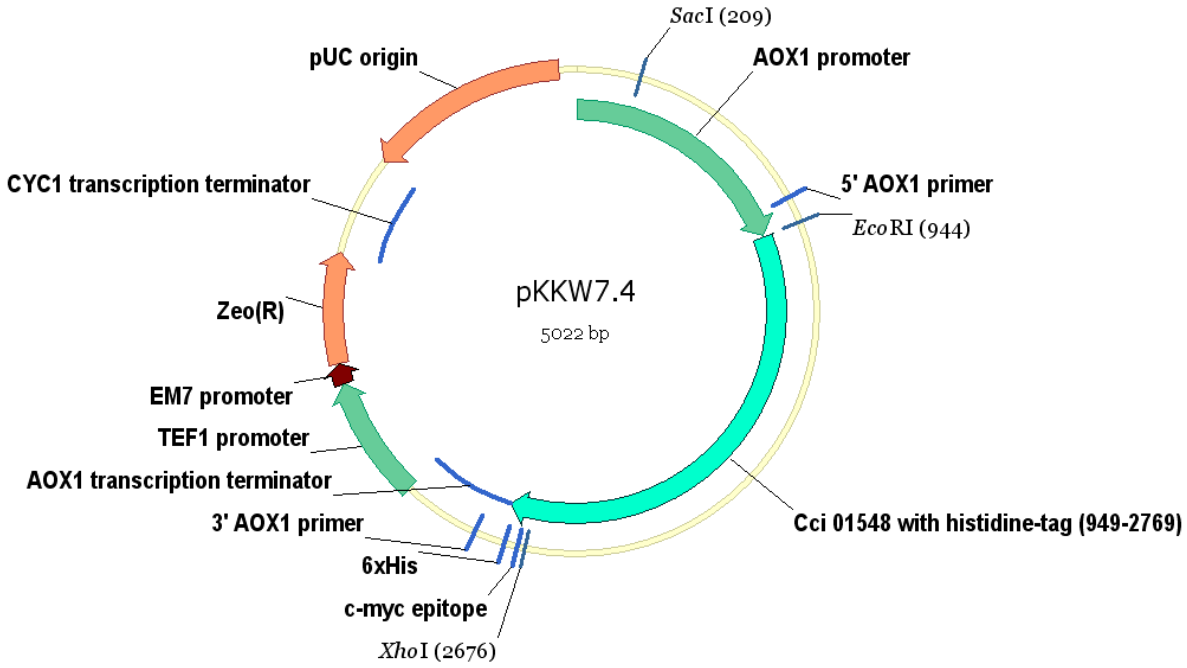

Figure 18 **Vector scheme** for pKKW6.3

with:

Cci 01548 with histidine tag: Modified CcPDH (Gene of interest)
3' AOX1 primer: 3AOX (see 12.6)
5' AOX1 primer: 5AOX (see 12.6)
6xHis: Histidine-tag
AOX1 promoter: *P. pastoris* promoter
AOX1 transcription terminator: *P. pastoris* terminator
c-myc epitope: Myc-tag
CYC1 transcription terminator: Terminator
EcoRI: Restriction site
EM7 promotor: *E. coli* promoter
pUC origin: Origin of replication
SacI: Restriction site
TEF1 promotor: *P. pastoris* promotor
XhoI: Restriction site
Zeo(R): Zeocin™ resistance gene

12.6 Sequence of pKKW10.1

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCATTCTCACACATAA
GTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAAC
ACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCA
ACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCC
AAATGGCCCAAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACT
AAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTT
TGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTG
CACCTGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAA
GATTCTGGTGGGAATACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAAT
ATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTCATAATTGCGACT
GGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGA
AACGAGGAATTCATGGGAGTCTTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTAC
AACTCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTGCTGGTCCTGTCATT
GCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTGAGGCCGGTGGAGAAAGATACCCATTGTCTAAC
GAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATT
GGTCAAACTGGATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGA
ATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGATGGAAATGGGAG
AACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAAC
CCTGACGCTCATGGTTACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTT
CAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCAACTCAA
TACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTC
CACGTTTTGTTGAACACCTACGTCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTT
GCCGCCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCA
CCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATT
CCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCC
GTCGACGAGGCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATT
GTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACAC
ATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCC
GTCAAAATCAGATCCAACAACCCATTCGATCCACCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTG
GCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATT
ACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTT
AGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACC
GGATTGAGAGTTGCCGATGCCTCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGA
GAGAGAGCTGCCGATATCATTAAGGCCGACTGGTCTTAGCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAAC
TCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACT
GTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATT
TGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATTTT
GTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAA
TCATTCGAGTTTGATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGT
GCGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCA
TCGCCGTACCACTTCAAAACACCCAAGGACAGCATACTAAATTTTCCCTCTTTCTTTCCTCTAGGGTGTCGTTAATTAC
CCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTT
ATCACGTTTCTTTTTCTTGAAATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTTAATA
AACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCATTAGAA
AGAAAGCATAGCAATCTAATCTAAGGGGCGGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACG
ACAAGGTGAGGAACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGG
TCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACG
TGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGG
ACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCG
GCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGG
ACTGACACGTCCGACGGCGGCCCACGGGTCCCAGGCCTCGGAGATCCGTCCCCTTTTCCTTTGTCGATATCATGTAA
TTAGTTATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAA
GTCTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTTTTTTT
CTGTACAGACGCGTGTACGCATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTA
ATTTGCAAGCTGGAGACCAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCG
TTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGA
CTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATAC
CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTC
GTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTT
GAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTA
GGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTG
CTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGAC
GCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATC

Table 58 **Sequence** of pKKW10.1
CcPDH-mod is colored in **cyan**.

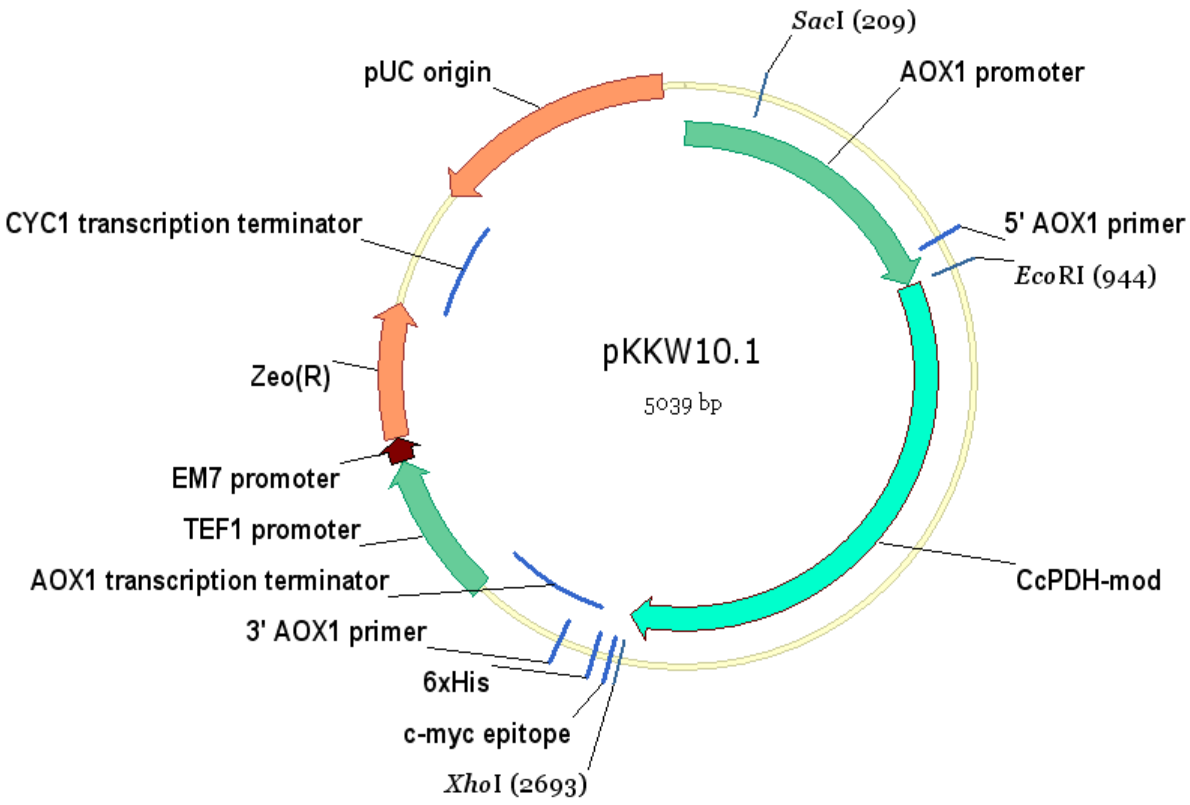

Figure 19 **Vector scheme** for pKKW10.1

with:

CcPDH-mod: Gene of interest
3' AOX1 primer: 3AOX (see 12.6)
5' AOX1 primer: 5AOX (see 12.6)
6xHis: Histidine-tag
AOX1 promoter: *P. pastoris* promoter
AOX1 transcription terminator: *P. pastoris* terminator
c-myc epitope: Myc-tag
CYC1 transcription terminator: Terminator
EcoRI: Restriction site
EM7 promotor: *E. coli* promoter
pUC origin: Origin of replication
SacI: Restriction site
TEF1 promotor: *P. pastoris* promotor
XhoI: Restriction site
Zeo(R): Zeocin™ resistance gene

12.7 Sequence of pKKW11.1

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCATTCTCACACATAA
GTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAAC
ACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCA
ACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCC
AAATGGCCCAAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACT
AAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTT
TGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTG
CACCTGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAA
GATTCTGGTGGGAATACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAAT
ATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTCATAATTGCGACT
GGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGA
AACGAGGAATTCATGGGAGTCTTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTAC
AACTCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTGCTGGTCCTGTCATT
GCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTGAGGCCGGTGGAGAAAGATACCCATTGTCTAAC
GAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATT
GGTCAAACTGGATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGA
ATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGATGGAAATGGGAG
AACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAAC
CCTGACGCTCATGGTTACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTT
CAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCAACTCAA
TACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTC
CACGTTTTGTTGAACACCTACGTCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTT
GCCGCCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCA
CCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATT
CCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCC
GTCGACGAGGCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATT
GTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACAC
ATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCC
GTCAAAATCAGATCCAACAACCCATTCGATCCACCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTG
GCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATT
ACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTT
AGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACC
GGATTGAGAGTTGCCGATGCCTCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGA
GAGAGAGCTGCCGATATCATTAAGGCCGACTGGTCTGCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTC
ATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGT
TCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTG
CCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTCATTTTGT
TTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATC
ATTCGAGTTTGATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTGC
GGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATC
GCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCC
GTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTAT
CACGTTTCTTTTTCTTGAAATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTTAATAAA
CGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCATTAGAAAG
AAAGCATAGCAATCTAATCTAAGGGGCGGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGAC
AAGGTGAGGAACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTC
GAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTG
ACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGAC
GAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGC
GAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGAC
TGACACGTCCGACGGCGGCCCACGGGTCCCAGGCCTCGGAGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAATT
AGTTATGTCACGCTTACATTCACGCCCTCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGT
CTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTTTTTTTCT
GTACAGACGCGTGTACGCATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAAT
TTGCAAGCTGGAGACCAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTT
TTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACT
ATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCT
GTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGT
TCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGA
GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGG
CGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCT
GAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTT
TGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGC
TCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATC

Table 59 **Sequence** of pKKW11.1
CcPDH-mod-HIS is colored in **cyan**.

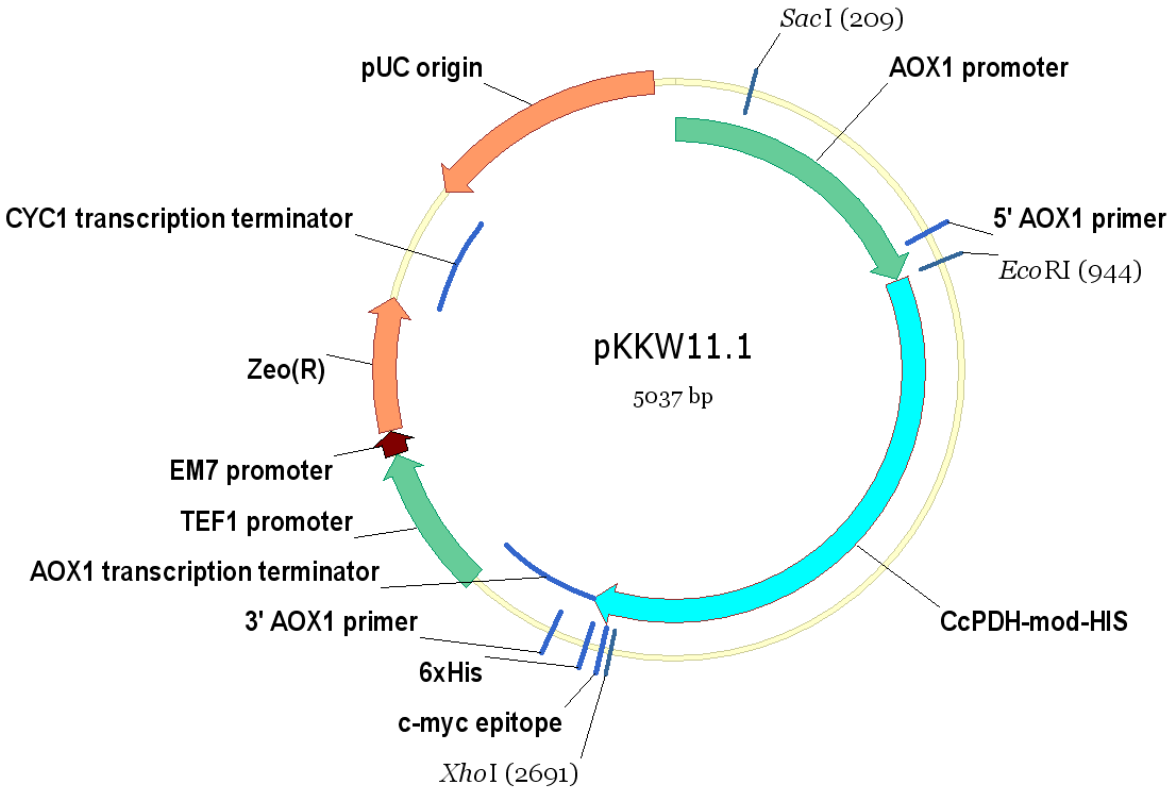

Figure 20 **Vector scheme** for pKKW11.1

with:

CcPDH-mod-HIS: Gene of interest
3' AOX1 primer: 3AOX (see 12.6)
5' AOX1 primer: 5AOX (see 12.6)
6xHis: Histidine-tag
AOX1 promoter: *P. pastoris* promoter
AOX1 transcription terminator: *P. pastoris* terminator
c-myc epitope: Myc-tag
CYC1 transcription terminator: Terminator
EcoRI: Restriction site
EM7 promotor: *E. coli* promoter
pUC origin: Origin of replication
SacI: Restriction site
TEF1 promotor: *P. pastoris* promotor
XhoI: Restriction site
Zeo(R): Zeocin™ resistance gene

12.8 Sequence of pPICZ B

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCATTCTCACACATAA
GTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAAC
ACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCA
ACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCC
AAATGGCCCAAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACT
AAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTT
TGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTG
CACCTGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAA
GATTCTGGTGGGAATACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAAT
ATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTCATAATTGCGACT
GGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGA
AACGAGGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACA
AAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCATTGAGTTTGTAGCCTTAGACA
TGACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATG
CCATTTGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTC
ATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGG
GAAAATCATTCGAGTTTGATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCG
TTTGTGCGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCC
GCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTTCCCTCTTTCTTCCTCTAGGGTGTCGTTA
ATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAA
TTTTTATCACGTTTCTTTTTCTTGAAATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGT
TAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCAT
TAGAAAGAAAGCATAGCAATCTAATCTAAGGGGCGGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATA
ATACGACAAGGTGAGGAACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGG
AGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGA
CGACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGG
CCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGA
GATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGA
GCAGGACTGACACGTCCGACGGCGGCCCACGGGTCCCAGGCCTCGGAGATCCGTCCCCCTTTTCCTTTGTCGATATCA
TGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAAC
CTGAAGTCTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTTT
TTTTTCTGTACAGACGCGTGTACGCATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGG
CTTTAATTTGCAAGCTGGAGACCAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGA
CAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCG
GATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGT
AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATC
GTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCG
CTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTG
GTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGT
CTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATC

Table 60 **Sequence** of pPICZ B

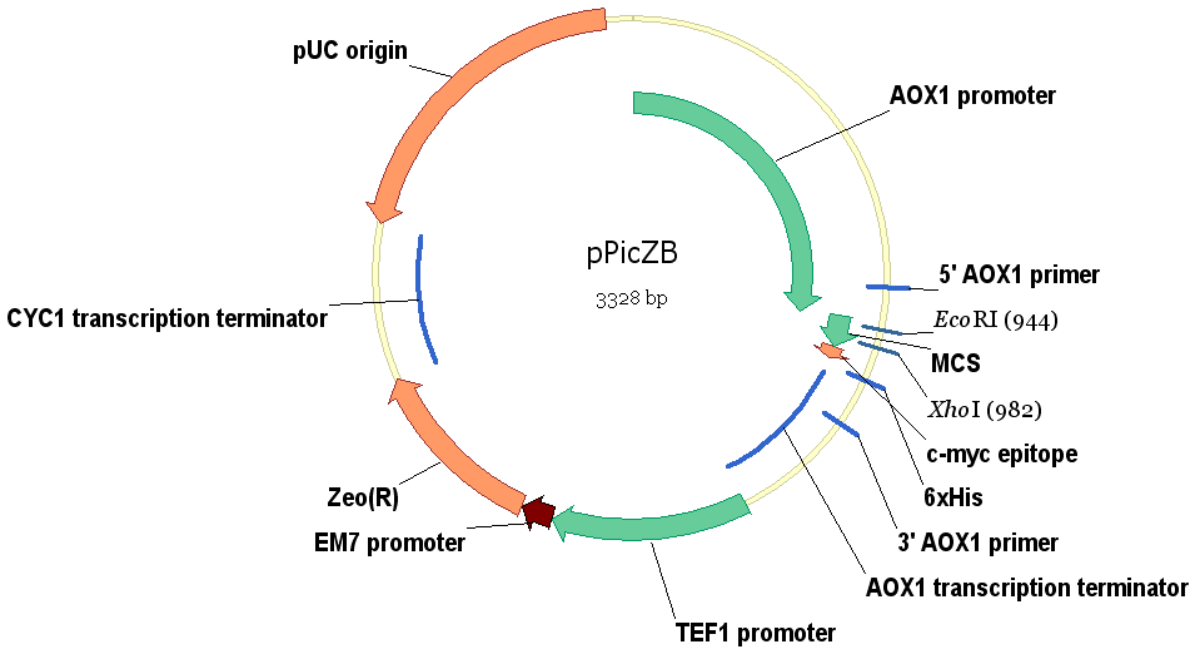

Figure 21 **Vector scheme** for pPICZ B

with:

3' AOX1 primer: 3AOX (see 12.6)
5' AOX1 primer: 5AOX (see 12.6)
6xHis: Histidine-tag
AOX1 promoter: *P. pastoris* promoter
AOX1 transcription terminator: *P. pastoris* terminator
c-myc epitope: Myc-tag
CYC1 transcription terminator: Terminator
EcoRI: Restriction site
EM7 promotor: *E. coli* promoter
MCS: Multi cloning site
pUC origin: Origin of replication
TEF1 promotor: *P. pastoris* promotor
XhoI: Restriction site
Zeo(R): Zeocin™ resistance gene

12.9 Sequences of Primers

Name	Sequence 5’ -> 3’
KKWp1s	TCACTATAGGGCGAATTGAAGGAAGGCC
KKWp1a	CACCCCTCGAGCTAAGACCAGTCGGC
KKWp2a	GAACTCGAGCAGACCAGTCGGCCTT
KKWp3s	CGACTGGTCTTAGCTCGAGGGAATTAACTG
KKWp3a	CTAAGACCAGTCGGCCTTAATGATATCCCG
KKWp4s	GCCGACTGGTCTGCTCGAGGCTTAATTAACT
KKWp4a	AGACCAGTCGGCCTTAATGATATCGGCAGC
KKWp5s	TGATAGCGAAGAGCTACAGCCGTCTAGAACAAAAACTCATCT
KKWp5a	AGATGAGTTTTTGTTCTAGACGGCTGTAGCTCTTCGCTATCA
KKWp6s	TGTTGGTTGAGGCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAG
KKWp6a	TCCACCGGCCTCAACCAACAAGACCTGGAA
3AOX	GCAAATGGCATTCTGACATCC
5AOX	GACTGGTTCCAATTGACAAGC

Table 61 **Sequences** of primers

12.10DNA-Sequence-Comparisons

12.10.1 pKKW6.3 with s1

```
1:   898 CGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTC  957
2:     1 CGAC-ACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTC   59

1:   958 TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAAC 1017
2:    60 TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAAC  119

1:  1018 TCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACT 1077
2:   120 TCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACT  179

1:  1078 GCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTT 1137
2:   180 GCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTT  239

1:  1138 GAGGCCGGTGGAGACAACGAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAGAGAAGA 1197
2:   240 GAGGCCGGTGGAGACAACGAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAGAGAAGA  299

1:  1198 TTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTGAACGGTAGA 1257
2:   300 TTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTGAACGGTAGA  359

1:  1258 ACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTC 1317
2:   360 ACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTC  419

1:  1318 TACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGA 1377
2:   420 TACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGA  479

1:  1378 TGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCC 1437
2:   480 TGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCC  539

1:  1438 GATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTTACGATGGTGAATTG 1497
2:   540 GATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTTACGATGGTGAATTG  599

1:  1498 TTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTC 1557
2:   600 TTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTC  659

1:  1558 AATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCA 1617
2:   660 AATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCA  719

1:  1618 ACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACT 1677
2:   720 ACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACT  779

1:  1678 GAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACGTCACCAGAGTCTTG 1737
2:   780 GAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACGTCACCAGAGTCTTG  839

1:  1738 GGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCTAGT 1797
2:   840 GGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCTAGT  899

1:  1798 CCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTT 1851
2:   900 CCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTT  953
```

Table 62 **DNA sequence comparison** of pKKW6.3 and s1
The expected pKKW6.3 (1) with the received sequence data from s1 (2). The **gene of interest CcPDH** is colored in cyan and the **mismatch** is colored in red.

12.10.2 pKKW6.3 with s2

```
1: 1861 CAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAA 1920
2:  968 CAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAA  909

1: 1921 TCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCC 1980
2:  908 TCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCC  849

1: 1981 ATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAA 2040
2:  848 ATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAA  789

1: 2041 TGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGT 2100
2:  788 TGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGT  729

1: 2101 AGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGT 2160
2:  728 AGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGT  669

1: 2161 GCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTG 2220
2:  668 GCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTG  609

1: 2221 TTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCA 2280
2:  608 TTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCA  549

1: 2281 CCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT 2340
2:  548 CCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT  489

1: 2341 ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCT 2400
2:  488 ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCT  429

1: 2401 ATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGT 2460
2:  428 ATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGT  369

1: 2461 GTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAAC 2520
2:  368 GTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAAC  309

1: 2521 GGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCC 2580
2:  308 GGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCC  249

1: 2581 TCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAG 2640
2:  248 TCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAG  189

1: 2641 AGAGCTGCCGATATCATTAAGGCCGACTGGTCTTAGCTCGAGCCGCGGCGGCCGCCAGCT 2700
2:  188 AGAGCTGCCGATATCATTAAGGCCGACTGGTCTTAGCTCGAGCCGCGGCGGCCGCCAGCT  129

1: 2701 TTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATC 2760
2:  128 TTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATC   69

1: 2761 ATCACTATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTAC 2820
2:   68 ATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACT-AC   10

1: 2821 GAGAAGACC 2829
2:    9 GAGAAGACC    1
```

Table 63 **DNA sequence comparison** of pKKW6.3 and s2

The expected pKKW6.3 (1) with the received sequence data from complementary s2 (2). The **gene of interest CcPDH** is colored in cyan and the **mismatch** is colored in red.

12.10.3 pKKW7.4 with s3

```
1:  898 CGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTC  957
2:    1 CGAC-ACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTC   59

1:  958 TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAAC 1017
2:   60 TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAAC  119

1: 1018 TCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACT 1077
2:  120 TCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACT  179

1: 1078 GCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTT 1137
2:  180 GCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTT  239

1: 1138 GAGGCCGGTGGAGACAACGAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAGAGAAGA 1197
2:  240 GAGGCCGGTGGAGACAACGAGGGAGACATCAACTTCGTCGTCCCTGGTTTCCAGAGAAGA  299

1: 1198 TTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTGAACGGTAGA 1257
2:  300 TTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTGGATTGAACGGTAGA  359

1: 1258 ACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTC 1317
2:  360 ACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCACAAACGGAATGGTC  419

1: 1318 TACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGA 1377
2:  420 TACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCACTGGAGATGACGGA  479

1: 1378 TGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCC 1437
2:  480 TGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAATGGGTCTTGCCTGCC 539

1: 1438 GATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTTACGATGGTGAATTG 1497
2:  540 GATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTTACGATGGTGAATTG  599

1: 1498 TTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTC 1557
2:  600 TTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAGTTCAGGACAATTTC  659

1: 1558 AATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCA 1617
2:  660 AATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACATTGGAGCCTGTCCA  719

1: 1618 ACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACT 1677
2:  720 ACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCGCTTTCGTTTCCACT  779

1: 1678 GAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACGTCACCAGAGTCTTG 1737
2:  780 GAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACGTCACCAGAGTCTTG  839

1: 1738 GGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCTAGT 1797
2:  840 GGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCGCCGACTCCGCTAGT  899

1: 1798 CCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCA 1857
2:  900 CCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTGGTGCTTTTGGATCA  959

1: 1858 CCACAAATC 1866
2:  960 CCACAAATC  968
```

Table 64 **DNA sequence comparison** of pKKW7.4 and s3
The expected pKKW7.4 (1) with the received sequence data from s3 (2). The **gene of interest CcPDH-HIS** is colored in cyan and the **mismatch** is colored in red.

12.10.4 pKKW7.4 with s4

```
1: 1861 CAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAA 1920
2:  966 CAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAA  907

1: 1921 TCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCC 1980
2:  906 TCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCC  847

1: 1981 ATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAA 2040
2:  846 ATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAA  787

1: 2041 TGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGT 2100
2:  786 TGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGT  727

1: 2101 AGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGT 2160
2:  726 AGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGT  667

1: 2161 GCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTG 2220
2:  666 GCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTG  607

1: 2221 TTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCA 2280
2:  606 TTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCA  547

1: 2281 CCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT 2340
2:  546 CCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT  487

1: 2341 ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCT 2400
2:  486 ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCT  427

1: 2401 ATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGT 2460
2:  426 ATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGT  367

1: 2461 GTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAAC 2520
2:  366 GTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAAC  307

1: 2521 GGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCC 2580
2:  306 GGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCC  247

1: 2581 TCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAG 2640
2:  246 TCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAG  187

1: 2641 AGAGCTGCCGATATCATTAAGGCCGACTGGTCTGCTCGAGCCGCGGCGGCCGCCAGCTTT 2700
2:  186 AGAGCTGCCGATATCATTAAGGCCGACTGGTCTGCTCGAGCCGCGGCGGCCGCCAGCTTT  127

1: 2701 CTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCAT 2760
2:  126 CTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCAT   67

1: 2761 CATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTACGA 2820
2:   66 CATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACT-ACGA    8

1: 2821 GAAGACC 2827
2:    7 GAAGACC    1
```

Table 65 **DNA sequence comparison** of pKKW7.4 and s4

The expected pKKW7.4 (1) with the received sequence data from complementary s4 (2). The **gene of interest CcPDH-HIS** is colored in cyan and the **mismatch** is colored in red.

12.10.5 pKKW10.1 with s5

```
1:   898 CGACAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTC  957
2:     1 CGAC-ACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTC   59

1:   958 TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAAC 1017
2:    60 TTTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAAC  119

1:  1018 TCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACT 1077
2:   120 TCTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACT  179

1:  1078 GCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTT 1137
2:   180 GCTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTT  239

1:  1138 GAGGCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAGACATCAACTTCGTCGTCCCT 1197
2:   240 GAGGCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAGACATCAACTTCGTCGTCCCT  299

1:  1198 GGTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACT 1257
2:   300 GGTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACT  359

1:  1258 GGATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCC 1317
2:   360 GGATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCC  419

1:  1318 ACAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTC 1377
2:   420 ACAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTC  479

1:  1378 ACTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAA 1437
2:   480 ACTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAA  539

1:  1438 TGGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGT 1497
2:   540 TGGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGT  599

1:  1498 TACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGA 1557
2:   600 TACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGA  659

1:  1558 GTTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAAC 1617
2:   660 GTTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAAC  719

1:  1618 ATTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACC 1677
2:   720 ATTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACC  779

1:  1678 GCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTAC 1737
2:   780 GCTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTAC  839

1:  1738 GTCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCC 1797
2:   840 GTCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCC  899

1:  1798 GCCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCT 1857
2:   900 GCCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAG-TCGTCTTGTCTGCT  958

1:  1858 GGTGCTTTTGGATCACCACAAATCTTGTTGA  1888
2:   959 GGTGCTTTTGGATCACCACAAATCTTGTTGA   989
```

Table 66 **DNA sequence comparison** of pKKW10.1 and s5

The expected pKKW10.1 (1) with the received sequence data from s5 (2). The **gene of interest CcPDH-mod** is colored in cyan, the **mismatches** are colored in red and the mutated sequence is colored in **green**.

12.10.6 pKKW10.1 with s7

```
1: 1876 CAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAA 1935
2:  966 CAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAA  907

1: 1936 TCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCC 1995
2:  906 TCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGATCACCCTGCTTCATTCGCC  847

1: 1996 ATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAA 2055
2:  846 ATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAA  787

1: 2056 TGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGT 2115
2:  786 TGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCTCACTACATTGTCTGGAGT  727

1: 2116 AGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGT 2175
2:  726 AGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGATGACCAAACCGCTCCTGGT  667

1: 2176 GCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTG 2235
2:  666 GCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTG  607

1: 2236 TTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCA 2295
2:  606 TTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCCAACAACCCATTCGATCCA  547

1: 2296 CCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT 2355
2:  546 CCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACATTTTGGCCTTCGTTGAGGGT  487

1: 2356 ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCT 2415
2:  486 ATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAAGGACCACGTTGTTGCTCCT  427

1: 2416 ATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGT 2475
2:  426 ATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGAACAACAATTGAGAAACGGT  367

1: 2476 GTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAAC 2535
2:  366 GTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAAC  307

1: 2536 GGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCC 2595
2:  306 GGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGGATTGAGAGTTGCCGATGCC  247

1: 2596 TCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAG 2655
2:  246 TCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGCTGTTTACGTTATCGGAGAG  187

1: 2656 AGAGCTGCCGATATCATTAAGGCCGACTGGTCTTAGCTCGAGCCGCGGCGGCCGCCAGCT 2715
2:  186 AGAGCTGCCGATATCATTAAGGCCGACTGGTCTTAGCTCGAGCCGCGGCGGCCGCCAGCT  127

1: 2716 TTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATC 2775
2:  126 TTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATC   67

1: 2776 ATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTAC 2835
2:   66 ATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACT-AC    8

1: 2836 GAGAAGA 2842
2:    7 GAGAAGA    1
```

Table 67 **DNA sequence comparison** of pKKW10.1 and s7

The expected pKKW10.1 (1) with the received sequence data from complementary s7 (2). The **gene of interest CcPDH-mod** is colored in cyan and the **mismatch** is colored in red.

12.10.7 pKKW11.1 with s6

```
1:   899 GACAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTCT  958
2:     1 GAC-ACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTCT   59

1:   959 TTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAACT 1018
2:    60 TTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAACT  119

1:  1019 CTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTG 1078
2:   120 CTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTG  179

1:  1079 CTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTG 1138
2:   180 CTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTG  239

1:  1139 AGGCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAGACATCAACTTCGTCGTCCCTG 1198
2:   240 AGGCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAGACATCAACTTCGTCGTCCCTG  299

1:  1199 GTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTG 1258
2:   300 GTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTG  359

1:  1259 GATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCA 1318
2:   360 GATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCA  419

1:  1319 CAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCA 1378
2:   420 CAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCA  479

1:  1379 CTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAAT 1438
2:   480 CTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAAT  539

1:  1439 GGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTT 1498
2:   540 GGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTT  599

1:  1499 ACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAG 1558
2:   600 ACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAG  659

1:  1559 TTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACA 1618
2:   660 TTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACA  719

1:  1619 TTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCG 1678
2:   720 TTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCG  779

1:  1679 CTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACG 1738
2:   780 CTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACG  839

1:  1739 TCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCG 1798
2:   840 TCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCG  899

1:  1799 CCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTG 1858
2:   900 CCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAG-TCGTCTTGTCTGCTG  958

1:  1859 GTGCTTTT 1866
2:   959 GTGCTTTT  966
```

Table 68 **DNA sequence comparison** of pKKW11.1 and s6

The expected pKKW11.1 (1) with the received sequence data from s6 (2). The **gene of interest CcPDH-mod-HIS** is colored in cyan, the **mismatches** are colored in red and the mutated sequence is colored in **green**.

12.10.8 pKKW11.1 with s8

```
1: 1902 TCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGG 1961
2:  937 TCCTAGAGAGGAATTGGAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGG  878

1: 1962 TAAAAACTTGCAGGATCACCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATC 2021
2:  877 TAAAAACTTGCAGGATCA-CCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATC  819

1: 2022 TCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATT 2081
2:  818 TCCTGCCGTCGACGAGGCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATT  759

1: 2082 GACTGACCCTGGATCTCACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTT 2141
2:  758 GACTGACCCTGGATCTCACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCA-TTT  700

1: 2142 CCAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTC 2201
2:  699 CCAAGAGTACCCAGATGACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTC  640

1: 2202 TGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATC 2261
2:  639 TGGTTCTGGTCCAACCGTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATC  580

1: 2262 CGTCAAAATCAGATCCAACAACCCATTCGATCCACCTGTTATCGATTTGGGATTTTTGAC 2321
2:  579 CGTCAAAATCAGATCCAACAACCCATTCGATCCACCTGTTATCGATTTGGGATTTTTGAC  520

1: 2322 CCACAGATACGACATTTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATACTTTGC 2381
2:  519 CCACAGATACGACATTTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATACTTTGC  460

1: 2382 TGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACC 2441
2:  459 TGGAGATGGATTCAAGGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACC  400

1: 2442 ATTGGAGGAAATCGAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGG 2501
2:  399 ATTGGAGGAAATCGAACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGG  340

1: 2502 ATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGT 2561
2:  339 ATCCGTTGCTATGAGTGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGT  280

1: 2562 CAAGGGAGCCACCGGATTGAGAGTTGCCGATGCCTCCATTATGCCATACATCACTACTGG 2621
2:  279 CAAGGGAGCCACCGGATTGAGAGTTGCCGATGCCTCCATTATGCCATACATCACTACTGG  220

1: 2622 TCACACTGTCGGTGCTGTTTACGTTATCGGAGAGAGAGCTGCCGATATCATTAAGGCCGA 2681
2:  219 TCACACTGTCGGTGCTGTTTACGTTATCGGAGAGAGAGCTGCCGATATCATTAAGGCCGA  160

1: 2682 CTGGTCTGCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGA 2741
2:  159 CTGGTCTGCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGA  100

1: 2742 GGATCTGAATAGCGCCGTCGACCATCATCATCATCATCATTGAGTTTGTAGCCTTAGACA 2801
2:   99 GGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAGTTTGTAGCCTTAGACA    40

1: 2802 TGACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGAC 2841
2:   39 TGACTGTTCCTCAGTTCAAGTTGGGCACT-ACGAGAAGAC    1
```

Table 69 **DNA sequence comparison** of pKKW11.1 and s8
The expected pKKW11.1 (1) with the received sequence data from complementary s8 (2). The **gene of interest CcPDH-mod-HIS** is colored in cyan and the **mismatches** are colored in red.

12.10.9 KKW10.1.1 with s9, s10, s11 and s12

```
1:       GACAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTCT
2:     1 GAC-ACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGAGGAATTCATGGGAGTCT  59

1:       TTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAACT
2:    60 TTAGTAGAATCGCCTTGTCCGGTTTGTACATCAATTTGGCTTTGGGAGCCGTTTACAACT 119

1:       CTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTG
2:   120 CTGTTGCTGAATTGCCAACCGATGTCGAGTTCGACTTCATCGTCGCTGGAGGTGGTACTG 179

1:       CTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTG
2:   180 CTGGTCCTGTCATTGCCTCTAGATTGGCCGAAAACCCAGATTTCCAGGTCTTGTTGGTTG 239

1:       AGGCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAGACATCAACTTCGTCGTCCCTG
2:   240 AGGCCGGTGGAGAAAGATACCCATTGTCTAACGAGGGAGACATCAACTTCGTCGTCCCTG 299
3:     1                                                 TCGTCCCTG   9

1:       GTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTG
2:   300 GTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTG 359
3:    10 GTTTCCAGAGAAGATTGTCCTCTTCCTACAATTGGGGATTCCAAACCATTGGTCAAACTG  69

1:       GATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCA
2:   360 GATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCA 419
3:    70 GATTGAACGGTAGAACCTTGAACTACGCTAGAGGAAAAGTCTTGGGAGGATCTTCCTCCA 129

1:       CAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCA
2:   420 CAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCA 479
3:   130 CAAACGGAATGGTCTACAATAGAGGATCCGCTCAGGATTACAATAGATGGGCAAATGTCA 189

1:       CTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAAT
2:   480 CTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAAT 539
3:   190 CTGGAGATGACGGATGGAAATGGGAGAACTTGTTGCCATCAATCAAAAGAGGTGAAAAAT 249

1:       GGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTT
2:   540 GGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTT 599
3:   250 GGGTCTTGCCTGCCGATGGTAGATCTGTCGATGGAGCCTACAACCCTGACGCTCATGGTT 309

1:       ACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAG
2:   600 ACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAG 659
3:   310 ACGATGGTGAATTGTTGATCACCAACTTCAACACTCCTCCAACCGACTTCGATAGAAGAG 369

1:       TTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACA
2:   660 TTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACA 719
3:   370 TTCAGGACAATTTCAATGAGGAGTTCCCATTCTGCTTGGACGTCAACGACGGTAACAACA 429

1:       TTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCG
2:   720 TTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCG 779
3:   430 TTGGAGCCTGTCCAACTCAATACACTATTGGATACGGTGAGAGATCATCTGCTGCTACCG 489

1:       CTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACG
2:   780 CTTTCGTTTCC                                               790
3:   490 CTTTCGTTTCCACTGAGCATAGAAACAGACCAAACTTCCACGTTTTGTTGAACACCTACG 549
4:   916                                         CGTTTTGTTGAACA-CTACG 898
```

```
1:       TCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCG
3:  550 TCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCG 609
4:  897 TCACCAGAGTCTTGGGAACCGGTGACAATGCCTTGGACTTTAGAACAATCGAGGTTGCCG 838

1:       CCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTG
3:  610 CCGACTCCGCTAGTCCTAGACAAACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTG 669
4:  837 CCGACTCCGCTAGTCCTAGAC-AACAATCGTTGCTTCAAAGGAGGTCGTCTTGTCTGCTG 779

1:       GTGCTTTTGGATCACCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATT-G
3:  670 GTGCTTTTGGATCACCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATTTG 729
4:  778 GTGCTTTTGGATCACCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATT-G 720
5:  976  TGCTTTTGGATCACCACAAATCTTGTTGAATAGTGGTATCGGTCCTAGAGAGGAATT-G 919

1:       GAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGAT
3:  730 GAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGAT 789
4:  719 GAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGAT 660
5:  918 GAGGAAGTCGGAGTTGAATCCGTTTTGGACATTCCAGATGTCGGTAAAAACTTGCAGGAT 859

1:       CACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAG
3:  790 CACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAG 849
4:  659 CACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAG 600
5:  858 CACCCTGCTTCATTCGCCATGTGGTTGGCCAACGGTCAACCATCTCCTGCCGTCGACGAG 799

1:       GCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCT
3:  850 GCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCT 909
4:  599 GCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCT 540
5:  798 GCTGAGGCTTTTGCTCAATGGCAACAGAATAGATCCGGTCCATTGACTGACCCTGGATCT 739

1:       CACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGAT
3:  910 CACTACATTGTCTGGAGTAGAATCCCTG                                 937
4:  539 CACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGAT 480
5:  738 CACTACATTGTCTGGAGTAGAATCCCTGCCAACGCTTCCATTTTCCAAGAGTACCCAGAT 679

1:       GACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACC
4:  479 GACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACC 420
5:  678 GACCAAACCGCTCCTGGTGCTCCACACATTGAATTGGCCATCTCTGGTTCTGGTCCAACC 619

1:       GTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCC
4:  419 GTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCC 360
5:       GTTGCTGCTTCTGTCTTGTTGTTGAACCCTGCCTCAAGAGGATCCGTCAAAATCAGATCC 559

1:       AACAACCC-ATTCGATCCACCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACAT
4:  359 AACAACCC-ATTCGATCCACCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACAT 301
5:  558 AACAACCCCATTCGATCCACCTGTTATCGATTTGGGATTTTTGACCCACAGATACGACAT 499

1:       TTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAA
4:  300 TTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAA 241
5:  498 TTTGGCCTTCGTTGAGGGTATTAGAAGTGCCTGGAGATACTTTGCTGGAGATGGATTCAA 439

1:       GGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGA
4:  240 GGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGA 181
5:  438 GGACCACGTTGTTGCTCCTATTACTGCCAACCCAGACACTACACCATTGGAGGAAATCGA 379
```

```
1:       ACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAG
4:  180 ACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAG 121
5:  378 ACAACAATTGAGAAACGGTGTCGGAACCACTTTGCATGTTAGTGGATCCGTTGCTATGAG 319

1:       TGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGG
4:  120 TGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGG  61
5:  318 TGCTAGAGGAGCTTCAAACGGTGTTTTGGACCCAGACTTGAAAGTCAAGGGAGCCACCGG 259

1:       ATTGAGAGTTGCCGATGCCTCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGC
4:   60 ATTGAGAGTTGCCGATGCCTCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGC   1
5:  258 ATTGAGAGTTGCCGATGCCTCCATTATGCCATACATCACTACTGGTCACACTGTCGGTGC 199

1:       TGTTTACGTTATCGGAGAGAGAGCTGCCGATATCATTAAGGCCGACTGGTCTTAGCTCGA
5:  198 TGTTTACGTTATCGGAGAGAGAGCTGCCGATATCATTAAGGCCGACTGGTCTTAGCTCGA 139

1:       GCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGC
5:  138 GCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGC  79

1:       GCCGTCGACCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCA
5:   78 GCCGTCGACCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCA   19

1:       GTTCAAGTTGGGCACT
5:   18 GTTCAAGTTGGGCACT    3
```

Table 70 **DNA sequence comparison** of KKW10.1.1 with s9, s10, s11 and s12

The expected gene coding sequence of genomic DNA from KKW10.1.1 between 5AOX and 3AOX (1) compared with the received sequence data from s9 (2), s12 (3), complementary s11 (4) and complementary s10 (5). The **gene of interest CcPDH-mod** is colored in cyan, the **mismatches** are colored in red and the mutated sequence is colored in **green**.

12.11Protein Database Search with CcPDH as Query

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
XP_001833871.2	pyranose dehydrogenase [*Coprinopsis cinerea* okayama7#130]	1132	99%	0	98%
XP_001833865.1	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	560	99%	2E-157	50%
XP_001833863.2	pyranose dehydrogenase [*Coprinopsis cinerea* okayama7#130]	550	96%	2E-154	50%
XP_001835456.1	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	522	96%	5E-146	48%
XP_001833868.1	hypothetical protein CC1G_01545 [*Coprinopsis cinerea* okayama7#130]	514	99%	1E-143	48%
XP_001841576.2	pyranose dehydrogenase [*Coprinopsis cinerea* okayama7#130]	497	98%	2E-138	44%
XP_001833867.2	pyranose dehydrogenase [*Coprinopsis cinerea* okayama7#130]	486	98%	5E-135	44%
XP_001833869.2	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	478	98%	1E-132	44%
XP_002912280.1	hypothetical protein CC1G_13811 [*Coprinopsis cinerea* okayama7#130]	456	97%	4E-126	41%
XP_002912279.1	hypothetical protein CC1G_13810 [*Coprinopsis cinerea* okayama7#130]	456	98%	4E-126	44%
XP_001831050.2	pyranose dehydrogenase [*Coprinopsis cinerea* okayama7#130]	446	89%	5E-123	43%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
XP_001836102.2	pyranose dehydrogenase [*Coprinopsis cinerea* okayama7#130]	437	96%	3E-120	41%
XP_001835073.2	pyranose dehydrogenase [*Coprinopsis cinerea* okayama7#130]	427	96%	2E-117	43%
XP_001834862.2	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	412	94%	6E-113	41%
XP_001835052.2	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	410	96%	3E-112	41%
AAZ94874.1	pyranose dehydrogenase [*Leucoagaricus meleagris*]	407	98%	3E-111	40%
XP_002910136.1	glucose dehydrogenase short protein [*Coprinopsis cinerea* okayama7#130]	399	98%	7E-109	39%
AAW82996.1	pyranose dehydrogenase [Leucoagaricus meleagris]	395	98%	7E-108	40%
XP_001834863.2	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	390	98%	4E-106	39%
AAF31169.1	aryl-alcohol oxidase precursor [*Pleurotus pulmonarius*]	390	98%	4E-106	38%
AAW92124.1	pyranose dehydrogenase [*Agaricus bisporus*]	385	99%	8E-105	38%
AAW92123.1	pyranose dehydrogenase [*Agaricus xanthodermus*]	385	96%	8E-105	41%
ADD14021.1	aryl-alcohol oxidase [*Pleurotus eryngii*]	385	98%	1E-104	38%
AAC72747.1	aryl-alcohol oxidase precursor [*Pleurotus eryngii*]	385	98%	1E-104	37%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
XP_002910635.1	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	383	94%	4E-104	40%
3FIM_B	Chain B, Crystal Structure Of Aryl-Alcohol-Oxidase From *Pleurotus eryingii*	382	93%	8E-104	38%
XP_001829694.2	hypothetical protein CC1G_11430 [*Coprinopsis cinerea* okayama7#130]	381	87%	1E-103	38%
AAW82998.1	pyranose dehydrogenase [*Leucoagaricus meleagris*]	380	97%	3E-103	39%
XP_001836103.1	hypothetical protein CC1G_12262 [*Coprinopsis cinerea* okayama7#130]	355	82%	1E-95	40%
XP_001882478.1	predicted protein [*Laccaria bicolor* S238N-H82]	354	98%	2E-95	34%
XP_003027068.1	hypothetical protein SCHCODRAFT_79386 [*Schizophyllum commune* H4-8]	352	99%	1E-94	36%
XP_001834845.2	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	349	97%	8E-94	36%
XP_001831908.2	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	347	89%	3E-93	39%
XP_001884302.1	predicted protein [*Laccaria bicolor* S238N-H82]	347	98%	4E-93	36%
XP_001833981.2	aryl-alcohol oxidase [*Coprinopsis cinerea* okayama7#130]	345	98%	9E-93	37%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
XP_002475146.1	aryl-alcohol oxidase-like protein [*Postia placenta* Mad-698-R]	345	92%	1E-92	37%
XP_001879270.1	predicted protein [*Laccaria bicolor* S238N-H82]	329	94%	9E-88	37%
XP_002390204.1	hypothetical protein MPER_10557 [*Moniliophthora perniciosa* FA553]	325	87%	1E-86	36%
XP_002910135.1	alcohol dehydrogenase [*Coprinopsis cinerea* okayama7#130]	307	88%	3E-81	36%
NP_935088.1	choline dehydrogenase [*Vibrio vulnificus* YJ016]	273	94%	6E-71	35%
XP_002389049.1	hypothetical protein MPER_11873 [*Moniliophthora perniciosa* FA553]	271	72%	3E-70	37%
ZP_06053464.1	choline dehydrogenase [*Grimontia hollisae* CIP 101886]	267	93%	3E-69	33%
ZP_01306234.1	putative choline dehydrogenase [*Oceanobacter sp.* RED65]	264	93%	3E-68	35%
YP_167582.1	GMC family oxidoreductase [*Ruegeria pomeroyi* DSS-3]	264	93%	3E-68	36%
YP_320380.1	glucose-methanol-choline oxidoreductase [*Anabaena variabilis* ATCC 29413]	264	93%	3E-68	35%
XP_003041895.1	hypothetical protein NECHADRAFT_87097 [*Nectria haematococca* mpVI 77-13-4]	263	98%	6E-68	32%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
YP_366469.1	glucose-methanol-choline oxidoreductase [*Burkholderia sp.* 383]	262	93%	1E-67	34%
YP_001171160.1	putative alcohol dehydrogenase [*Pseudomonas stutzeri* A1501]	261	93%	2E-67	35%
EFP98317.1	choline dehydrogenase [*Vibrio caribbenthicus* ATCC BAA-2122]	260	93%	4E-67	34%
ZP_07024031.1	glucose-methanol-choline oxidoreductase [*Alicycliphilus denitrificans* BC]	256	93%	7E-66	35%
ZP_05060389.1	alcohol dehydrogenase [gamma proteobacterium HTCC5015]	256	93%	9E-66	33%
ADP98420.1	glucose-methanol-choline oxidoreductase [marine bacterium HP15]	255	93%	1E-65	34%
ZP_07375284.1	choline dehydrogenase [*Ahrensia sp.* R2A130]	255	94%	1E-65	34%
CBA30511.1	Alcohol dehydrogenase [acceptor] [*Curvibacter* putative symbiont of *Hydra magnipapillata*]	255	94%	1E-65	33%
YP_973212.1	glucose-methanol-choline oxidoreductase [*Polaromonas naphthalenivorans* CJ2]	255	93%	1E-65	33%
YP_001022991.1	putative choline dehydrogenase lipoprotein oxidoreductase [*Methylibium petroleiphilum* PM1]	255	93%	2E-65	34%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
EFN64444.1	Glucose dehydrogenase [acceptor] [*Camponotus floridanus*]	253	94%	5E-65	34%
ZP_05067451.1	alcohol dehydrogenase [*Octadecabacter antarcticus* 238]	252	94%	1E-64	33%
XP_002390024.1	hypothetical protein MPER_10770 [*Moniliophthora perniciosa* FA553]	252	93%	1E-64	32%
XP_001834836.1	hypothetical protein CC1G_08481 [*Coprinopsis cinerea* okayama7#130]	252	60%	1E-64	40%
YP_002942230.1	glucose-methanol-choline oxidoreductase [*Variovorax paradoxus* S110]	251	93%	2E-64	33%
YP_003643276.1	glucose-methanol-choline oxidoreductase [*Thiomonas intermedia* K12]	251	93%	2E-64	33%
XP_001648298.1	glucose dehydrogenase [*Aedes aegypti*]	251	93%	3E-64	32%
ZP_01894771.1	glucose-methanol-choline oxidoreductase [*Marinobacter algicola* DG893]	250	93%	5E-64	33%
BAJ27189.1	putative oxidoreductase [*Kitasatospora setae* KM-6054]	249	93%	7E-64	35%
ZP_05781295.1	alcohol dehydrogenase (acceptor) [*Citreicella sp.* SE45]	249	94%	7E-64	34%
ACV84069.1	DddA [*Halomonas sp.* HTNK1]	249	92%	7E-64	34%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
YP_267420.1	GMC family oxidoreductase [*Colwellia psychrerythraea* 34H]	249	93%	7E-64	32%
ZP_05052326.1	GMC oxidoreductase family [*Octadecabacter antarcticus* 307]	249	94%	1E-63	33%
YP_001265285.1	glucose-methanol-choline oxidoreductase [*Sphingomonas wittichii* RW1]	249	93%	1E-63	34%
ZP_07021322.1	glucose-methanol-choline oxidoreductase [*Alicycliphilus denitrificans* BC]	248	93%	1E-63	33%
XP_001607948.1	PREDICTED: similar to ENSANGP00000012169 [*Nasonia vitripennis*]	248	94%	1E-63	33%
YP_617373.1	glucose-methanol-choline oxidoreductase [*Sphingopyxis alaskensis* RB2256]	248	93%	1E-63	35%
XP_002388786.1	hypothetical protein MPER_12156 [*Moniliophthora perniciosa* FA553]	247	75%	4E-63	36%
XP_002426463.1	glucose dehydrogenase precursor, putative [*Pediculus humanus corporis*]	247	94%	4E-63	35%
ZP_01035570.1	oxidoreductase, GMC family protein [*Roseovarius sp.* 217]	247	94%	4E-63	34%
XP_001998692.1	GI23491 [*Drosophila mojavensis*]	246	94%	6E-63	34%
XP_312382.2	AGAP002557-PA [*Anopheles gambiae* str. PEST]	246	94%	6E-63	34%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
XP_002166501.1	PREDICTED: similar to choline dehydrogenase [*Hydra magnipapillata*]	246	93%	7E-63	34%
ZP_01904700.1	citrate synthase [*Roseobacter sp.* AzwK-3b]	246	93%	9E-63	34%
ZP_01901081.1	oxidoreductase, GMC family protein [*Roseobacter sp.* AzwK-3b]	245	94%	1E-62	33%
ZP_01754024.1	glucose-methanol-choline oxidoreductase [*Roseobacter sp.* SK209-2-6]	245	93%	1E-62	33%
YP_002822699.1	putative glucose-methanol-choline oxidoreductase [*Rhizobium sp.* NGR234]	245	93%	1E-62	33%
BAB07804.1	polyethylene glycol dehydrogenase [*Sphingopyxis macrogoltabida*]	245	93%	1E-62	31%
YP_003277340.1	glucose-methanol-choline oxidoreductase [*Comamonas testosteroni* CNB-2]	245	93%	2E-62	33%
XP_002056410.1	glucose dehydrogenase [*Drosophila virilis*]	244	94%	2E-62	34%
ADQ00130.1	glucose-methanol-choline oxidoreductase [marine bacterium HP15]	244	93%	3E-62	33%
YP_003410784.1	glucose-methanol-choline oxidoreductase [*Geodermatophilus obscurus* DSM 43160]	244	94%	4E-62	33%
ZP_05085589.1	alcohol dehydrogenase (acceptor) [*Pseudovibrio sp.* JE062]	244	93%	4E-62	34%

NCBI Reference Sequence	Description	Total Score	Query Coverage	E Value	Max Ident
YP_002278603.1	glucose-methanol-choline oxidoreductase [*Rhizobium leguminosarum* bv. trifolii WSM2304]	244	93%	4E-62	34%
XP_001651431.1	glucose dehydrogenase [*Aedes aegypti*]	244	94%	4E-62	34%
XP_001851210.1	glucose dehydrogenase [*Culex quinquefasciatus*]	243	94%	5E-62	33%
YP_998315.1	glucose-methanol-choline oxidoreductase [*Verminephrobacter eiseniae* EF01-2]	243	93%	5E-62	32%
YP_002779312.1	putative L-sorbose dehydrogenase [*Rhodococcus opacus* B4]	243	93%	6E-62	34%
XP_623443.2	PREDICTED: similar to Glucose dehydrogenase isoform 1 [*Apis mellifera*]	243	94%	6E-62	33%
ZP_05450190.1	alcohol dehydrogenase (acceptor) [*Brucella neotomae* 5K33]	243	93%	8E-62	31%
ZP_05162503.1	alcohol dehydrogenase (acceptor) [*Brucella suis* bv. 5 str. 513]	243	93%	8E-62	31%
ZP_03784461.1	Choline dehydrogenase [*Brucella ceti* str. Cudo]	243	93%	8E-62	31%
XP_394879.3	PREDICTED: similar to Glucose dehydrogenase [*Apis mellifera*]	243	93%	8E-62	33%
ZP_07047542.1	glucose-methanol-choline oxidoreductase [*Comamonas testosteroni* S44]	242	93%	1E-61	33%

Table 71 **Protein database search** with CcPDH as query

12.12Culture Collection

12.12.1 *E. coli* Culture Collection

No.	Plasmid	Gene/Genotype	Medium	T [°C]	Storage Box/Place
B108	pPICZ B	-	LB	37	#1 F1-F4
B208	pKKW1.2	PDH aus *Coprinosis cinerea*	LB-Kan	37	#4 C1-C4
B225	pKKW6.3	PDH of *Coprinopsis cinerea* in pPICZ B Vektor	LB-Zeo	37	#5 C5-C8
B226	pKKW7.4	PDH of *Coprinopsis cinerea* with HIS-Tag in pPICZ B Vector	LB-Zeo	37	#5 D1-D4
B236	pKKW10.1	PDH (Broad Institute Sequence) of *Coprinopsis cinerea* in pPICZ B Vector	LB-Zeo	25	#5 G5-G8
B237	pKKW11.1	PDH (Broad Institute Sequence) of *Coprinopsis cinerea* with HIS-Tag in pPICZ B Vector	LB-Zeo	25	#5 H1-H4

Table 72 **Culture collection** of *E. coli*

12.12.2 *P. pastoris* Culture Collection

No.	Genotype/Phenotype	Plasmid used for Transformation	Medium	T [°C]	Storage Box/Place
Y41	KKW6.3.1 (PDH from *Coprinopsis cinerea*, Zeocin™ resistance)	pPICZ B	YPD-Zeo	25	#2 H3-H5
Y42	KKW7.4.1 (PDH from *Coprinopsis cinerea* with HIS-Tag, Zeocin™ resistance)	pPICZ B	YPD-Zeo	25	#2 H6-H8
Y47	KKW10.1.1 (PDH from *Coprinopsis cinerea*, Zeocinresistence)	pPICZ B	YPD-Zeo	30	#3 B5-B7
Y48	KKW11.1.1 (PDH from *Coprinopsis cinerea* with HIS-Tag, Zeocinresistence)	pPICZ B	YPD-Zeo	30	#3 B8-C2

Table 73 **Culture collection** of *P. pastoris*

12.13 Sample Results from "Protein Expression with All Modified Organisms"

Culture Number	t after induction [h]	OD_{600} calc. (see 8.11)	Protein [mg/ml] (see 8.12)	PDH activity [U/ml] (see 8.13.1)
1	32.5	13.8	0.024	0.147
1	50.0	15.8	0.046	0.224
1	71.5	18.5	0.059	0.370
1	90.0	20.4	0.045	0.417
1	118.3	21.3	0.072	0.634
1	141.2	24.2	0.099	0.760
1	169.3	25.3	0.127	0.984
1	192.5	29.4	0.145	0.859
1	212.3	24.1	0.199	1.079
1	240.8	29.5	0.203	1.275
1	258.5	-	0.184	1.088
2	32.5	13.1	0.031	0.291
2	50.0	16.9	0.056	0.398
2	71.5	19.1	0.057	0.611
2	90.0	20.2	0.058	0.863
2	118.3	22.7	0.068	1.157
2	141.2	24.1	0.080	1.305
2	169.3	24.9	0.089	1.639
2	192.5	27.0	0.098	1.511
2	212.3	22.2	0.117	1.791
2	240.8	26.6	0.109	2.028
2	258.5	-	0.118	1.703
3	32.5	13.8	0.027	0.018
3	50.0	14.5	0.047	0.009
3	71.5	17.0	0.043	0.012
3	90.0	18.7	0.043	0.019
3	118.3	20.3	0.061	0.015

Culture Number	t after induction [h]	OD_{600} calc. (see 8.11)	Protein [mg/ml] (see 8.12)	PDH activity [U/ml] (see 8.13.1)
3	141.2	21.3	0.057	0.016
3	169.3	23.8	0.076	0.013
3	192.5	28.2	0.061	0.019
3	212.3	25.9	0.082	0.013
3	240.8	31.7	0.071	0.018
3	258.5	-	0.076	0.015
4	32.5	12.9	0.046	0.020
4	50.0	14.9	0.078	0.010
4	71.5	15.4	0.093	0.004
4	90.0	16.4	0.105	0.018
4	118.3	17.0	0.124	0.002
4	141.2	19.1	0.137	0.013
4	169.3	19.3	0.158	0.016
4	192.5	19.1	0.163	0.015
4	212.3	14.8	0.178	0.012
4	240.8	19.1	0.166	0.023
4	258.5	-	0.158	0.012
5	32.5	11.7	0.075	0.016
5	50.0	14.8	0.119	0.015
5	71.5	18.2	0.171	0.012
5	90.0	20.5	0.199	0.014
5	118.3	20.7	0.230	0.020
5	141.2	21.4	0.288	0.019
5	169.3	22.3	0.321	0.012
5	192.5	27.7	0.345	0.020
5	212.3	21.2	0.374	0.012
5	240.8	26.6	0.362	0.017
5	258.5	-	0.360	0.015
6	32.5	13.6	0.046	0.022

Culture Number	t after induction [h]	OD_{600} calc. (see 8.11)	Protein [mg/ml] (see 8.12)	PDH activity [U/ml] (see 8.13.1)
6	50.0	15.2	0.074	0.016
6	71.5	16.5	0.102	0.014
6	90.0	17.9	0.117	0.018
6	118.3	19.8	0.137	0.016
6	141.2	22.8	0.147	0.018
6	169.3	23.0	0.174	0.010
6	192.5	26.0	0.167	0.017
6	212.3	20.1	0.178	0.015
6	240.8	25.2	0.179	0.020
6	258.5	-	0.173	0.015
7	32.5	12.6	0.058	0.017
7	50.0	17.3	0.072	0.017
7	71.5	16.3	0.096	0.015
7	90.0	19.2	0.084	0.022
7	118.3	21.2	0.113	0.015
7	141.2	22.5	0.151	0.012
7	169.3	23.8	0.165	0.012
7	192.5	26.8	0.168	0.019
7	212.3	22.6	0.169	0.017
7	240.8	27.2	0.170	0.018
7	258.5	-	0.169	0.016
8	32.5	14.2	0.027	0.043
8	50.0	17.0	0.042	0.075
8	71.5	19.2	0.047	0.141
8	90.0	20.8	0.064	0.164
8	118.3	24.4	0.074	0.204
8	141.2	24.4	0.085	0.237
8	169.3	25.5	0.101	0.236
8	192.5	28.7	0.101	0.221

Culture Number	t after induction [h]	OD_{600} calc. (see 8.11)	Protein [mg/ml] (see 8.12)	PDH activity [U/ml] (see 8.13.1)
8	212.3	25.8	0.109	0.226
8	240.8	29.2	0.109	0.246
8	258.5	-	0.115	0.234

Table 74 **Sample Results** from “Protein Expression with All Modified Organisms”

12.14 Sample Results from "Protein Expression with KKW6.3.1 and KKW7.4.1"

t after Induction [h]	Set pH	Remark
-98.0	5	Inoculation
-31.2	5	Batch finished
-1.7	5	Glycerol fed batch finished
22.1	5	Sampling
41.1	5	Sampling
43.0	3	pH change
64.3	3	Sampling
88.8	3	Sampling
89.8	7	pH change
113.3	7	Sampling
114.3	5	pH change
136.8	5	Sampling
167.8	5	Sampling and harvesting

Table 75 **Time table** from "Protein Expression with KKW6.3.1 and KKW7.4.1"

Culture Number	t after Induction [h]	OD_{600} calc. (see 8.11)	Wet Cell Mass [g/l]	PDH Activity [U/ml] (see 8.13.1)	Remark
1	-98.0	102	43.2		Preculture properties
1	-31.2	37.9	76.4	0.013	
1	-1.7	97.3	157	0.075	
1	22.1	109	139	0.007	
1	41.1	116	140	0.009	
1	64.3	95.4	148	0.011	
1	88.8		176	0.009	
1	113.3		199	0.013	
1	136.8		167	0.013	
1	167.8		192	0.002	
2	-98.0	66.4	28.5		Preculture properties
2	-31.2	36.9	74.0	0.013	
2	-1.7	90.8	180	0.005	
2	22.1	105	126	0.011	
2	41.1	120	153	0.012	
2	64.3	103	153	0.010	
2	88.8		184	0.008	
2	113.3		207	0.006	
2	136.8		216	0.035	
2	167.8		216	0.014	
3	-98.0	76.4	34.8		Preculture properties
3	-31.2	41.9	94.3	0.017	
3	-1.7	97.3	185	0.010	
3	22.1	115	150	0.013	
3	41.1	119	160	0.010	
3	64.3	101	156	0.005	
3	88.8		191	0.008	

3	113.3		215	0.010	
3	136.8		214	0.021	
3	167.8		214	0.035	
4	-98.0	91.5	47.4		Preculture properties
4	-31.2	36.6	89.2	0.009	
4	-1.7	89.6	168	0.050	
4	22.1	103	131	0.253	
4	41.1	114	141	0.470	
4	64.3	98.0	147	0.010	
4	88.8		179	0.008	
4	113.3		206	>1.000	
4	136.8		217	>1.000	
4	167.8		217	>1.000	

Table 76 **Sample Results** from “Protein Expression with KKW6.3.1 and KKW7.4.1”

12.15Complete Work Experience List

Work experience	
Dates	October 2010 onwards February – June 2010
Occupation or position held	Tutor
Main activities and responsibilities	Teaching and direction of students in a practical course in enzyme technology
Name and address of employer	Universität für Bodenkultur, 33, Gregor Mendel-Straße, 1180, Vienna, Austria
Type of business or sector	Education
Dates	October 2008 – February 2009 October 2007 – July 2008
Occupation or position held	Tutor
Main activities and responsibilities	Teaching and direction of students in an organic chemical course
Name and address of employer	Universität Wien, 1, Dr. Karl Lueger Ring, 1010, Vienna, Austria
Type of business or sector	Education
Dates	February 2004 – October 2007 April – September 2002
Occupation or position held	Office employee
Main activities and responsibilities	- Data updating - Price calculation
Name and address of employer	PBS Austria, 37, Vogelweiderstraße, 4600, Wels, Austria
Type of business or sector	Wholesaling
Dates	October – November 2006
Occupation or position held	Airport worker
Main activities and responsibilities	- Loading of aeroplanes with luggage
Name and address of employer	Flughafen Wien AG, 1, Postfach, 1300 Wien-Flughafen, Austria
Type of business or sector	Aeronautics
Dates	July – August 2001 July – August 2000 July – August 1999
Occupation or position held	Steel worker
Main activities and responsibilities	- Inspection - Material separation - Cleaning duties
Name and address of employer	VOEST-ALPINE PERSONALSERVICE GMBH, 31, Stahlstraße, 4030, Linz, Austria
Type of business or sector	Heavy industry
Dates	July – August 1998
Occupation or position held	Warehouser
Main activities and responsibilities	- Stocking - Acceptance of goods
Name and address of employer	PBS-LOGISTIK, 37, Vogelweiderstraße, 4600, Wels, Austria
Type of business or sector	Wholesaling

13 REFERENCES

13.1 Literature

1. **Kittl, Roman, et al.** Molecular cloning of three pyranose dehydrogenase-encoding genes from *Agaricus meleagris* and analysis of their expression by real-time RT-PCR. *Current Genetics.* 2008, 53, pp. 117-127.

2. **Peterbauer, Clemens K. and Volc, Jindřich.** Pyranose dehydrogenases: biochemical features and perspectives of technological applications. *Applied Microbiology and Biotechnology.* 2010, 85, pp. 837-848.

3. **Ferreira, Patricia, et al.** Aryl-alcohol Oxidase Involved in Lignin Degradation. *The Journal of Biological Chemistry.* 2009, Vol. 284, 37, pp. 24840-24847.

4. **Campbell, Neil A. and Reece, Jane B.** *Biology.* 7th Edition. s.l.: Pearson Education, 2004.

5. **Kües, Ursula.** Life History and Developmental Processes in the Basidiomycete *Coprinus cinereus*. *Microbiology and Molecular Biology Reviews.* 2000, 64, pp. 316-353.

6. **Moncalvo, Jean-Marc, et al.** One hundred and seventeen clades of euagarics. *Molecular Phylogenetics and Evolution.* 2002, 23, pp. 357-400.

7. **Macauley-Patrick, Sue, et al.** Heterologous protein production using the *Pichia pastoris* expression system. *Yeast.* 2005, 22, pp. 249-270.

8. **Lee, Jonas and Kim, Sung-Hou.** High-throughput T7 LIC vector for introducing C-terminal poly-histidine tags with variable lengths without extra sequences. *Protein Expression and Purification.* 2009, 63, pp. 58-61.

9. **Volc, Jindřich, et al.** Pyranose 2-dehydrogenase, a novel sugar oxidoreductase from the basidiomycete fungus *Agaricus bisporus*. *Archives of Microbiology.* 1997, 167, pp. 119-125.

10. **Sygmund, Christoph, et al.** Characterization of pyranose dehydrogenase from *Agaricus meleagris* and its application in the C-2 specific conversion of D-galactose. *Journal of Biotechnology.* 2008, 133, pp. 334-342.

11. **Perkins, David N., et al.** Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* 1999, 18, pp. 3551-3567.

12. **Bradford, Marion M.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* 1976, 72, pp. 248-254.

13. **Kujawa, Magdalena, et al.** Properties of pyranose dehydrogenase purified from the litter-degrading fungus *Agaricus xanthoderma*. *The FEBS Journal.* 2007, 3, pp. 879-894.

14. **Guillén, Francisco, Martínez, Angel T. and Martínez, María Jesús.** Substrate specificity and properties of the aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. *The FEBS Journal.* 1992, 209, pp. 603-611.

15. **Van Hecke, Wouter, et al.** Biocatalytic cascade oxidation using laccase for pyranose 2-oxidase regeneration. *Bioresource Technology.* 2009, 100, pp. 5566-5573.

16. **Altschul, Stephen F., et al.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research.* 1997, 25, pp. 3389-3402.

13.2 Figure Credits

Figure 1 http://www.fermentas.com

Figure 2 http://www.bio-rad.com

14 ABREVIATIONS

A	Adenine
A. bisporus	*Agaricus bisporus*
A. meleagris	*Agaricus meleagris*
AAO	Aryl-alcohol oxidase
AbPDH	*Agaricus bisporus* PDH sequence
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AmPDH	*Agaricus meleagris* PDH sequence
AOX	Alcohol oxidase
BBGM	Basal batch with glycerol medium
BisTris	Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan
BMMY	Buffered methanol-complex medium for yeast
C	Cytosine
C. cinerea	*Copinopsis cinerea*
C. cinereus	*Coprinus cinereus*
CcPDH	*Coprinopsis cinerea* PDH sequence
CcPDH-HIS	*Coprinopsis cinerea* PDH sequence with histidine tag
CcPDH-mod	*Coprinopsis cinerea* PDH modified sequence
CcPDH-mod-HIS	*Coprinopsis cinerea* PDH modified sequence with histidine tag
CDH	Cellobiose dehydrogenase
E. coli	*Escherichia coli*
EC	Enzyme-Commission number
EDTA	Ethylenediaminetetraacetic acid
G	Guanine
GFM	Glycerol feed medium
Glc	Glucose
Kan	Kanamycin
LB	Luria Bertani
MNS	Micro nutrient solution
MOPS	3-(N-morpholino)propanesulfonic acid
ORF	Open reading frame

P. pastoris	*Pichia pastoris*
P2O	Pyranose 2-oxidase
PDH	Pyranose dehydrogenase
RB	Restriction enzyme buffer
RE	Restriction enzyme
RO water	Reverse osmosis water
SDS	Sodium dodecyl sulfate
T	Thymine
TAE	Tris acetate EDTA
TEMED	Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
Zeo	Zeocin™